

**A TOXICITY TEST FOR THE EFFECTS OF CHEMICALS ON THE NON-TARGET  
SUBMERSED AQUATIC MACROPHYTE, *MYRIOPHYLLUM SIBIRICUM* KOMAROV**

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**by**

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## ABSTRACT

### A TOXICITY TEST FOR THE EFFECTS OF CHEMICALS ON THE NON-TARGET SUBMERSED AQUATIC MACROPHYTE, *MYRIOPHYLLUM SIBIRICUM* KOMAROV

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A laboratory toxicity testing system with *Myriophyllum sibiricum* Komarov (northern watermilfoil) was developed for predicting the effects of pesticides and other phytotoxic chemicals upon non-target submersed macrophytes. Features of this toxicity test that enhance standardisation include a chemically defined medium, artificial sediment and inoculation of each tube with an axenic *M. sibiricum* macrophyte segment. It is a static, partial life cycle laboratory toxicity test that determines the toxicant effect on plant growth over fourteen days. Endpoints measured included growth rate, plant height, root number and length, fresh and dry weight, plant area, oxygen production, change in membrane integrity, chlorophyll *a*, chlorophyll *b* and carotenoid content.

Once the toxicity testing system was established, eight herbicides (atrazine, 2,4-D, diquat, fluridone, glyphosate, hexazinone, metolachlor and triclopyr) and two reference toxicants ( $ZnCl_2$  and phenol) were tested on the growth and development of *Myriophyllum sibiricum*. The toxicity test successfully detected the toxic effects of the reference toxicants and the technical herbicides at their maximum label rates (MLR) as applied to water 15 cm in depth. Once the MLR was determined to be toxic, serial dilution tests were conducted to establish no observable effect levels (NOEL) and IC50s (concentration to inhibit the parameter by 50%). If stimulation was observed in the endpoint parameter, SC20s (concentration to stimulate the parameter by 20%) were calculated. Compared with the results from other aquatic plant species, *M. sibiricum*, as tested in this axenic toxicity test, was highly sensitive to the phytotoxic effects of 2,4-D, fluridone, glyphosate, triclopyr and phenol. This static, axenic toxicity test is a fast, simple and accurate method that can determine possible effects of new herbicides and other phytotoxic chemicals on non-target submersed macrophytes.

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## 1. INTRODUCTION

Rooted aquatic macrophytes are important components of the aquatic ecosystem because they contribute to primary productivity, generate oxygen, affect flow patterns (Dennis, 1984), provide habitat and food for other organisms (Dewey, 1986), stabilise the sediment (Lembi and Netherland, 1990), are utilised by detritivores (Wallace, 1989), are involved in nutrient cycling (Pimentel and Edwards, 1982) and improve water quality (Catallo, 1993; Hook, 1993). When herbicides are intentionally sprayed to control aquatic weeds and algal blooms or when pesticides unintentionally enter the waterway through atmospheric fallout, soil erosion, industrial effluent, sewage discharge, spills (McEwen and Stephenson, 1979), drift from aerial or ground applications (Akesson and Yates, 1964), or runoff from pesticide treated agricultural, urban or recreation areas (Harris and Miles, 1975), non-target plants can be adversely affected. A non-target plant is defined by the United States Environmental Protection Agency (U.S. EPA)<sup>1</sup> as a plant outside the area of intended application or a plant that is not the target of the pesticide application (Swanson and Peterson, 1988).

Pesticides are any substance or mixture of substances that are used to control or destroy any pest, or used as a plant regulator, defoliant or desiccant (Hayes, 1975). Environmental testing is required for all new pesticides. Presently, there are standardised tests for other aquatic organisms including fish, invertebrates, amphibians, algae and floating aquatic macrophytes but no structured test exists for non-target rooted aquatic macrophytes. The development of a test for a rooted aquatic macrophyte would be a beneficial addition to the existing pesticide testing requirements.

A toxicity test is considered to be a standardised test procedure used to determine the effects of a test chemical upon a test organism, whereas, a bioassay is a standardised test procedure that uses a biological organism to determine the concentration of a test chemical (ASTM, under consideration). These tests may be used for the preliminary assessment of chemicals (Mason, 1988) and the study of the activity, persistence and movement of a chemical or mixture of chemicals through the environment (Martin, 1973; Santelmann, 1977). In order for a toxicity test to be effective; it must be rapid, reproducible, sensitive, not space requiring and inexpensive.

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<sup>1</sup> Acronyms are defined in Appendix 8.1 (see page 398).

There should also be a correlation between the laboratory test and environmental effect. The test species should be easily obtained, produce a uniform response (Hess, 1980), be economically or ecologically important and be readily cultured in the laboratory (Mason, 1988).

The primary objective of this research was to develop a simple tissue culture toxicity testing system for testing pesticides and other phytotoxic chemicals upon non-target aquatic macrophytes. Because of its ubiquitous nature and importance in the Canadian environment, *Myriophyllum sibiricum* Komarov (northern watermilfoil)<sup>2</sup> was selected as an aquatic macrophyte suitable for tissue culturing and testing in a laboratory toxicity test. The axenic aquatic macrophyte toxicity test was designed to be a replicable and repeatable system. Features of this toxicity test that enhance standardisation include a chemically defined medium and artificial sediment and inoculation of each tube with an axenic macrophyte segment. It is a static, partial life cycle laboratory toxicity test that tests the toxicant effect over fourteen days. Every second day, plant shoot height was measured to allow for the development of growth curves. Endpoints measured included total plant height, root number and length, fresh weight, dry weight, plant area, amount of oxygen produced, change in membrane integrity, chlorophyll *a*, chlorophyll *b* and carotenoid content.

Once the toxicity testing system was established, the phytotoxicity of eight pesticides (atrazine, 2,4-D, diquat, fluridone, glyphosate, hexazinone, metolachlor and triclopyr) and two reference toxicants (ZnCl<sub>2</sub> and phenol) to the growth and development of *Myriophyllum sibiricum* was determined. The chemicals tested represent a wide array of different modes of phytotoxic action, as follows:

- Atrazine - photosystem II inhibitor;
- 2,4-D - RNA, DNA and protein disrupters; auxin mimic;
- Diquat (dibromide salt) - contact membrane toxin;
- Fluridone - carotenoid synthesis inhibitor used in aquatic situations;
- Glyphosate - disrupter of aromatic amino acid synthesis;
- Hexazinone - photosystem II inhibitor used in forestry
- Metolachlor - growth inhibitor

- Triclopyr - RNA, DNA and protein disrupters; auxin mimic

The toxicity test successfully detected the toxic effects of all the tested technical pesticides at their maximum label rates (MLR). Once the MLR was determined to be toxic, dilution series tests were conducted to establish No Observable Effect Levels (NOEL) and IC50s (concentration to inhibit the parameter by 50%). If stimulation of the endpoint parameter occurred, SC20s (concentration to stimulate the parameter by 20%) were calculated. Some pesticides have a low water solubility and had to be dissolved in a solvent prior to testing. Methanol was determined to be the solvent with the smallest toxic effect upon *Myriophyllum* growth and development when tested at a rate of 0.4% (v/v).

This static, axenic toxicity test is a fast, simple and accurate method that can determine possible effects of new herbicides and other phytotoxic chemicals on non-target submersed macrophytes. Since this toxicity test has been proven to have a high predictive value for macrophytes, regulatory officials may wish to include it with the algae and *Lemna* (duckweed) tests in order to have a more reliable procedure for predicting possible pesticide effects upon non-target aquatic plants.

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<sup>2</sup> Throughout this document, at the first mention of an organism, the full species name with authority is given. The species' common name, if available, follows in parentheses. A full listing of all the organisms cited in this document can be found in Appendix 8.2 (see page 399).

## **2. LITERATURE REVIEW**

### **2.1 PESTICIDE USE**

Since the 1940's, pesticides have been used extensively to control weed, insect and fungal pests in North America and Western Europe (Freemark and Boutin, 1995). Currently, there are over 500 pesticides registered for use in over 5 000 formulations. Pesticides are used extensively in Canada in agriculture, forestry and urban areas (Government of Canada, 1991). In terms of total volume sprayed, land area treated (Freemark and Boutin, 1995) and total sale (Harris and Miles, 1975), herbicides are now the most widely used pesticides . In Canada, 21.6 million hectares of farmland were treated with herbicides in 1990 (Freemark and Boutin, 1994) with the three prairie provinces using more than the rest of Canada combined (Government of Canada, 1991). The advantages of pesticide use include increased crop yield (Greig-Smith, 1992; Pimentel and Levitan, 1986), reduced labour cost and time (Freemark and Boutin, 1995) and unblemished produce with a longer storage life. The use of pesticides is economically beneficial but depending upon the type of pesticide, use pattern, application method and weather conditions, as little as 1% of the applied pesticide has been reported to actually reach the target organism (Pimentel and Edwards, 1982; Pimentel and Levitan, 1986). According to some estimates, only 5% of postemergence herbicides applied to corn actually reaches the target weed (Pimentel and Levitan, 1986). If the product is persistent, mobile or volatile, it is more likely to reach non-target areas and affect non-target organisms. The pattern of pesticide use, including geographical location, soil characteristics, type of crop to be treated and seasonal timing of spraying, determines which non-target organisms are likely to be exposed, and how frequently and during which life stage(s) this exposure might occur (Freemark and Boutin, 1994). The application method is extremely important in determining how much pesticide enters non-target areas. During a ground application, the pesticide is usually confined to the target area but during an aerial application, non-crop habitats may be sprayed directly or are more likely to be affected by pesticide drift (Freemark and Boutin, 1995). Formulation type also affects the loss of pesticides from the target area. Up to five percent of a surface applied wettable powder formulation may be lost from the site of application depending upon weather and slope (Wauchope, 1978). The meteorological conditions (e.g., wind, rain, temperature) during and after the pesticide

application can determine the amount of pesticide that is applied to and remains in the target area to interact with the target organism (Pimentel and Levitan, 1986; Wauchope, 1978). Soil particles with adsorbed pesticides can enter aquatic systems by wind and water erosion. Pesticides have been detected in groundwater and supplies of domestic drinking water. This contamination may be caused by accidental spills or leaching. Once the groundwater is contaminated, the pesticides may enter surface water bodies where the groundwater emerges. Pesticides travel with the surface freshwater and eventually reach the Great Lakes, and the Atlantic and Pacific Oceans (Government of Canada, 1991). Specific details regarding the chemical and physical properties of the pesticides used in this study can be found in Chapters 5.3 and 5.7.

The amount of pesticides entering aquatic systems could be reduced by changes in agricultural practices, such as changes in tillage systems and the use of buffer zones. For example, atrazine rates up to 3.4 kg/ha have been used in conventionally tilled corn but in no-till corn production atrazine rates of 1.1 to 2.0 kg/ha have been recommended (Glotfelty *et al.*, 1984). Conservation tillage systems, such as chisel, ridge-plant and no-till, reduced both runoff and soil loss (Buttle, 1990; Edwards *et al.*, 1980; Sauer and Daniel, 1987). Intercropping or strip cropping reduced water, soil and herbicide losses by 66%, 76% and 91%, respectively. Pre-plant incorporated applications further reduced herbicide loss (Hall *et al.*, 1983). Subsurface drains were another effective method of controlling pesticide loss into waterways (Southwick *et al.*, 1990b). The use of untreated buffer zones around major water arteries minimised the loss of pesticides into those water bodies (Merkle and Bovey, 1974). Buffer zones between 50 and 100 metres should protect non-target aquatic areas from aerially sprayed pesticides (Feng *et al.*, 1990; Payne, 1992; Payne and Thompson, 1992).

## 2.2 NON-TARGET AQUATIC EFFECTS

Aquatic ecosystems can be highly affected by pesticides. The type of aquatic environment can influence the pesticide exposure, with lotic systems (e.g., rivers) usually having a shorter length of exposure than lentic systems (e.g., ponds, swamps, sloughs, bogs) where the standing water has a long residence time (Pimentel and Edwards, 1982). Richards and Baker (1993) determined that the size of the river influenced the pesticide exposure with small rivers being affected by high pesticide concentrations for a short time period while larger rivers are exposed to moderate

pesticide concentrations for a longer period. Short term toxicant exposure causes direct acute toxic responses and long term exposure is responsible for chronic and ecosystem level effects (Richards and Baker, 1993).

Non-target organisms may be affected directly by the chemical or affected indirectly through changes in the food chain and ecosystem structure. Ecosystem level effects, including changes in species diversity and nutrient cycling (Pimentel and Edwards, 1982), may also occur. If plants are adversely affected by toxicants, environments can be severely altered because plants are the primary producers in all ecosystems. In order to determine the impact of chemicals upon non-target systems, most countries have developed testing guidelines.

## 2.3 EXISTING GUIDELINES

### 2.3.1 Canadian Guidelines

The Pest Management Regulatory Agency (PMRA)<sup>1</sup> of Health Canada has been responsible for pesticide registration in Canada since 1995. Their mandate includes protecting human health and the environment by minimising the risks associated with pesticides (Health Canada, 1997). Environment Canada recently proposed a four tiered testing scheme to evaluate the effect of pesticides upon non-target plants (Boutin *et al.*, 1993). Tier 1 tests are conducted to assess the phytotoxic potential of a technical pesticide to a single plant species under a worst-case scenario. This worst-case scenario encompasses the effects of a direct overspray of non-target habitats. For submersed aquatic macrophyte species, this is the concentration resulting from the application at the maximum recommended label rate (MLR) into a 15 cm deep column of water. This rate is termed the expected environmental concentration (EEC) (Boutin *et al.*, 1993) and is the definition applied in this document. The definition of EEC varies depending upon the type of species and application method (Boutin *et al.*, 1993). Another method of deriving the EEC is to calculate 20% of the MLR as applied to a pond with a surface area of 0.01 ha and a volume of 50 000 L. This method assumes that only 20% of a pesticide applied in a terrestrial environment will reach the aquatic environment (Kent *et al.*, 1991). If the EEC is toxic, progression to Tier II is required. Tier II is designed to quantify the magnitude of the toxic response by assessing toxicity over a range of concentrations. From dose-response curves, calculation of the NOEC (no observable effect concentration) and EC50 levels is recommended. Tier III includes

whole plant life cycle tests with submersed and emergent aquatic plants and tests with formulated pesticides. On a case-by-case basis, Tier IV testing requires microcosm, mesocosm or field testing with multispecies and community systems (Boutin *et al.*, 1993).

Canada has no defined test protocol for assessing the effects of pesticides on non-target aquatic macrophytes (Environment Canada, 1989; Swanson and Peterson, 1988). Pesticide registration in Canada is governed by the Pest Control Products Act under the jurisdiction of the Pest Management Regulatory Agency (PMRA). In certain cases, algal and macrophyte screening is conducted for herbicides following the Organisation for Economic Co-operation and Development (OECD) and U.S. Environmental Protection Agency (EPA) test guidelines (Swanson and Peterson, 1988). Algal screening may also be conducted following the recently published growth inhibition test (Environment Canada, 1992) that determines the effects of toxicants upon the freshwater alga, *Selenastrum capricornutum* Printz., using a microplate technique. Environment Canada (1989) has initiated the development of tests applicable to the Canadian environment.

### 2.3.2 U.S. Environmental Protection Agency Criteria

Present guidelines set up by the U.S. EPA involve a three-tier system of testing for non-target aquatic plants. The first tier consists of five day algal growth inhibition tests on four species (a freshwater green (*Selenastrum capricornutum*) (U.S. EPA, 1971; 1985a), a freshwater diatom (no species recommended), a marine diatom (*Skeletonema costatum* (Greville) Cleve) and a blue-green (*Anabaena flos-aquae* Bréb.)) (Swanson and Peterson, 1988) and a fourteen day floating aquatic macrophyte (*Lemna* spp. (duckweed)) growth inhibition test (Swanson and Peterson, 1988; U.S. EPA, 1985b). Concentrations used are the maximum label rate or three times the predicted environmental level of technical grade material. Tier two tests are only conducted if the results from tier one are detrimental ( $\geq$ EC50) to one or more aquatic species. This stage uses five dosages on those species that showed toxic effects in the first stage of testing. Progression to the final tier occurs only if the maximum recommended application quantity or the anticipated exposure is  $\geq$ EC50 for any one species examined in tier two; the pesticide is to be applied directly to water or enters via the discharge of treated water; the pesticide is to be used in a forest system; or, the

pesticide is for use in areas containing endangered species. Field tests of at least two weeks duration with the same concentrations of end-use products as in tier two are the basis of tier three (Swanson and Peterson, 1988). For terrestrial plant species, the EPA recommends that germination tests are conducted on six species from at least four families (Fletcher *et al.*, 1990).

### 2.3.3 Organisation for Economic Co-operation and Development

The European Community is in the process of harmonising the pesticide registration process between countries. Pesticide registration within the European Community will include a single approval for an active ingredient as suitable for incorporation into plant protection products and separate approvals for each product in the individual countries. Currently, the OECD (Organisation for Economic Co-operation and Development) guidelines are frequently incorporated into an individual country's registration procedures (Greig-Smith, 1992).

Testing conducted by the OECD is also based upon a three-tiered system: the basic level, the confirmatory level and the definitive level. A three day algal growth inhibition test with five doses is the only non-target plant test in the first step. In the second stage, the OECD recommends growth inhibition tests on additional algal species and growth, reproductive and LC50 (lethal concentration to fifty percent of the population) tests on *Lemna* and other aquatic vascular macrophytes. No precise tests have been developed for the third tier but tests with confined natural communities, aquarium tests with artificial communities and tests with separate trophic levels are recommended. The OECD recommends that any new guidelines that are established include as many different species as possible (Swanson and Peterson, 1988). The OECD is presently updating their testing guidelines to include a *Lemna* test in the basic level in order to address the need for aquatic vascular plant testing. It recommends that the algal tests in the basic level be changed to multispecies tests run in parallel to increase the sensitivity of the response (OECD, 1989). The OECD requires that germination and early seedling growth tests be conducted on at least three terrestrial plant species from three families (Fletcher *et al.*, 1990).

### 2.3.4 Problems with Existing Guidelines

There are several problems associated with the present pre-registration testing. Among the major problems are a lack of species diversity and the fact that the species

used are not always ecologically important (Swanson and Peterson, 1988; Swanson, 1989; Fletcher *et al.*, 1990). The aquatic tests require the use of one to four algal species and a species of *Lemna* (Swanson and Peterson, 1988). There is a large variation between the toxicological responses of different species (Blanck *et al.*, 1984; Peterson *et al.*, 1994; Slooff *et al.*, 1983; Wangberg and Blanck, 1988). The algal and *Lemna* tests used may not reflect the herbicidal effects upon submersed aquatic macrophytes (Fowler, 1977; Swanson, 1989). Most studies comparing algal sensitivity to rooted macrophytes have shown that the macrophyton is much more sensitive to the pesticide. *Lemna* is a good species for the examination of pesticide drift and for the study of the effects of surface films at the air-water interface but these results are not directly transferable to rooted aquatic plants. Based upon the differences in toxicity between aquatic plant species, it would be beneficial to implement a test battery approach utilising a wider taxonomic range of organisms for pesticide registration (Blanck, 1984; Blanck and Björnsäter, 1988; Lewis, 1995; Swanson *et al.*, 1991). The predictive value of a test battery improves with size and more than six representative species should be used (Blanck, 1984). Standardisation of new and existing test protocols is required (Fleming *et al.*, 1991; Wang and Freemark, 1995). There is a definite need for a toxicity test with a submersed aquatic macrophyte.

The term ecological importance needs to be defined in the context of the Canadian environment. This definition can be based upon numerical dominance, productivity, geographical distribution, or importance in food webs, habitat structure, or determining biochemical fate (Swanson, 1989). It is important that ecologically relevant species be used in toxicity testing (Calow, 1992).

#### 2.4 CRITERIA FOR NEW ENVIRONMENTAL TESTS

Any new toxicity test developed for aquatic macrophytes should be quick, simple, inexpensive and reproducible (Swanson and Peterson, 1988). The chosen species should be sensitive to pollutants, ecologically important, available commercially, well characterised taxonomically and rapidly growing under laboratory conditions using well defined growth media. An additional criterion that would be beneficial is a growth habit that lends itself to easy measurement at the endpoint, such as dry weight, leaf whorl counts, frond number or frond length. Several rooted aquatic macrophytes have been tested using long term field tests, enclosure tests or laboratory cultures but with wide

variations in methods, duration and endpoint. Possible candidate species include *Vallisneria americana* Michx. (wild celery), *Hydrilla verticillata* (L.f.) Royle, *Potamogeton perfoliatus* L. (redhead-grass), *P. pectinatus* L. (sago pondweed), (Swanson, 1989), *P. crispus* L. (curlyleaf pondweed) (personal communication with C. Boutin<sup>3</sup>), *P. richardsonii* (A. Benn.) Rydb. (Richardson pondweed), *Myriophyllum exalbescens* Fernald (northernwater milfoil) (personal communication with M.C. Gadsby<sup>4</sup>), *M. verticillatum* L. (whorled milfoil) (personal communication with C. Boutin<sup>3</sup>), *Ranunculus subrigidus* W.B. Drew (water buttercup), *Utricularia vulgaris* L. (bladderwort) and *Ceratophyllum demersum* L. (coontail) (personal communication with M.C. Gadsby<sup>4</sup>). To be acceptable, any new test should be quick, simple, inexpensive and reproducible (Swanson and Peterson, 1988).

## 2.5 TYPES OF BIOLOGICAL TESTING

There is an ongoing controversy about the advantages and disadvantages of the different types of biological testing. Single species laboratory toxicity tests, multi-species laboratory toxicity tests, microcosms, mesocosms, limnocorals and field testing are all methods of determining the effects of toxic chemicals upon organisms and biological systems. All of these methods have advantages and disadvantages (Neuhold, 1986) as summarised below.

### 2.5.1 Laboratory Toxicity Tests

The laboratory toxicity test has many advantages because there is complete control over the environmental conditions, such as growth media, light, temperature, dissolved oxygen and humidity. Other factors including competition for nutrients and space and standardisation of dilution water can be controlled. The isolation of the effects caused by the toxicant from other variables is one of the main advantages of the laboratory toxicity test. There is no environmental impact when the toxicity tests are conducted within a laboratory (Martin, 1973). The laboratory toxicity test may help reveal the toxicant's mode of action (Lovett-Doust *et al.*, 1994b).

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Laboratory toxicity tests can be subdivided into static and flow-through types. The static toxicity test maintains the initial concentration of test chemical throughout the duration of the test. Problems involving degradation, volatilisation, or precipitation of the test substance; chemical interactions in the solution; deposition of the test material onto the sides of the test container; uptake by the test organism; and interactions between the test materials and test organism exudates may occur. There are advantages to using a static test that include ease of initiation and operation, minimal amount of space and equipment required and the short testing span (Martin, 1973; Rand and Petrocelli, 1985a).

The flow-through toxicity test can more closely approximate natural conditions because there is a continual input of test solution. Compared to static toxicity tests, flow-through toxicity tests require a considerable amount of sophisticated equipment, more space, more test chemical to replace the amount lost in the effluent (Martin, 1973) and more labour since fresh nutrient medium and toxicant solutions should be prepared every day (Patrick, 1973). The disposal of large quantities of nutrient medium spiked with test material may be problematic for highly toxic compounds.

The other main distinction between types of laboratory tests is determined by the length of the testing period and by the type of endpoints analysed. In an acute toxicity test, lethality is the most common endpoint measured within 48 to 120 hours after test initiation. LC50s or other percentages of lethal concentrations (e.g., LC95 or LC5) are determined at the end of acute toxicity tests. An application factor or limit of safety is usually applied to the acute toxicity data. Traditionally, an application factor of 0.01 is applied to substances that are cumulative. A chronic toxicity test can run for several months to a year and sublethal effects, such as respiration, reproduction, metabolism and growth can be measured. Subtle chronic effects may remove the organism as a viable member of the system. At the end of a chronic test, the no observable effect limit (NOEL) can be determined (Martin, 1983; Rand and Petrocelli, 1985a).

### 2.5.2 Microcosms

A standardised aquatic microcosm (SAM) has been developed and evaluated with copper sulphate (Harrass and Taub, 1985; Taub, 1993) and atrazine (Stay *et al.*, 1985). The SAM consists of 4-L glass jars filled with 3 L of a sterile liquid medium plus

an artificial sediment. Each microcosm is spiked with ten species of algae (*Anabaena cylindrica* Lemm., *Chlamydomonas reinhardtii* Dang., *Chlorella vulgaris* Beij., *Nitzschia kuetzingiana* Hilse (= *N. pusilla* Grunov), *Scenedesmus obliquus* (Turp.) Kützing, *Selenastrum capricornutum*, *Ankistrodesmus* sp., *Lyngbya* sp., *Stigeoclonium* sp. and *Ulothrix* sp.) on Day 0 and five animal species (*Daphnia magna* Straus., *Hyalella azteca* Strassure, *Cypridopsis* sp., *Philodina* sp. and hypotrich protozoans) on Day 4 (ASTM, 1991a; Harrass and Taub, 1985; Stay *et al.*, 1985; Taub, 1993). Chemical treatment is made on Day 7 and observations are made for 63 days. The use of microcosms allows for the examination of changes in primary productivity and biomass, changes in algal and zooplankton species dominance and diversity, system recovery following deactivation of the toxicant and observation of indirect or secondary effects. The size limitations of the SAM introduce some drawbacks including the lack of a macrophyte population and a vertebrate predator, lack of a realistic sediment, lack of an anaerobic area and a high surface-to-volume ratio. The algal species affected in a SAM stressed with copper sulphate were not necessarily the same species affected in field studies (Harrass and Taub, 1985).

A similar microcosm was developed by Metcalf *et al.* (1971). They used radioactive insecticides to model pesticide movement and fate in terrestrial and aquatic systems. Glass aquaria containing 7 L of nutrient water were inoculated with terrestrial plants, algae, invertebrates and fish. The experiment was terminated after 33 days and the path of the radioactivity was determined (Metcalf *et al.*, 1971). Cairns and Cherry (1993) summarised the different types of multi-species tests that can be used in the laboratory.

### 2.5.3 Mesocosms

The next more complex type of biological testing involves the use of mesocosms, in-situ enclosures, experimental ponds or limnocorrals. These enclosed areas of a naturally occurring system allow for the examination of toxic effects upon organisms under conditions that mimic natural ones. The enclosures help maintain the desired treatment concentration and prevent the spread of the contaminant into the whole system. Their disadvantages include their size limitation so that some portion of the ecosystem is not present (e.g. large fish or macrophytes) and they restrict the normal movement of organisms and nutrients (Lovett-Doust *et al.*, 1994b).

Mesocosms have been used to study the environmental fate of pesticides (Detenbeck *et al.*, 1996; Heimbach *et al.*, 1994; Muir *et al.*, 1985; Solomon *et al.*, 1988) and the effect of pesticides upon biological communities (deNoyelles *et al.*, 1994; deNoyelles and Kettle, 1985; Detenbeck *et al.*, 1996; Dewey, 1986; Fairchild *et al.*, 1994a; Hamilton *et al.*, 1988; Heimbach *et al.*, 1994; Huggins *et al.*, 1994). Cairns and Cherry (1993) summarised different types of multi-species mesocosm tests used in field situations and the collection of papers edited by Graney *et al.* (1994) describes mesocosm regulations, experimental design, statistical analysis and case studies.

#### 2.5.4 Field Testing

The final step in evaluating the environmental impact of a compound is field or ecosystem testing. This is the most complex and expensive step but it may be the most ecologically relevant (Lovett-Doust *et al.*, 1994b). Lovett-Doust *et al.* (1994a) used the aquatic macrophyte *Vallisneria americana* in field studies of aquatic contaminants. They determined that this species accumulated contaminants and could be used as a biomonitoring organism (Lovett-Doust *et al.*, 1994a).

Toxicological results collected from field studies might reflect what was observed in laboratory experiments but laboratory results should not be extrapolated to field situations (Chapman, 1995a; 1995b). Field collected results correlated well with laboratory microcosm studies for changes in invertebrate species richness but the results did not correlate as well for algal growth (Niederlehner *et al.*, 1990). Changes in algal dominance and productivity were observed in both laboratory microcosms and field studies (Harrass and Taub, 1985).

Using the PHYTOTOX data base, Fletcher *et al.* (1990) examined 230 published papers and compared the toxic response (EC50) of thirteen terrestrial plant species to seventeen chemicals when they were tested in both the field and greenhouse. It was determined that there was not much variability between field and lab results. There was more variability between the mean sensitivity ratios of terrestrial plant species, which means that different plant species from different plant families are likely to exhibit different sensitivities to the same phytotoxic compound and that closely related species are likely to have similar toxicities (Fletcher *et al.*, 1990).

### 2.5.5 Comparison between Aquatic Plants and Other Organisms

Several authors have investigated whether the testing of other aquatic organisms (i.e., fish and invertebrates) is sufficient to negate the need for algal and duckweed testing. Different species are sensitive to different types of compounds (Blanck, 1984; Lewis, 1993; Neuhold, 1986). Theoretically, plants should be more sensitive to herbicides and insects should be more sensitive to insecticides. In a comparison of toxicity data for algae, fish and invertebrates, Kenaga and Moolenaar (1979) erroneously concluded that the animals were more sensitive indicators than the plants. Miller *et al.* (1985) compared the toxicity of 2,4-D to *Selenastrum capricornutum* and *Daphnia magna*. The green alga was affected by statistically lower concentrations (95.8 mg/L) than the daphnia (> 240 mg/L). *Lemna minor* L. (common duckweed) was compared to *Ceriodaphnia dubia* (invertebrate) and *Pimephales promelas* (fathead minnow) and it was found to be more sensitive to the effluent from a herbicide plant (Taraldsen and Norberg-King, 1990). Wang (1986a) found that the chronic values determined from animal toxicity testing were not always sensitive enough to protect aquatic plants.

Despite this ongoing controversy, all types of biological testing systems can have a place within the framework of pesticide regulation and environmental protection. However, the development and validation of a static, partial life cycle laboratory toxicity test for a submersed macrophyte is definitely needed and could fit into the regulatory scheme at Tier I or Tier II.

### 2.6 ESTABLISHED TISSUE CULTURING

Tissue culturing of terrestrial plants is a method that has been used successfully to detect pesticide effects, such as metabolism, mode of action and resistance. Cell cultures require little space, lack permeability barriers, reflect whole plant effects and are easy to monitor (Gressel, 1979). Herbicide metabolism studies have been conducted in tissue cultures of *Zea mays* L. (maize) (Ezra and Gressel, 1982; Ezra *et al.* 1982; 1983), soybean (*Glycine max* (L.) Merr.) (Scheel and Sandermann, 1977; 1981), wheat (*Triticum aestivum* L.) (Scheel and Sandermann, 1981), parsley (*Petroselinum hortense* Hoffm.) (Scheel and Sandermann, 1977) and white clover (*Trifolium repens* L.) (Smith, 1979). Studies comparing the effects of herbicides on intact plants to those of cell cultures have shown that the culture reflects what is occurring in the intact plant

(Harrison *et al.*, 1983; Swisher and Corbin, 1982; 1983) but there might be some protective mechanisms in the whole plant that are lacking in cell cultures. The use of several clones is advantageous because it provides a limited amount of genetic variability, enhancing repeatability and provides a year round supply of plant material (Biemacki *et al.*, 1997; Fleming *et al.*, 1991; Lovett-Doust *et al.*, 1993). No specific culture media or protocol exists for tissue culturing of aquatic plants.

## 2.7 ALGAL PROTOCOLS

Currently, the majority of standardised aquatic plant protocols are for algae (ASTM, 1990; 1993; Blanck and Björnsäter, 1988; Environment Canada, 1992; U.S. EPA, 1971; 1985a). "Algae" is an inclusive term used for a large number of photosynthetic eukaryotes of varying form and complexity, the majority of which are truly aquatic (Bold *et al.*, 1980; Palmer, 1977). A listing of the common algal species used in toxicity tests is presented in Table 1. Algae are useful organisms for toxicity testing because they are small, require little laboratory space and are easy to culture in the laboratory (Addison and Bardsley, 1968). The standardised pesticide toxicity tests for algae are quick, simple, inexpensive and reproducible (Swanson and Peterson, 1988).

### 2.7.1 Standard Bottle Test

In 1971, the U.S. EPA developed a static bottle test for algal toxicity tests (Standard Bottle Test). The purpose of this test was to provide an established guideline for the proper collection and manipulation of samples to provide accurate and reproducible results. It was established for use in evaluation of materials, waste water and receiving water. Algal stock solutions are maintained at  $24 \pm 2$  °C under cool-white fluorescent lighting ( $4\ 304$  lux  $\pm$  10%). The pH of the enriched nutrient medium is maintained below 8.5 by continuous shaking of the flask or by ventilating the flask with an air or air/carbon dioxide mixture (U.S. EPA, 1971). A series of culture flasks is prepared with an inoculate of the algal stock solution. Toxicant is added in a concentration series (Wood, 1975). Untreated control flasks are cultured under the same light and temperature regime as the treated flasks (Parrish, 1985). Daily, relative growth of the algal population is measured by one of several methods: optical density with a spectrophotometer, manual count with a hemocytometer or plankton counting cell, or electronic cell count using a Coulter counter (Wood, 1975). This is a chronic test

Table 1: Algal species used in toxicity testing.		
Species	Division	Reference
<b>Recommended by the U.S. EPA</b>		
<i>Selenastrum capricornutum</i> *	Chlorophycophyta (Green)	ASTM (1993)
		Blanck and Björnsäter (1988)
		U.S. EPA (1971)
		Environment Canada (1992)
		Payne and Hall (1979)
		Swanson and Peterson (1988)
<i>Skeletonema costatum</i>	Chrysophycophyta (Diatom)	Blanck and Björnsäter (1988) Swanson and Peterson (1988)
<i>Anabaena flos-aquae</i>	Cyanochloronta (Blue-green)	Blanck and Björnsäter (1988)
		U.S. EPA (1971)
		Swanson and Peterson (1988)
<i>Microcystis aeruginosa</i> Kütz.	Blue-green	U.S. EPA (1971)
		Giddings <i>et al.</i> (1983)
		Payne and Hall (1979)
<b>Commonly Used by Other Agencies</b>		
<i>Chlorella pyrenoidosa</i> Chick.	Green	Kratky and Warren (1971)
		Sikka and Pramer (1968)
		Thomas <i>et al.</i> (1973)
<i>Chlorella vulgaris</i>	Green	Addison and Bardsley (1968)
		Birmingham and Colman (1983)
<i>Chlamydomonas eugametos</i> Moewus (= <i>C. sphagnophila</i> Pascher)	Green	Hess (1980)
		Loeppky and Tweedy (1969)
<i>Chlamydomonas reinhardtii</i>	Green	Blanck and Björnsäter (1988)
		Loeppky and Tweedy (1969)
<b>Occasionally Used</b>		
<i>Asterionella formosa</i> Hass.	Diatom	Blanck and Björnsäter (1988)
		Mason (1988)
<i>Scenedesmus quadricauda</i> (Turp.) Bréb.	Green	Giddings <i>et al.</i> (1983)
<i>Cosmarium botrytis</i> (Menegh.) Ralfs	Desmid	Giddings <i>et al.</i> (1983)
<i>Chlorella elliposidea</i> Gerneck	Green	Sumida <i>et al.</i> (1977)
<i>Chlorella sorokiniana</i> Shihira and Krauss	Green	St. John (1971)
<i>Navicula pelliculosa</i> (Kützing) Hilse in Rabenhorst	Diatom	Birmingham and Colman (1983)
		Hughes <i>et al.</i> (1988)
<i>Nitzschia palea</i> (Kützing) W. Smith	Diatom	Giddings <i>et al.</i> (1983)
<i>Euglena gracilis</i> Klebs	Euglenophycophyta (Euglenid)	Sikka and Pramer (1968)
* This species is easiest to culture, use (Mason, 1988) and is most frequently tested (Lewis, 1993; 1995).		

because by the end of the experimental period the algal population has reached a maximum growth rate and cell density (Parrish, 1985).

At the end of the test (96 hours to 14 days (Giddings *et al.*, 1983)), maximum specific growth or maximum standing crop can be determined. The maximum specific growth rate for an individual flask is the largest specific growth rate occurring at any time during incubation. Maximum specific growth rate occurs during the logarithmic phase of growth, usually between days 0 and 5, so it is necessary for measurements (cell counts) to be made daily (U.S. EPA, 1971). The maximum standing crop is the maximum algal biomass achieved during incubation. Biomass can be determined by direct determination of dry or fresh weight, by cell count using absorbance (spectrophotometer or colorimeter), by chlorophyll measurements (extraction or direct fluorometric determination), or by total cell carbon (carbon analyser) (U.S. EPA, 1971; Mason, 1988). At this point, the concentration of the toxicant can be semi-logarithmically plotted against the specific growth rate or daily cell count. LD50, IC50, EC50 or other appropriate average response can be calculated (Wood, 1975). There is some controversy regarding whether biomass or growth rate is the better end point. It has been observed that there is appreciable variation between the EC50 values for these two end points. Nyholm (1985) statistically compared the EC50 values and concluded that growth rate is theoretically superior and shows less variation between and within tests than biomass.

#### 2.7.1.1 Problems with the Standard Bottle Test

The flask algal assays can be time-consuming, labour intensive and not efficient when a high sample turnover is required (St. Laurent *et al.*, 1992). The use of these static algal culture tests can also result in several problems. The composition of the growth medium can change throughout the experiment. There can be pH shifts, nutrient level decline, algal metabolite accumulation in the medium and bacterial development on the walls of the test container. Physiological changes can occur in the algae as they adapt to their new environment. Photolysis, hydrolysis, biodegradation and volatilisation may change the concentration and chemical form of the pesticide during the toxicity test (Giddings *et al.*, 1983). Many algal tests reported in the literature seem to have been conducted under conditions of poor gas exchange and variable physical and chemical parameters. This produces results that vary up to three orders of magnitude, even for identical test species (Nyholm and Källqvist, 1989).

## 2.7.2 Alternative Algal Toxicity Test Methods

### 2.7.2.1 Algal Microplate Technique

Microplates have been suggested as an alternative method to the Standard Bottle Test. This is the algal test method recommended by Environment Canada (Environment Canada, 1992). U-bottomed polystyrene 96-well microplates can be used and filled in a random fashion with algae and toxicant, along with control wells. The peripheral wells are not filled because an edge-effect phenomenon increases evaporation rate from the perimeter wells. This test can either be static, with no renewal of the growth medium (Environment Canada, 1992; Blaise, 1986; Blaise *et al.*, 1986), or semistatic, with the liquid growth medium renewed every 24 hours. Renewal of the toxicant-spiked medium ensures a more constant pH, toxicant and nutrient concentration (Radetski *et al.*, 1995). After four days of incubation, a Coulter counter (Environment Canada, 1992; Blaise, 1986; Blaise *et al.*, 1986; Radetski *et al.*, 1995) or fluorimeter (Blanck and Björnsäter, 1988; Caux *et al.*, 1992) is used to measure cell growth. In a comparison with the flask technique, this method was as effective in determining pesticide toxicity, with the added benefit of being more cost and space efficient and more precise (Blaise *et al.*, 1986; St. Laurent *et al.*, 1992). The microplate technique allows numerous algal species to be tested simultaneously in a test battery approach so that reasonable estimates of algal toxicity can be made. Toxicity estimates are not limited to one or a few test organisms (Blanck and Björnsäter, 1988).

### 2.7.2.2 Physiological Algal Techniques

Giddings *et al.* (1983) proposed another alternative method to the Standard Algal Test. They suggested that the measurement of the short-term effects of the toxicant upon the rates of a physiological process, like photosynthesis, would result in more reliable data because the problems with the Standard Bottle Test would be avoided. Five mL aliquots of the algal culture were added to twenty mL glass scintillation vials, to which the pesticide had been added. After 2 hours of incubation C-14 labelled sodium bicarbonate ( $\text{NaHCO}_3$ ) were added to each vial. Following another 2 hour incubation period, carbon fixation was terminated and the quantity of C-14 assimilated was determined by liquid scintillation counting. This endpoint, amount of C-14 assimilated, can be rapidly and precisely measured and the whole toxicity test procedure requires

little time and space (Giddings *et al.*, 1983). Additional information on mirco-algal toxicity tests and pesticide effects on algae can be found in Wright (1978).

## 2.8 EXISTING MACROPHYTE RESEARCH

### 2.8.1 Floating Macrophytes

There are several standardised testing procedures for floating aquatic macrophytes (ASTM, 1991b; U.S. EPA, 1985b). Duckweed is the common name for the plant family containing the world's smallest flowering plants, all of which are aquatic (Sculthorpe, 1971). The two genera used in toxicity testing are *Lemna* and *Spirodela* (Table 2) (Wang, 1990a). *Lemna* is a good genus for the examination of pesticide drift and for the study of the effects of surface films at the air-water interface (Swanson, 1989; Taraldsen and Norberg-King, 1990). Since *Lemna* usually reproduces asexually, clones may be used in testing to avoid genetic variability (Bishop and Perry, 1981).

Species	Reference
<i>Lemna minor</i> L.	Bishop and Perry (1981)
	Huber <i>et al.</i> (1982)
	Lockhart <i>et al.</i> (1983; 1989)
	O'Brien and Prendeville (1978; 1979)
	Parker (1965)
	Sutton <i>et al.</i> (1969)
	Taraldsen and Norberg-King (1990)
	Wang (1991)
<i>L. gibba</i> L. (inflated duckweed)	Ensley <i>et al.</i> (1994)
	Greenberg <i>et al.</i> (1992)
	Huang <i>et al.</i> (1991; 1993)
	Wang (1990b)
<i>L. valdiviana</i> Philippi.	Wang (1990a)
<i>L. polyrrhiza</i> L. (= <i>Spirodela polyrrhiza</i> (L.) Scheid)	Charpentier <i>et al.</i> (1987)
<i>L. perpusilla</i> Torr.	Clark <i>et al.</i> (1981)
<i>Spirodela polyrrhiza</i> (L.) Schleiden	Wang (1990a)

A static duckweed test is recommended for use by registration agencies and is similar to the algal flask test (ASTM, 1991b; U.S. EPA, 1985b). Duckweed has also been studied in renewal and flow-through experiments. The flow-through and renewal systems are useful for samples containing volatile or biodegradable compounds. A variety of test vessels have been used including glass beakers, flat-bottomed test tubes, jars, Erlenmeyer flasks and culture dishes. Frond, plant, and root numbers, dry or fresh biomass, root length, frond diameter, C-14 uptake and chlorophyll production are some of the end points of duckweed toxicity tests (Wang, 1990a).

The usual static duckweed test involves growing twenty fronds in a Petri plate for 120 hours at a temperature of 27 - 28 °C and a continuous photoperiod (86  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Frond increase per culture vessel is the standard end point (Wang, 1990a). This static duckweed test can be plagued with problems similar to the algal Standard Bottle Test, especially changes in toxicant and medium composition due to chemical and biological processes. Despite these minor problems, the static test is widely used because the initial set-up and maintenance is simple (Bishop and Perry, 1981).

Taraldsen and Norberg-King (1990) compared this static duckweed test to a renewal method that used change in chlorophyll production as an endpoint. Their alternative method involved filling 30 mL polystyrene plastic cups with 15 mL of test solution. Twelve fronds were added to each container. All test solutions were renewed daily and the number of fronds counted. After 96 hours, chlorophyll *a*, *b* and *c* and pheophytin *a* concentrations were determined with a spectrophotometer. Chlorophyll content was a more sensitive indicator of injury than was total frond count. This renewal test avoids many of the problems involved in the static test, as there is little change in pesticide concentration or medium consistency, since the solution is changed daily.

A flow-through system for *Lemna* was proposed by Bishop and Perry (1981). Dilution water flows from the reservoir into the diluter mixing chamber where mixing with the nutrient stock and test material occurs. The nutrient stock and toxicant are pumped from their holding tanks into the diluter mixing chamber. The material is then delivered from the diluter mixing chamber to the flow-splitter distribution tubes, controlled by a timer and switch. The test chambers have an overflow notch that keeps their volume at approximately 400 mL. This system keeps the pesticides at a constant concentration

but involves a large amount of space, an intensive set-up period (Bishop and Perry, 1981) and a large volume of clean dilution water (Mason, 1988).

## 2.8.2 Emergent and Submergent Macrophytes

There has been a recent increase in research conducted on aquatic macrophytes other than duckweed. Three other standardised tests for macrophytes are currently being developed and perfected. Researchers at Monsanto Company, St. Louis, Missouri have developed and recently published an emergent macrophyte testing protocol with the American Society for Testing and Materials (ASTM, 1996). Monsanto began testing with wetland plant species in 1992 in order to assist with wetland evaluation (personal communication with R. Powell<sup>5</sup>). They have screened numerous emergent wetland macrophytes species including *Oryza sativa* L. (domestic rice), *Spartina pectinata* Link. (prairie cordgrass), *Phalaris arundinacea* L. (reed canary grass), *Polygonum muhlenbergh* G. (nodding smartweed), *Scirpus acutus* Muhl. (hardstem bulrush), *Typha latifolia* L. (narrowleaf cattail), *Iris versicolor* L. (blue water iris), *Trifolium repens* L. (white clover), *Zizania aquatica* L. (giant wild rice), *Alisma plantago aquatica* L. (water plantain), *Onobrychis viciaefolia* Scop. (Sainfoin), *Carex rostrata* Stokes. (beaked sedge) and *Juncus effusus* L. (soft rush), listed from best to worst for toxicant screening. Tubers, rhizomes or seeds of these species are planted in plastic pots (Powell, 1993; Powell *et al.*, 1996) containing an artificial sediment, such as the one developed by Walsh *et al.* (1991a) or a natural soil (Powell *et al.*, 1996) and then placed into large trays filled with half strength Hoagland's solution (Hoagland and Arnon, 1938). Once the plants have grown for 2 - 6 weeks, they are placed into trays containing the nutrient solution spiked with different concentrations of the test compound. The test solutions are renewed three times per week. Depending upon the species being tested, plants are harvested 2 - 6 weeks after the initial exposure to the test chemical (Powell, 1993; Powell *et al.*, 1996). Endpoints examined included wet weight, dry weight and plant height but differences were not noticeable until after a long exposure (Powell, 1993). Leaf tissue residue has also been determined. Currently,

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<sup>5</sup> R. Powell, Monsanto Company, 800 North Lindbergh Boulevard - U4G, St. Louis, MO, 63167, (314) 694-5074. This address was only valid until May 31, 1997 but all mail will be forwarded to R. Powell's new address when it is established.

domestic rice is recommended for toxicity testing because the seeds are easily obtainable, the species is easily cultured in the greenhouse, it is economically important and produces consistent results. Chlorophyll a is being emphasised as the most sensitive endpoint, except that more time and equipment are needed to conduct this analysis (Powell *et al.*, 1996; personal communication with R. Powell<sup>5</sup>).

Rice has been also been used by other researchers. The toxicity of effluents to rice seed germination and asexual reproduction of *Lemna minor* was examined by Wang (1991). It was recommended that both aquatic plant species be tested because their sensitivities were effluent specific.

Nelson<sup>6</sup> and her research group at the National Biological Service in conjunction with Carolina Ecotox, Inc., (J. Hughes<sup>7</sup> and M. Alexander<sup>8</sup>) are working on the development and evaluation of another pesticide toxicity test for submersed macrophytes using an aquarium culturing and beaker testing system. They have tested numerous macrophyte genera including *Lemna*, *Ceratophyllum*, *Myriophyllum*, *Egeria* (elodea) and *Najas* (naiad) with four pesticides (atrazine, alachlor, metolachlor and metribuzin) (Fairchild *et al.*, 1994b) and *Vallisneria* with metribuzin (Nelson and Fairchild, 1994). The plants were grown in 1 L beakers containing 750 mL (Fairchild *et al.*, 1994b) of freshwater algal medium (ASTM, 1990). The static test lasted 14 days, after which blotted wet weights were determined. The species were ranked, in order of decreasing sensitivity, *Ceratophyllum* > *Najas* > *Lemna* > *Egeria* > *Myriophyllum* (Fairchild *et al.*, 1994b). This range of sensitivities would be valuable when making wetland management decisions (Nelson and Fairchild, 1994).

This group is currently developing an ASTM protocol, where it is recommended that static toxicity tests be conducted with either *Ceratophyllum demersum* or *Myriophyllum heterophyllum* Michx. In this assay, plants are grown in a 10% Hoagland's solution (M. Nelson<sup>6</sup>, personal communication) buffered with 200 mg/L NaHCO<sub>3</sub> (Byl and Klaine, 1991). Five 7 cm apical segments are placed in each test beaker and allowed to incubate for 14 days at 25 ± 1 °C under a photoperiod of 16 hours light. Each concentration is replicated four times. The endpoints recommended are wet weight for

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*C. demersum* and total root number for *M. heterophyllum* (M. Nelson<sup>6</sup>, personal communication).

Biernacki *et al.* (1997) recently developed a laboratory toxicity test with *Vallisneria americana* Michx. (wild celery) for testing contaminated sediment. This toxicity test involves planting *V. americana* shoots from different genetic lines into glass jars containing sediment from different sites. Six jars with sediment from the same site are placed into three replicate aquaria and exposed to the natural photoperiod. The plants were grown in dechlorinated tap water without additional nutrients. After one week of exposure, the plants were measured for the number of leaves, length and width of each leaf, number of roots, diameter and length of each root and the biomass of leaves and roots. This toxicity test successfully detected the effects of contaminated sediments on the different genetic lines of *V. americana* (Biernacki *et al.*, 1997).

Other lab and field experiments using pesticides have been conducted but there is great variability in species tested and method utilised. For example, laboratory studies were conducted on *Cabomba caroliniana* Gray (fanwort), *Elodea canadensis* Michx. (American elodea), *Egeria densa* Planch. (Brazilian elodea), *Myriophyllum spicatum* L. (Eurasian watermilfoil), *Vallisneria americana* and *Potamogeton perfoliatus* to examine the effects of atrazine, metribuzin and glyphosate on their growth. In these experiments, four or five cuttings or tubers were grown in 3.8 L glass jars on 5 cm of soil and filled with one-quarter strength Hoagland and Arnon's solution No. 2 with twice the prescribed amount of iron. A 14 hour photoperiod ( $35 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 20 °C were the environmental conditions used. Plants were grown for three to six weeks in the herbicide solutions. Every seven days, stem lengths were measured and a final dry weight was determined. When plants were grown from tubers, the herbicide had no effect for the first several weeks because the plant was utilising stored food reserves (Forney and Davis, 1981).

Toxicant effects upon oxygen production in *Potamogeton perfoliatus*, *Ruppia maritima* L. (widgeon grass), *Myriophyllum spicatum* and *Zannichellia palustris* L. (horned pondweed) have been studied by Jones and Winchell (1984). Individual plants were grown in 300 mL Wheaton bottles containing river water and the toxicant and incubated for two hours at 23 °C with a light intensity of  $180 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

Jones and Estes (1984) conducted a study on detached *P. perfoliatus* leaves to determine if soil adsorbed herbicide affected photosynthesis. Individual leaves were covered with herbicide treated soil, placed on wire racks and illuminated with  $115 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at  $25^\circ\text{C}$  for four hours.

Fowler (1977) examined pesticide effects upon the vigour of *Potamogeton pectinatus* and *Myriophyllum verticillatum*. Plants, grown from turions<sup>9</sup>, were placed in 3-L glass jars filled with deionized water and herbicide. They were kept in a greenhouse under natural light conditions (between July and February) at maximum daily temperatures of  $17.5$  to  $29^\circ\text{C}$ . Two people rated the plant vigour on a scale from 0 (dead) to 9 (healthy). Both species showed chlorosis, necrosis and eventual death at high pesticide concentrations. Even the control plants of *P. pectinatus* showed signs of deterioration (Fowler, 1977). Thus, visual rating scales can be variable.

The effect of flurprimidol, paclobutrazol and uniconazole (gibberellin synthesis inhibitors) was determined on *Myriophyllum spicatum* and *Hydrilla verticillata*. Four centimetre apical tips were placed into 250 mL Erlenmeyer flasks containing 150 mL of modified Gerloff's medium for the *M. spicatum* and 10% Hoagland's medium for the *H. verticillata*. The media were autoclaved and buffered with filtered  $\text{NaHCO}_3$  at a concentration of  $2.3\cdot 10^{-3}$  M. The plants were incubated at a constant temperature of  $25 \pm 1^\circ\text{C}$ , a light fluence rate of  $400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and a photoperiod of 16 hours light and 8 hours dark. The *M. spicatum* cultures were continuously aerated with 0.5%  $\text{CO}_2$  enriched air (Netherland and Lembi, 1992).

Microcosm studies with aquatic macrophytes have also been conducted. Kemp *et al.* (1985) exposed *Potamogeton perfoliatus* and *Myriophyllum spicatum* to herbicides in microcosms and then monitored oxygen evolution and above ground biomass accumulation (shoots $\cdot\text{m}^{-2}$  and shoot length). Each microcosm consisted of a 50-L glass aquarium (32 x 36 x 48 cm) filled with estuarine water and 8 cm of sediment. A 14 hour photoperiod of  $150 - 200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}$  and a temperature of  $21.5 \pm 1.5^\circ\text{C}$  was maintained. Ten plants (6 - 14 cm stem length and 2 - 6 cm rhizome length) were planted in each aquarium. Water was continually recirculated. Oxygen evolution was monitored twice

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<sup>9</sup> Turion: a form of asexual reproduction in which a bud, formed as a dense cluster of apical leaves, survives the winter (Sculthorpe, 1971).

weekly while the plant density and shoot lengths were measured every three to four weeks during the eleven week experiment (Kemp *et al.*, 1985). Microcosms were used to examine the effects of herbicides on *Potamogeton pectinatus*. These microcosms consisted of 18.9 L plastic buckets containing peat, crushed oyster shell, white sand and synthetic freshwater. Each static microcosm contained three plants that were exposed to continuous lighting at  $70 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}$  (Fleming *et al.*, 1991).

In a field experiment, Brooker and Edwards (1973) examined the effects of an herbicide upon biomass accumulation in *Potamogeton pectinatus* and *Myriophyllum spicatum*. The herbicide was applied from a boat using a hand operated sprayer. Biomass was estimated by comparing light attenuation in the plant beds to that in plant free areas, over the summer.

The above examples illustrate the wide variety of methods used to examine the effects of herbicides upon aquatic macrophyton. Field experiments (Brooker and Edwards, 1973) and large scale aquaria studies (Kemp *et al.*, 1985) are not practical until the third tier of pre-registration testing (Swanson and Peterson, 1988). These studies are extremely time consuming and require large growth rooms or permission to conduct field trials. The field studies could be unpredictable in their toxic effects on other organisms within the ecosystem. Jones and Estes (1984) have explored the use of detached plant leaves for aquatic toxicity tests. However, since photosynthesis is more readily reduced in separated leaves than in leaves on intact plants, this approach does not seem adequate. Other laboratory studies (Forney and Davis, 1981; Fowler, 1977; Jones and Winchell, 1984) require a lot of laboratory space and are time consuming. Aquatic macrophytes are notably hard to culture in the laboratory (Fowler, 1977) and special light and temperature regimes are often required. Since it is difficult for any toxicity test with whole, rooted aquatic macrophytes to meet many of these criteria, this toxicity test was developed using an axenic tissue culture of macrophytes to determine non-target effects.

The data presented in this literature review suggest that toxicity testing can be species dependent and that several types of toxicity tests with species from different taxonomic classifications should be employed in order to correctly assess the possible environmental impact. The lack of a standardised toxicity test with a submersed aquatic macrophyte is also evident.

### **3. OBJECTIVES**

There were two main objectives to this research project:

- i) to develop a laboratory toxicity test for a non-target submersed macrophyte that could be used to detect the effects of phytotoxic compounds (Section 4);
- ii) to use pesticides and reference toxicants to validate the effectiveness of this submersed macrophyte toxicity test to compare its sensitivity with existing aquatic plant toxicity tests (Section 5).

The first objective was subdivided into:

- i) selection of an appropriate non-target rooted aquatic macrophyte (Section 4.1);
- ii) to develop numerous endpoint parameters that were effective at detecting minor changes in the growth and development of the aquatic plant species in the laboratory (Section 4.3.2);
- iii) to develop an appropriate laboratory culturing technique;
  - a) to determine sterilisation procedure for field collected plants (Section 4.3.1);
  - b) to determine the best laboratory environmental conditions (Section 4.3.3.1);
  - c) to compare rooting substrates (Section 4.3.3.2);
  - d) to perfect a liquid growth medium for culturing the aquatic macrophyte in the laboratory (Section 4.3.4); including:
    - i) to select an appropriate carbon source (Section 4.3.5);
    - ii) to determine if the presence of a buffer improved growth (Section 4.3.6);
    - iii) to chose the best pH for maximum growth (Section 4.3.7);
    - iv) to determine the best level of iron chelator in the liquid medium (Section 4.3.8);
  - e) to determine the best length of time for the toxicity test so that changes in plant growth could be detected (Section 4.4).

Once the toxicity testing methods were perfected, the methods were summarised (Section 4.6), and were submitted to the American Society for Testing and Materials (ASTM) for inclusion in their Annual Book of ASTM Standards (Appendix 8.6).

The axenic toxicity test for *Myriophyllum sibiricum* is currently being reviewed by the main committee of ASTM (see p. 415 for more details on the ASTM balloting procedure).

Validation of the toxicity test (second objective) involved:

- i) determination of whether formulated or technical compounds should be used in the toxicity test (Section 5.3);
- ii) to determine if there was an interaction between pesticides and the chelator used in the liquid growth medium (Section 5.4);
- iii) to determine the least toxic solvent to use for herbicides with a low water solubility (Section 5.5);
- iv) to compare new statistical methods for analysis of continuous statistical data with the existing statistical guidelines to ensure that the most appropriate statistical method was used in analysing the toxicity data (Section 5.6);
- v) pesticide validation was conducted using numerous herbicides with different modes of phytotoxic action (Section 5.7) and for each herbicide, the toxicity was compared with the data in the scientific literature for the other aquatic plant toxicity tests;
- vi) validation of the effectiveness of the toxicity test was conducted using both an organic and an inorganic reference toxicant compound (Section 5.8). The sensitivity of the submersed aquatic plant toxicity test was compared with the sensitivity of the other published aquatic plant toxicity tests.

## 4. DEVELOPMENT OF THE *MYRIOPHYLLUM SIBIRICUM* TOXICITY TEST

### 4.1 SPECIES SELECTION

The *Myriophyllum* genus belongs to the family Haloragaceae and contains 39 (Cook, 1985) or 40 species (Sutton, 1985) with a world-wide distribution. Ten species are native to North America. Taxonomy within this dicotyledonous genus can be difficult because of phenotypic plasticity. Most of the species differences are based upon flower or fruit characteristics (Cook, 1985; Sutton, 1985). The unisexual flowers are produced above the water surface and are wind pollinated (Cook, 1985). Of the species found in North America, *M. spicatum* (Eurasian watermilfoil), *M. sibiricum* (northern watermilfoil) and *M. verticillatum* (whorled watermilfoil) have often been confused taxonomically and some authors have suggested that they are varieties or subspecies of the same species. Based upon morphological and distributional patterns, and physiological differences, they should be separate taxa (Couch and Nelson, 1985; Löve, 1961; Nichols, 1984). Morphological differences between these species include the shape and length of the floral bracts, the shape and size of the bracteoles, the number of pairs of leaf divisions (Gerber and Les, 1994; Löve, 1961; Nichols, 1984; Patten, 1954; Sculthorpe, 1971) and the dried stem colour (Löve, 1961; Patten, 1954). Morphological plasticity is common and has been demonstrated in *M. spicatum* (Eurasian watermilfoil) and *M. sibiricum* (northern watermilfoil). When grown on a nutrient-poor substrate, the leaves of *M. spicatum* converged in appearance towards the leaves of *M. sibiricum* grown on nutrient-rich substrate (Aiken and Picard, 1980, Ceska and Ceska, 1985). *M. sibiricum* and *M. verticillatum* are geographically distributed at northern latitudes where there is a 0 °C January isotherm because they require cold weather for successful turion formation (Couch and Nelson, 1985). Leaf morphology and turion size and shape in these two species are different (Aiken and Walz, 1979; Gerber and Les, 1994; Weber and Nooden, 1974). The slender, cylindrical turions of *M. sibiricum* (northern watermilfoil) are covered by leaves much smaller than normal leaves (Weber, 1972a) while the turions produced by *M. verticillatum* (whorled watermilfoil) are club-shaped and enclosed by leaves similar to normal leaves (Weber and Nooden, 1974). Turions of *M. verticillatum* are released after the production of an abscission layer, whereas, *M. sibiricum* turions lack this layer and remain attached to the plant until it decays (Weber, 1972a; Aiken and Walz, 1979). Food reserve utilisation in the autumn differs between

these species (Aiken and Walz, 1979). *M. spicatum* (Eurasian watermilfoil) does not produce turions (Couch and Nelson, 1985; Löve, 1961; Patten, 1954) and thus overwinters as a root crown, mobilising reserves downward but the turion producing species mobilise their reserves into the turion (Aiken and Walz, 1979). These species can also be distinguished by their flavonoid patterns. All the chromatograms of *M. sibiricum* (northern watermilfoil) showed two different anthocyanins and the chromatograms were variable emphasising that this species is abundant and polymorphic. Chromatograms of *M. spicatum* showed very little variation suggesting a common origin of introduction into North America from Europe (Ceska and Ceska, 1985).

In 1919, Fernald (1919) described the North American taxon, *M. exalbescens*. It has been shown that *M. exalbescens* is the same species as *M. sibiricum*, which was named in 1914 by Komarov. Therefore, Komarov's name, *M. sibiricum* Komarov, has priority (Aiken and Cronquist, 1988; Ceska and Ceska, 1986; Ricketson, 1989). *M. sibiricum* is a circumpolar species that has been found in Siberia, Kamchatka, Scandinavia and North America (Ceska and Ceska, 1985). Dolgovskaya *et al.* (1994) are in the process of delineating the *M. sibiricum* range in the former Soviet Union. *M. sibiricum* is the most common *Myriophyllum* species in British Columbia. This species is characterised by its entire or shallowly cut floral bracts that are shorter than the fruits, whitish stems, leaves with up to 12 pairs of segments and the formation of turions (Ceska and Ceska, 1985). Lowering temperatures in the autumn initiates the formation of turions on lateral branches. The leaf segments on the turions are fewer, shorter and thicker than normal leaves and are very rich in starch (Arber, 1963; Sculthorpe, 1971). An upward translocation of food reserves occurs and the turion becomes heavier and sinks to the sediment (Aiken and Walz, 1979). Bud activation and turion growth normally occurs in March or April but can be induced by warm temperature. The dwarf leaves spread away from the axis and the apical bud develops into an erect shoot and adventitious roots form from the basal nodes of the turion (Arber, 1963; Sculthorpe, 1971) producing a U-shaped base to the new plant (Aiken and Walz, 1979).

*M. sibiricum* has a broad ecological range since it can be found in eutrophic waters, marl lakes, slightly alkaline lakes and brackish water (Ceska and Ceska, 1985). It is ecologically important since it provides food and shelter for other organisms (Fink, 1994). The seeds and foliage provide 0.5 to 5% of waterfowl diet while the seeds are

0.5 - 2% of the diet of marsh and shore birds (Martin *et al.*, 1951). Martin and Uhler (1951) grouped *M. spicatum* and *M. exalbescens* together when they examined the types of aquatic vegetation utilised as food by game ducks. Together, they were the most widely eaten of the *Myriophyllum* species. *Myriophyllum* species are also important in nutrient cycling and reducing the erosional impact of wind and wave action (Sutton, 1985).

As previously discussed (Section 2.4), the aquatic macrophyte species selected for use in the toxicity test should be sensitive to toxicants, ecologically important with a considerable geographic distribution and abundance, available commercially, well characterised taxonomically and grow rapidly under laboratory conditions using well defined nutrient media. Another beneficial criterion is a growth habit that lends itself to easy measurement, such as dry weight, leaf whorl counts or plant height (Swanson, 1989; Martin, 1973). The organism(s) selected should have the potential to be exposed to the substance under examination (Martin, 1973). *Myriophyllum sibiricum* fulfils these requirements.

#### 4.2 COLLECTION AND MAINTENANCE OF STOCK PLANTS

Numerous *M. sibiricum* turions (Figure 1) were collected from Puslinch Lake (15 km southwest of Guelph, Ontario, just south of Hwy. 401) on November 2 and 25, 1990. The turions were collected from the shallows and stored in water filled plastic bags for transport to the University of Guelph laboratory. The turions were washed in distilled water and stored in the dark at 4 °C until needed.

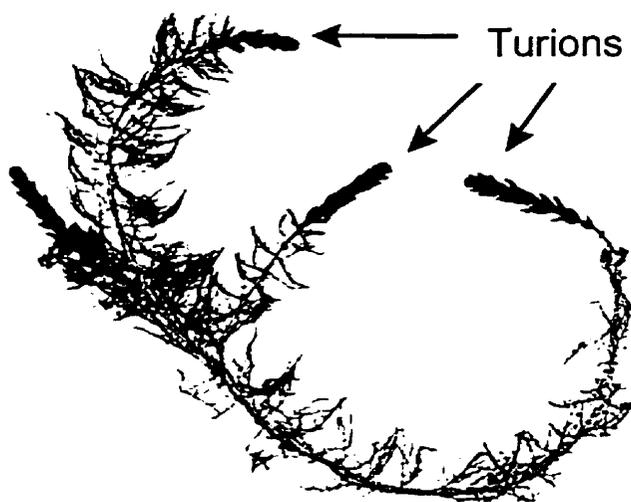


Figure 1: Cylindrical shaped turions of *Myriophyllum sibiricum* collected from Puslinch Lake, Ontario.

Twenty L aquaria were established containing 5 cm of sediment and 18-L of distilled water. Ten *M. sibiricum* turions were placed in the sediment on December 6, 1990. This aquarium was constantly aerated and maintained at a temperature of 15 °C to ensure a constant supply of actively growing individuals.

The *M. sibiricum* used in this study was identified following the taxonomic key in Gleason and Cronquist (1991). Voucher specimens are stored in the University of Guelph Herbarium (Access Number 77543).

### 4.3 TOXICITY TEST DEVELOPMENT

#### 4.3.1 Axenic Culturing Technique

##### 4.3.1.1 Introduction

It is a common practice to use an axenic plant culture when testing the direct effects of toxicants upon a plant species. Axenic plant toxicity tests have been conducted with algae (ASTM, 1993; Christy *et al.*, 1981; Day and Hodge, 1996; Environment Canada, 1992; Miller *et al.*, 1978), floating aquatic macrophytes (ASTM, 1991b; Cowgill and Milazzo, 1987; Day and Hodge, 1996; Greenberg *et al.*, 1992) and submersed aquatic plant species (Bird, 1993; Christopher and Bird, 1992; Fleming *et al.*, 1991; Hinman and Klaine, 1992; Selim *et al.*, 1989). An axenic testing system is designed to determine the direct effect of the test material upon the test species. There is nothing except the plant within the test system that could degrade or otherwise change the test chemical. This type of test is especially valuable during the initial stages of examining a new compound (e.g., pesticide evaluation and registration (Tier 1 and Tier 2)) (Boutin *et al.*, 1993; 1995).

Aquarium culturing techniques can have problems including algal, bacterial and aphid infestations. Aphid infestation can be alleviated by placing a weighted Nytex™ screen, or equivalent, on top of the submersed plants for 24 to 48 hours (personal communication with M. Nelson<sup>6</sup>). Algal problems can be controlled by regular cleaning of the aquaria system or use of algicides. In microcosms, it was recommended that testing be terminated after 20 to 30 days due to algal colonisation of the microcosm walls. At this point, the untreated microcosms were no longer similar to the ecosystems they were emulating (Cragg and Fry, 1984). Bacterial contamination of aquaria culture can decrease the nutrient availability (Hoffmann *et al.*, 1984). In these systems, there is no way to predict how the microorganism or insect infestations will interact with the nutrient solution, test chemical or test species. Even researchers who have used aquarium culture have mentioned the benefits of utilising an axenic system for physiological studies (Hoffmann *et al.*, 1984). For these reasons, it was decided to use

axenic plants for testing. This would ensure that any change in plant vigour was the direct effect of the toxicant upon the test species. This type of information may be of value for pesticide regulators at the Tier 1 stage and for risk assessment evaluators.

Filamentous algae can have a deleterious effect upon the growth of submersed macrophytes as was observed in the study by Ozimek *et al.* (1991). A decrease in new shoot growth, fresh weight and chlorophyll *a* production was observed in *Elodea canadensis* and *Potamogeton pectinatus* when they were grown in the presence of the filamentous algae (*Cladophora glomerata* (L.) Kütz.) as compared to macrophytes grown in the absence of algae. The algae affected the macrophytes regardless of the culture conditions, which included floating in water versus rooted in sediment, high versus low nutrient condition and a 20 or 30 day growth period (Ozimek *et al.*, 1991). Bacteria damaged the epidermal cell wall, invaded the tissues of *P. crispus* leaves (Rogers and Breen, 1981) and caused cuticular erosion and epidermal pitting in *P. pectinatus* leaves (Howard-Williams *et al.*, 1978). *Lemna minor* plants inoculated with a natural population of bacteria showed a higher level of senescence than axenic populations (Underwood and Baker, 1991). Fungal populations on *Myriophyllum spicatum* caused a reduction in yield and death of a few epidermal cells (Smith *et al.*, 1989).

Stem fragments of *Myriophyllum* species have the natural ability to regenerate shoots and roots under appropriate growing conditions (Sutton, 1985). Based upon the above points, it was decided that it would be beneficial to culture *Myriophyllum sibiricum* axenically.

#### 4.3.1.2 Methods

Different concentrations of a sodium hypochlorite (NaOCl) and Tween® 20 solution were tested for their ability to surface sterilise *Myriophyllum* tissue over different time periods. The NaOCl concentrations were varied at 1, 2, and 3% (w/v) while the concentration of Tween® 20, a surfactant, was kept constant at 0.01%. The immersion time of the plant tissue in the disinfectant solution varied from 5, 10, 15, 20, 25 and 45 minutes. The plant tissue, submerged in the disinfectant, was gently agitated for the appropriate time period. The plant segments were placed into glass culture tubes (100 x 25 mm) containing 80 mL of Murashige and Skoog medium (Murashige and Skoog,

1962) and incubated at 25 °C and 16 hours of light for several days to weeks until contamination was observed or the plant was considered axenic.

Axenic conditions were confirmed by culturing medium and plant tissue on separate agar plates. Both trypticase soy agar (TSA) and potato dextrose agar (PDA) plates at concentrations of 1, 2, 5, 10, 20, 50 and 100% strength were used to check for the presence of microorganisms.

Throughout the time that this *Myriophyllum* toxicity test was being developed and evaluated, all stock and experimental plants were monitored for fungal and bacterial contamination.

#### 4.3.1.3 Results and Discussion

When the sterilisation process was unsuccessful, contamination by algae, fungi or bacteria was evident. Leaving the plant segments in the disinfectant solution for too long a time period (e.g. 25 or 45 min) resulted in plant death or slow recovery from the adverse effects of the disinfectant. Low concentrations of NaOCl (1 and 2%) and short time periods (5, 10 or 15 min) did not successfully produce axenic plants. A 3% NaOCl solution with 0.01% Tween® 20 for 20 min was selected as the most reliable method of producing axenic *Myriophyllum* segments. For other *Myriophyllum* species, a lower concentration of 1.05% (v/v) NaOCl and a shorter sterilization time of 12 minutes was successful (Kane and Albert, 1987; 1989a; 1989b; Kane and Gilman, 1991; Kane *et al.*, 1991). A longer sterilisation time (30 min) and a stronger NaOCl solution (5.25%) were required to effectively produce axenic *Potamogeton pectinatus* tubers (Madsen, 1985).

All concentrations of TSA and PDA were successful at detecting contamination but full strength TSA was used in all subsequent experiments.

Maintaining an axenic culture is the most challenging aspect of this toxicity testing procedure. After an axenic culture had been established (August 1991), random contamination was observed. The contamination that was observed over the course of this research is graphically depicted in Figure 2 for stock plant transfers and Figure 3 for experimental plant transfers. Contamination was usually evident within two to ten days after transferring the plants. In both stock plant cultures and plants for experimental use, contamination was occasionally observed as a cloudy appearance in the liquid medium (bacterial contamination) or a conglomeration of fungal hyphae at the

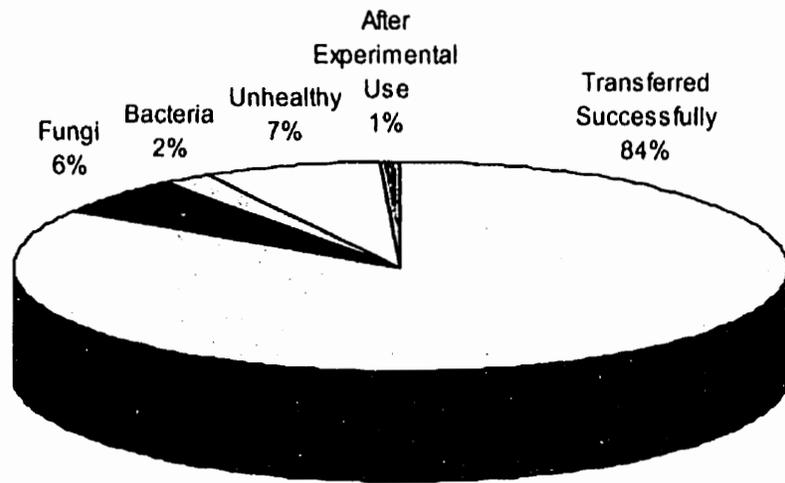
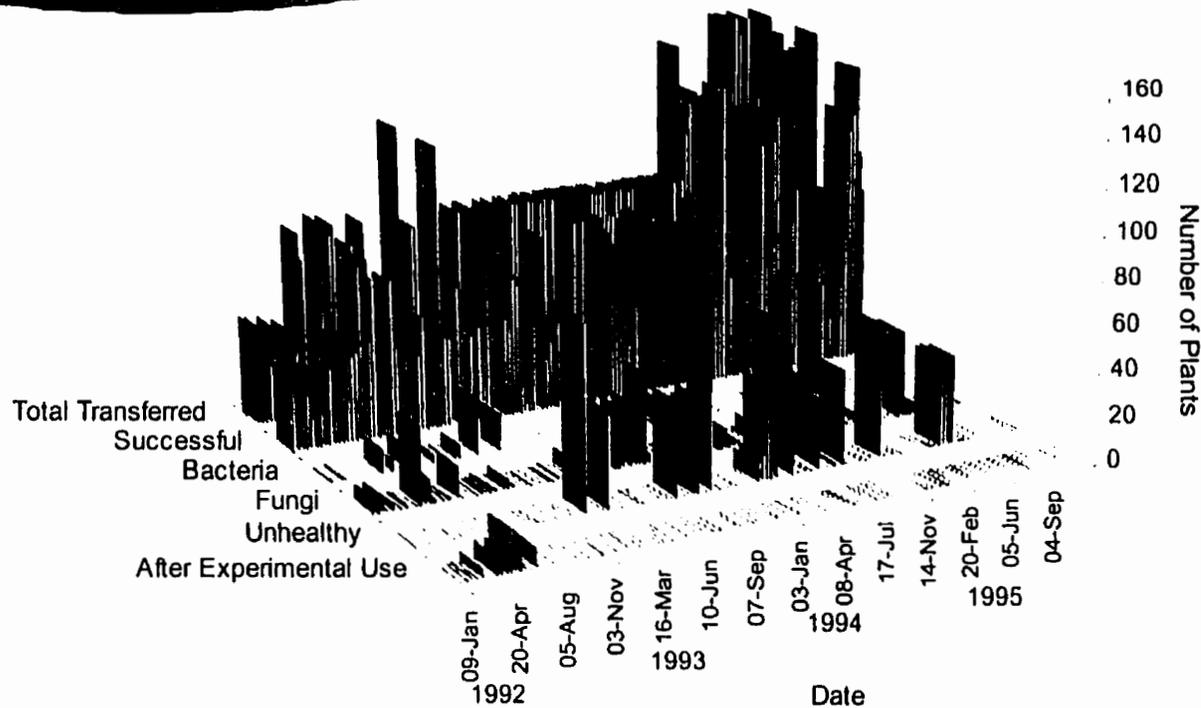


Figure 2: The contamination observed in *Myriophyllum sibiricum* plants that were transferred for use as stock plants. The pie graph demonstrates the percent of each type of contamination based on all the plants transferred. The bar graph breaks down the contamination based upon the date the plants were transferred into fresh medium. Plants were discarded based upon contamination by bacteria or fungi. Originally, plants were placed back into the test tubes but this usually resulted in contamination. The plants that were classified as unhealthy showed signs of epinasty and stem disfiguration.



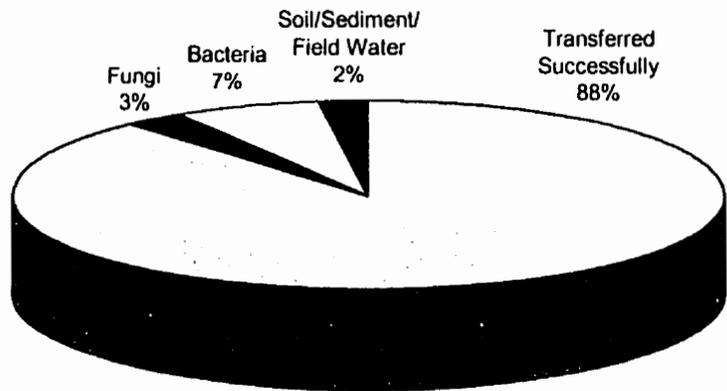
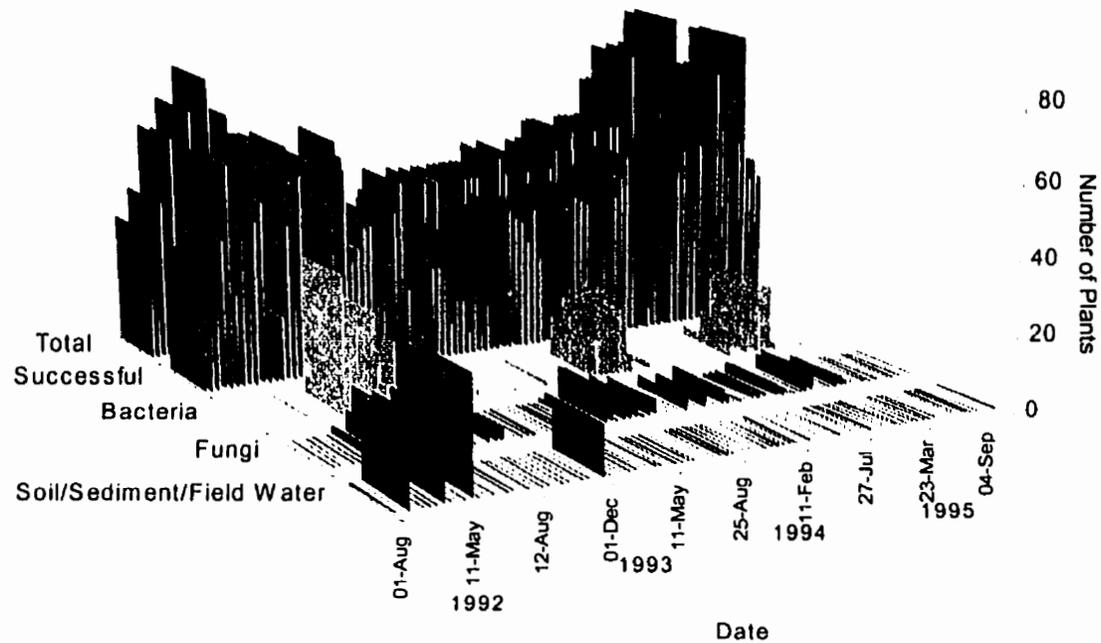


Figure 3: Graphical representation of the contamination observed in experimental *Myriophyllum sibiricum* plants. The pie graph demonstrates the percent of each type of contamination based on all the plants transferred. The bar graph breaks down the contamination by date of experiment initiation. Plants were discarded based upon contamination by bacteria or fungi. Some of the contamination was caused by sediment or field collected water that was improperly sterilised.



medium/air interface, floating in the medium or adhering to the *Myriophyllum* leaf surface (fungal contamination). One of the bacterial contaminants was identified as *Pseudomonas maltophilia*<sup>10</sup> and the two most common fungal contaminants were *Penicillium* spp. and *Fusarium* spp.<sup>11</sup>. Initially, if the stock plant that was being segmented contained small, healthy buds, the portion of the plant that was not transferred into fresh medium was placed back into the original tube and medium. This practise commonly resulted in contamination of the old plant segment and was discontinued as of June 8, 1992. Other plants were classified as unhealthy if they demonstrated epinasty including stem disfiguration. See Section 4.5.2 for a discussion of possible causes of this epinasty. A total of 10 561 stock plants were transferred on 140 occasions. Some type of contamination was observed on 89 occasions and 1 702 plants were discarded (Figure 2). Experimental plants were transferred 94 times for a total of 3 994 plants. Bacterial/fungal contamination was found on 38 occasions and 471 plants were discarded (Figure 3). In addition to contamination from fungi and bacteria, plants were discarded from experiments based on contamination from incorrectly sterilised soil, sediment (See Section 4.3.3.2.1) or field water (plant exposure data not included in this document). It is also evident in Figure 2 and Figure 3 (3-D graphs) that there is a seasonal pattern to the contamination. Fungal and bacterial contamination of both the experimental and stock plants peaked in the spring/summer months. This could have been caused by increased environmental bacterial and fungal spores or by inexperienced student assistants not properly trained in aseptic technique. To prevent or limit the amount of contamination, the laminar flow hood should be cleaned on a regular basis (every six months) and all personnel should be properly trained in aseptic technique (See Section 4.6.9).

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<sup>10</sup> Identified by S. Fessenden, Department of Microbiology, University of Guelph, Guelph, ON, N1G 2W1. (519) 824-4120.

<sup>11</sup> Identified by E. Szijarto and Dr. G. Barron, Department of Environmental Biology, University of Guelph, Guelph, ON, N1G 2W1. (519) 824-4120.

### 4.3.2 Endpoint Selection

The following section describes the endpoints that were examined for use with the *Myriophyllum sibiricum* axenic toxicity test. There are numerous possible physiological and morphological endpoints that could be utilised to assess the toxicity of chemicals to this aquatic plant species. The phytotoxic effects of the test material were assessed on increase in plant height, growth rate, fresh or dry weight, number and total length of roots, chlorophyll *a*, chlorophyll *b*, carotenoids, membrane integrity and oxygen evolution. Peroxidase activity or chlorophyll fluorescence might be other endpoints that could be explored. Some of the endpoints, such as length and weight, required very little examination, while other endpoints, such as pigment content, required detailed study and explanation.

#### 4.3.2.1 Shoot Height, Root Number and Length

##### 4.3.2.1.1 Introduction

Plant height has been commonly measured in aquatic plant studies (Barko *et al.*, 1982; Nielsen and Sand-Jensen, 1991). Plant height of *Myriophyllum spicatum* and *Hydrilla verticillata* was found to be a more sensitive indicator of gibberellin synthesis inhibition than physiological parameters, such as photosynthesis, respiration and chlorophyll content (Netherland and Lembi, 1992).

It has been demonstrated that the roots of *Vallisneria americana* contain high concentrations of organic pollutants, which suggests that the toxicants are absorbed through the roots rather than through the shoots (Lovett-Doust *et al.*, 1994a). Thus, the development of roots in a macrophyte toxicity test could be an important endpoint. Stimulation of lateral shoot and root formation has been observed in *Myriophyllum sibiricum* and *Hydrilla verticillata* (Netherland and Lembi, 1992).

There is a debate regarding the function of aquatic plant roots. One school of thought maintains that the roots absorb a major portion of the plants' nutrients from the sediments (Bristow, 1975; Bristow and Whitcombe, 1971; Carignan and Kalff, 1980; Chambers *et al.*, 1989; Mantai and Newton, 1982) while the other theory suggests that the leaves and shoots absorb most of the required nutrients from the water column (Sutton, 1985). In a study of six species of aquatic plants, Denny (1972) determined that both roots and shoots adsorbed nutrients and that the proportions were species specific. For nutrient adsorption, shoots were more important for *Ceratophyllum*

*demersum* and *Potamogeton schweinfurthii* A. Benn., while roots were more important in *P. thunbergii* Cham & Schlecht. (Denny, 1972). *Myriophyllum brasiliense* Cambess. (parrotfeather) plants derived 90%, *M. spicatum* var. *exalbescens* plants adsorbed 59% and *Elodea densa* (Planch.) Caspary derived 74% of their phosphate via the roots (Bristow and Whitcombe, 1971). Roots of some aquatic plant species, including those of *Myriophyllum*, are spirally twisted, which is advantageous for anchoring the plant in the sediment in flowing water (Arber, 1963; Bristow, 1975). In *Myriophyllum aquaticum* (Vell.) Verdc. (parrotfeather), root hairs have been found on roots that grow above the water but not on submerged ones (Sutton, 1985). Within one week, roots formed at the nodes on 12 cm long *M. brasiliense* apical cuttings (Sutton *et al.*, 1969). The roots of *Elodea nuttallii* (Planch.) St. John absorbed micronutrients (Marquenie-van der Werff and Ernst, 1979). The roots and rhizomes of aquatic plants account for a large portion of the plant's biomass (Bristow and Whitcombe, 1971; Westlake, 1982). In submersed aquatic plants that absorb their CO<sub>2</sub> from the sediment, the root surface area is sometimes greater than the leaf surface area (Bowes, 1985). Other aquatic plants do not require extensive transport tissues (e.g., xylem and phloem) and root systems because they are surrounded by nutrients (Bowes, 1985; Bristow, 1975).

#### 4.3.2.1.2 Methods

To ensure that the growth of the *Myriophyllum sibiricum* plants in axenic culture was due to mitosis and not just cellular expansion, a preliminary experiment was conducted. The number of visible nodes and the number of nodes hidden in the apex were determined on 10 sacrificial 3 cm apical segments. Another set of 10 apical segments was placed into modified Andrews' medium in test tubes (2.5 x 15 cm), covered with clear plastic test tube closures and allowed to incubate for fourteen days. Refer to Table 17 and Table 26 for the chemical composition of modified Andrews' medium. Plant height and node number were measured during the incubation period. At the end of the fourteen days, the plants were harvested and plant height, number of visible and hidden nodes, and root number and length were determined. The number of visible nodes, number of hidden nodes and the total node number from the initial plants and from plants that had grown for 14 days were compared statistically using the Mann-Whitney U-test for nonparametric parameters.

For all subsequent experiments,  $3 \pm 0.2$  cm apical segments were used to start each experiment. At the end of the appropriate incubation period, the plant was removed from the liquid medium and placed on a paper towel. Working with only one plant at a time, callipers were used to measure the shoot height from the base of the cut stem to the tip of the apex. Any leaves extending above the top of the apex were not included in this measurement. The initial height measurement from Day 0 was subtracted from this final height measurement. The total number of roots per plants was counted and each root was individually stretched out and measured with the callipers. For each plant, the lengths of the roots were summed.

#### 4.3.2.1.3 Results and Discussion

*Myriophyllum sibiricum* in axenic culture grew by both cellular expansion and division. The initial 3 cm apical segments contained  $9.0 \pm 0.9$  visible nodes and  $4.7 \pm 0.5$  nodes hidden in the apical bud. At the end of the 14 day growing period, the plants increased in height by  $48.1 \text{ cm} \pm 5.3 \text{ cm}$ , which consisted of  $23.1 \pm 1.4$  visible nodes and  $6.7 \pm 1.0$  nodes hidden in the apex. The number of visible nodes, hidden nodes and the total number of nodes produced during the 14 day growth period was statistically greater than the numbers in the 3 cm segment. The 14 day old plants produced  $5.3 \pm 0.9$  roots with a total length of  $267.1 \pm 54.2$  mm.

Occasionally, the spiral roots were difficult to straighten. In general, the number of roots, total root length and plant length were useful and reliable endpoint parameters for the *M. sibiricum* toxicity test as demonstrated in the pesticide toxicity tests (Section 5.7). These three parameters were capable of detecting both stimulation and inhibition by toxicants. Christopher and Bird (1992) decided that root number was not a good measure of toxicity because only a few roots were produced during their five day tissue culture bioassay with *M. spicatum*.

Guilizzoni *et al.* (1984) found that *Myriophyllum spicatum* control plants more than double their initial length of 10 cm during the 32 day incubation period. The *M. sibiricum* apices cultured in this toxicity test almost tripled their initial length during the 14 day experimental period. Hoffmann *et al.* (1984) used 6 cm lateral and apical segments of *M. spicatum*, which after 35 days of growth in an aquarium culturing system had grown to an average length of  $65 \pm 5$  cm.

### 4.3.2.2 Growth Curve

#### 4.3.2.2.1 Introduction

It is common in botanical studies to plot morphological parameters, such as size, height or weight of an organism against time to produce a growth curve. The growth curve may be fitted with an analytical equation (Morris and Silk, 1992; Salisbury and Ross, 1985) or the area under the growth curve may be determined (ASTM, 1990; Blanck and Björnsäter, 1988; Boutin *et al.* 1993). Area under the growth curve is commonly determined in algal toxicity studies (Adams *et al.*, 1986). Growth rate has also been determined by subtracting the final value from the initial value and dividing this number by the length of time (Blanck and Björnsäter, 1988; Denny, 1972). Growth rate is commonly determined in chronic bioassays (Martin, 1973). Differences in growth rate may reflect differences in photosynthetic efficiency (Madsen *et al.*, 1993).

Determination of area under the growth curve was recommended as an endpoint for toxicity tests with microalgae (ASTM, 1990; Blanck and Björnsäter, 1988). In algal studies, it has been observed that there can be appreciable variation between the EC50 values for biomass and growth rate. Nyholm (1985) statistically compared the EC50 values and concluded that growth rate was theoretically superior and exhibited less variation between and within tests than biomass. Area under the growth curve was a sensitive parameter in *Selenastrum capricornutum* toxicity studies (Adams *et al.*, 1985; 1986).

An important advantage to the test tube system described in this thesis is that the measuring rod allows for measurement of shoot growth during the 14 day toxicity testing period. It has been observed that this contributes valuable information to the evaluation of toxicity. There are seven types of growth curves possible (Figure 4). Curve 1 shows the expected growth of control plants. The test material can have an immediate toxic effect that does not change over time (Curve 2). The test material may not inhibit growth but may or may not affect the other parameters examined (Curve 3). The treated plants may grow at a reduced rate but otherwise be unaffected (Curve 4). In some cases, the test chemical may appear to be initially toxic but *Myriophyllum* might metabolise the test chemical and the toxic effect is reduced (Curve 5). When there is recovery, the final plant height may not be significantly different from the control plant final height, which emphasises the importance of measuring plant growth during the 14

days. The test chemical can have a delayed toxic reaction wherein toxicity is not displayed until several days after test initiation (Curve 6). The last scenario is that plant height may be stimulated but there could be an inhibitory effect on weight or one of the other endpoint parameters (Curve 7). This type of data can be important in examining chemical metabolism or possible plant recovery from the effects of the test chemical. This type of data is not readily attained from the beaker or aquarium testing systems that have been proposed to determine toxicant effects upon other macrophyte species.

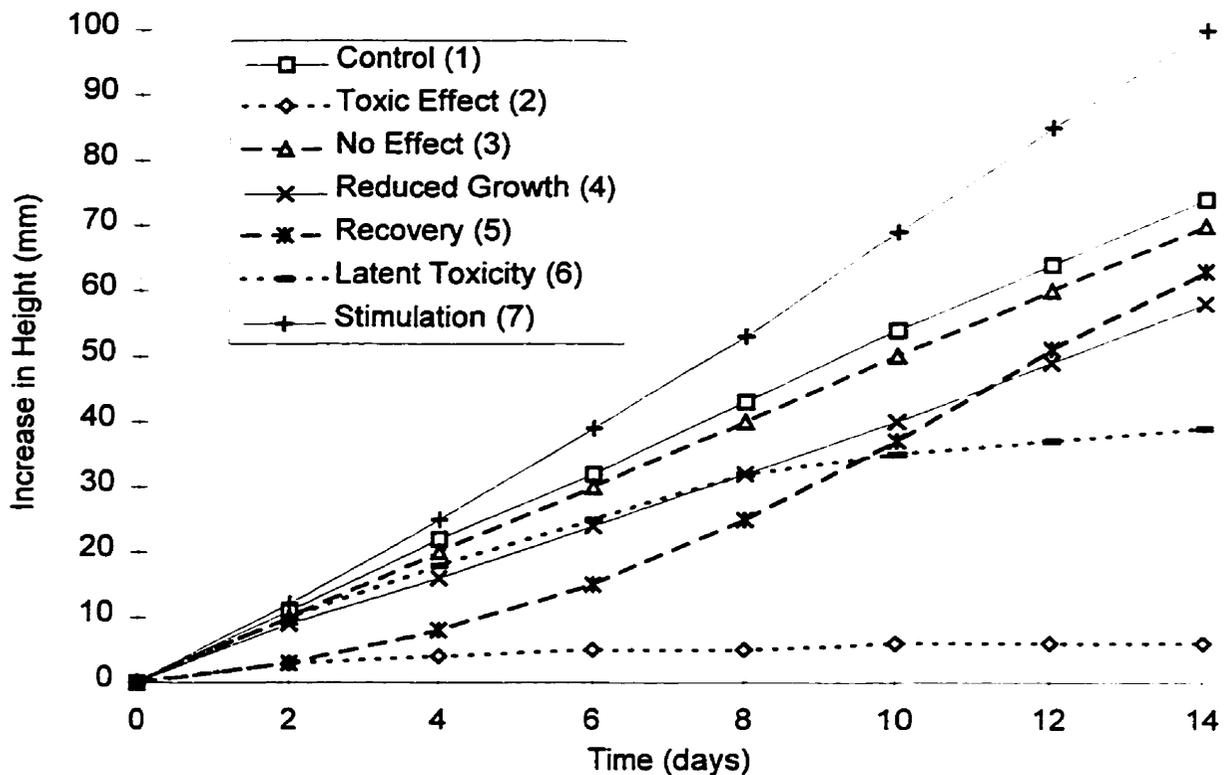


Figure 4: Hypothetical growth curves that may obtained during a *Myriophyllum sibiricum* toxicity test when the plants are exposed for 14 days to toxicants with different modes of action.

#### 4.3.2.2.2 Methods

Once a measuring rod system was developed, area under the growth curve was determined for all the *Myriophyllum sibiricum* toxicity tests. The glass measuring rod consisted of a 15 cm length of Westergren Blood Sedimentation Tube that was marked in mm. Once the 3 cm apical *Myriophyllum sibiricum* segment was placed into the liquid growth medium in the test tube (2.5 x 15 cm), the measuring rod was inserted into each

experimental test tube. The bottom end was pushed into the 3 g of Turface<sup>®12</sup> and the upper end was inserted into a 3.5 cm section of Tygon<sup>®</sup> tube (I.D. = 7 mm, O.D. = 10 mm) attached to the clear plastic test tube closure (I.D. = 25 mm, 38 mm in height). Initial plant height was measured to the nearest mm using the Westergren rod. The plants were incubated for fourteen days during which time the plant height was visually measured every second day with the assistance of the measuring rod.

Growth curves were plotted using the plant length data and the area under the growth curve was determined by:

$$Area = \sum_{i=2}^n \frac{IH_{i-1} + IH_i}{2} \cdot (T_i - T_{i-1}) ; n = 8 \quad (1)$$

where IH is the increase in height from the start of the experiment and T is the time at each subsequent measurement point, in hours from time zero (Boutin *et al.*, 1993).

#### 4.3.2.2.3 Discussion

Area under the growth curve was a repeatable and predictive endpoint parameter in the *Myriophyllum sibiricum* toxicity test. It was able to show toxicant stimulation and inhibition. Refer to the pesticide chapters (Section 5.7) for evidence of the value of this endpoint.

The benefit of utilising growth curves for macrophyte studies was demonstrated in the research conducted by Guilizzoni *et al.* (1984). They determined the effect of chromium on the growth of *Myriophyllum spicatum*. When shoot length and estimated shoot dry weight were graphed versus time, important information was added to their study. As compared to the control data, these curves showed slight height and weight stimulation by low Cr concentrations for 17 days, after which the control plants surpassed those in the 100 and 200 µg/L Cr concentrations. These concentrations produced growth curve lines similar to the hypothetical latent toxicity curve (Curve 6; Figure 4).

Hutber *et al.* (1979) identified three types of growth curves in their algal toxicity studies: algal growth at the same rate as the control for several hours followed by

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<sup>12</sup> Turface<sup>®</sup> was obtained from Aimcor, Applied Industrial Materials Corporation, 750 Lake Cook Road, Buffalo Grove, IL, 60089. 1-800-654-8793.

inhibition (Curve 6, Figure 4), complete inhibition from the time of pesticide addition (Curve 2, Figure 4) and growth at a rate lower than the control (Curve 4, Figure 4).

#### 4.3.2.3 Primary Productivity

##### 4.3.2.3.1 Introduction

Another common parameter in aquatic plant studies is the rate of primary productivity determined by measuring changes in dissolved oxygen (Grobbelaar *et al.*, 1992; Madsen *et al.*, 1991; Markager *et al.*, 1992). For example, Madsen *et al.* (1991) measured the short term oxygen production rates of seven aquatic macrophyte species. In plants, oxygen is produced during photosynthesis and utilised during respiration (Raven *et al.*, 1986; Salisbury and Ross, 1985). Oxygen production and carbon dioxide fixation normally follow a sinusoidal curve (Salisbury and Ross, 1985) with a circadian, diurnal or diel periodicity (Prézelin, 1992; Grobbelaar *et al.*, 1992). These processes are influenced by light (Grobbelaar *et al.*, 1992; Madsen *et al.*, 1991; Moeller, 1978; Salisbury and Ross, 1985). The success of photosynthesis can also be measured by the rate of carbon assimilation (Keeley and Sandquist, 1991; Moeller, 1978) or chlorophyll abundance (Kroon *et al.*, 1992; Markager *et al.*, 1992).

##### 4.3.2.3.2 Methods

In order to determine the amount of primary productivity occurring in the axenic *Myriophyllum* toxicity test, dissolved oxygen was measured during a 24 hour period on both healthy and unhealthy plants. *Myriophyllum sibiricum* plants were experimentally cultured for 14 days in full strength modified Andrews' medium with a 16 hour light period and an 8 hour dark period. For the healthy plants, 30 g/L of sucrose was added to the medium prior to autoclaving and for the unhealthy plants, no additional carbon was added to the growth medium. The amount of oxygen (%) in the liquid medium was measured with a Corning® Portable Checkmate Dissolved Oxygen Meter on an hourly basis. The other endpoint parameters, such as total plant height, root number and length, fresh weight, chlorophyll content, carotenoid content and membrane integrity were measured on all the plants after the dissolved oxygen was measured. The dissolved oxygen concentration in the medium was plotted over time and the mathematical software Mathcad® was used to fit a sinusoidal curve to the data for amplitude and period.

#### 4.3.2.3.3 Results and Discussion

The Mathcad® equations and the individual graphs for healthy and unhealthy plants may be found in Appendix 8.3 (page 404). For healthy plants, the amount of

### DO vs. Time for Healthy and Unhealthy Plants

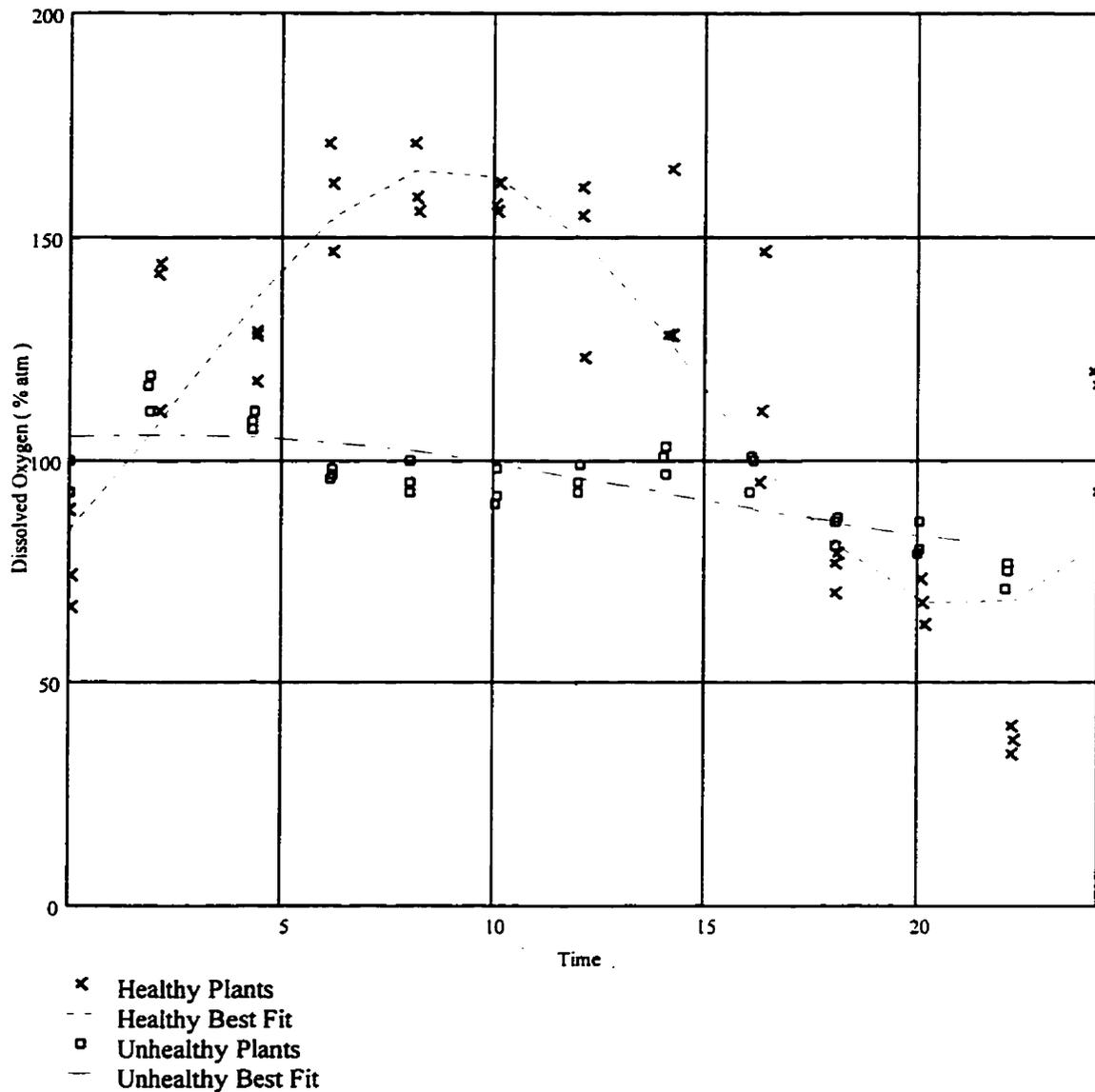


Figure 5: Percentage oxygen produced by *Myriophyllum sibiricum* plants cultured in modified Andrews' medium with 30 g/L sucrose (healthy plants) and plants cultured in modified Andrews' medium without sucrose (unhealthy plants). The plants were harvested over a 24 hour period during which time the plants were exposed to 16 hours of light and 8 hours of dark.

oxygen in the liquid medium fluctuated with a diurnal periodicity (Figure 5 and Figure 93 (Appendix 8.3.1)). When the light period started, the *M. sibiricum* plants started photosynthesising and reached a maximum dissolved oxygen concentration six hours into the light phase. Once the dark phase started, the plants utilised the oxygen in solution and the concentration decreased. The dissolved oxygen detected in the medium with the unhealthy plants fluctuated slightly (Figure 5 and Figure 94 (Appendix 8.3.2)).

The results from other endpoints are not presented because they were similar to those for healthy plants (with sucrose) and unhealthy plants (without sucrose) discussed in Section 4.3.5. Plant height, root number, root length and weight of the plants grown with sucrose were significantly greater than for the plants grown without sucrose. During the remaining toxicity tests, the photoperiod was regulated so that the light cycle would start at least four hours before harvesting the plants. To prevent equilibration with atmospheric oxygen, the dissolved oxygen concentration was measured immediately upon removing the laboratory sealant film from each test tube.

The increase in oxygen production during the day and decrease in oxygen in the surrounding medium during the dark phase has been observed in other studies (Markager *et al.*, 1992; Pahl-Wostl, 1992). In experiments with *Ruppia cirrhosa* (Petagna) Grande, oxygen concentration in solution increased to a maximum during the afternoon and decreased afterwards (Peñuelas and Menéndez, 1990). In closed flasks containing *Myriophyllum spicatum*, illumination induced oxygen supersaturation of the medium and the oxygen concentration decreased during the dark phase. Opening the flask during the light phase, resulted in oxygen concentration maintaining 100% saturation (Nalewajko and Godmaire, 1993). Even in the axenic bioassay system, *M. sibiricum* photosynthesises like other aquatic plants.

Light fluence rate can influence oxygen production by aquatic macrophytes (Madsen *et al.*, 1991) and algae (Grobbelaar *et al.*, 1992; Moeller, 1978) but this possible interaction was not examined in the *Myriophyllum sibiricum* axenic toxicity test.

The oxygen concentration in freshwater can range from near 0% to over 200% of air saturation (Bowes, 1985). Thus, the high levels of oxygen detected in the study with *Myriophyllum sibiricum* were not abnormal. Based on these results, dissolved oxygen was deemed an endpoint parameter that could detect environmental stress on *M. sibiricum* in this axenic toxicity test.

#### 4.3.2.4 Fresh and Dry Weight

##### 4.3.2.4.1 Introduction

Fresh and dry weight are parameters normally employed to estimate macrophyte biomass. Consistent fresh weight is hard to determine because water tends to adhere to the leaf surfaces and interleaf spaces. Suggested methods of removing the external water include using a domestic spin-drier, hanging the plants to dry (Westlake, 1974) or patting dry with paper towels (Byl and Klaine, 1991; Fairchild *et al.*, 1994b; Forsyth *et al.*, 1997; Madsen and Sand-Jensen, 1994). Occasionally, biomass production is determined. This measurement is determined by subtracting the initial fresh weight from the final weight, dividing this number by the initial fresh weight and multiplying the final product by one hundred percent (Fleming *et al.*, 1991). Biomass determination is impractical for axenic studies because an initial fresh weight of each axenic plant segment can not be obtained without risking contamination. Dry weight is determined by drying the plants at 105 °C for 24 hours (Westlake, 1974; Walsh *et al.*, 1991a) or 70 to 80 °C for 24 to 48 hours (Godmaire and Nalewajko, 1989; Guilizzoni *et al.*, 1984; Hoffmann *et al.*, 1984; Nalewajko and Godmaire, 1993; Salisbury and Ross, 1985) until a constant weight is reached. The dried plants will absorb up to 10% moisture from the atmosphere so they should be placed in a desiccator and allowed to cool to room temperature before being weighed (Westlake, 1974). Dry weight is an accurate and direct method of measuring toxicity to algal species (Lewis, 1995). Dry weight has also been used as an endpoint with emergent macrophytes but there was high variation between replicates (Powell *et al.*, 1996). In toxicological studies with two species of *Lemna*, Cowgill *et al.* (1989b) recommended dry weight as a more accurate endpoint than plant number, frond number, chlorophyll *a* or chlorophyll *b* (Cowgill *et al.*, 1989b). Submersed aquatic macrophytes require minor structural components because of the buoyancy provided by the water and therefore have a low dry weight to fresh weight ratios. Dry to fresh weight ratios of 1:15 are not uncommon in freshwater angiosperms (Bowes, 1985).

##### 4.3.2.4.2 Methods

Each replicate *Myriophyllum sibiricum* plant was removed from the liquid growth medium, patted dry on paper towels and the total fresh weight was determined. Because the plants were sectioned for pigment analysis (Section 4.3.2.6) and

membrane integrity (Section 4.3.2.7) parameters, a total dry weight could not be determined. The fresh weight of any additional plant material was determined and this extra portion was dried at 80 °C for at least 24 hours until a constant weight was reached. The dry weight of the plant tissue used for pigment analysis and membrane permeability were determined for some experiments.

#### 4.3.2.4.3 Results and Discussion

There was minor evaporative loss of water from the plants during the initial fresh weight determination because the plant tissue was sectioned for pigment and membrane integrity analysis. This emphasised the need to work quickly with the fresh plant material. This endpoint was reliable and repeatable. No fresh weight data are shown in this section because data summaries are presented with the other endpoint parameters in the results section of appropriate chapters. Dry weight data were determined for all experiments but are only presented in situations where there were significant effects.

The main fluctuation in weight occurred when the liquid growth medium used for culturing the *M. sibiricum* plants was changed from half strength M & S medium to full strength modified Andrews' medium. For example, the average control plant fresh weight determined from the last experiment started with plants cultured in M & S medium was  $581.3 \pm 39.4$  mg. Five months later, the 14 day control plant fresh weight for experimental plants started in modified Andrews' medium averaged  $714.5 \pm 38.1$  mg. This change in fresh weight affected the comparison of results from pesticide experiments that were repeated several months or years apart (see Sections 5.7.1.3.1, 5.7.2.3.1, 5.7.3.3.1, 5.7.4.3.1, 5.7.5.3.1 and 5.7.7.3.1).

#### 4.3.2.5 Plant Area

##### 4.3.2.5.1 Introduction

Plant area measures the surface area of the plant available for photosynthesis. For flat-leaved aquatic and terrestrial plants, the upper leaf surface is easily measured by photometric methods. For fine-leaved aquatic plants with photosynthetic stems, such as *Myriophyllum*, the whole surface area should be determined (Gerber *et al.*, 1994; Westlake, 1974). Minor changes in the surface area of dissected leaves can affect gas, light and nutrient exchange (Gerber and Les, 1994; Gerber *et al.*, 1994). Leaf surface area of finely dissected leaves may be determined by photographing the leaf, measuring

the length and diameter and calculating the area based on the area of a cone (Godmaire and Nalewajko, 1989; Nalewajko and Godmaire, 1993). By assuming that larger leaves of aquatic plants (e.g., *Potamogeton*) were elliptical, measurement of leaf length and breadth allowed for the determination of leaf area (Barko *et al.*, 1982). By taking a photograph of the plant, plant area may also be approximated. A range of standard curves, cut out of dark opaque card (Westlake, 1974) or wire (Gerber and Les, 1994; Gerber *et al.*, 1994) should also be photographed so that a standard calibration curve can be developed in case any of the plant area readings need to be converted (Westlake, 1974). The adsorption of dye onto a plants' surface can also be used to determine the leaf surface area of aquatic plants (Watala and Watala, 1994). There are also digitising and scanning computer programs that can be used to measure plant area (e.g., DAPPLE Image-Analysis System, OPTIMAS software) (Gerber and Les, 1994).

#### 4.3.2.5.2 Methods

Once this parameter was developed, the plants were photocopied for every experiment. Individual plants of *Myriophyllum sibiricum* were removed from the liquid growth medium, patted dry on paper towels and photocopied each plant onto translucent acetate photocopy sheets. A black construction paper circle with a known area was also photocopied onto each sheet. The appropriate plant label was placed next to each photocopied plant on the acetate sheet. The photocopied plants were cut off the acetate sheet and the area of the darkened image was determined using either a Li-Cor<sup>®</sup> LI-3000 Portable Area Meter or a Li-Cor<sup>®</sup> 3100 Area Meter. The area of every photocopied plant was determined five times and averaged because there were minor variations in the area measured on the acetates. Each photocopier was calibrated to the proper light intensity to mimic the actual area of the whole plant area.

#### 4.3.2.5.3 Results and Discussion

An example of the calibration of standard objects photocopied on the acetate sheet can be found in Table 3. For every sheet of plants photocopied, standard black objects were also copied. Most of the photocopied objects did not differ significantly from the actual calibration standard (Table 3). These examples demonstrate the accuracy of the photocopy method. On the rare occasion that the photocopied

Table 3: A comparison of black construction paper objects with photocopies of the same objects. All the photocopied objects did not differ significantly from the area of the original object.

Object	Circle			Square		
	#1	#2	#3	#1	#2	#3
Construction Paper	49.38 ± 0.07 <sup>a</sup>	50.09 ± 0.17 <sup>a</sup>	49.48 ± 0.12 <sup>a</sup>	15.05 ± 0.08 <sup>a</sup>	15.18 ± 0.04 <sup>a</sup>	14.90 ± 0.23 <sup>a</sup>
Photocopied Acetate	49.46 ± 0.17 <sup>a</sup>	50.26 ± 0.16 <sup>a</sup>	49.50 ± 0.04 <sup>a</sup>	15.03 ± 0.05 <sup>a</sup>	15.13 ± 0.06 <sup>a</sup>	14.98 ± 0.03 <sup>a</sup>

a Any two means in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$ . The data presented are the untransformed mean ± s.d. of five area meter readings.

Table 4: An example calibration of photocopied *Myriophyllum sibiricum* plants. For all four replicate plants, the plant area of plants photocopied at the photocopier setting that was second from the lightest setting did not differ significantly from the actual plant area.

Photocopier Setting	<i>Myriophyllum sibiricum</i> Plant Area			
	Plant #1	Plant #2	Plant #3	Plant #4
Actual Plant	16.00 ± 0.48 <sup>a</sup>	14.43 ± 0.65 <sup>a</sup>	9.16 ± 0.53 <sup>a</sup>	7.41 ± 0.39 <sup>a</sup>
Normal	20.33 ± 0.19 <sup>b</sup>	17.08 ± 0.09 <sup>b</sup>	12.54 ± 0.06 <sup>b</sup>	9.58 ± 0.14 <sup>b</sup>
Second Lightest	16.55 ± 0.22 <sup>a</sup>	13.46 ± 0.13 <sup>a</sup>	8.75 ± 0.19 <sup>a</sup>	6.90 ± 0.08 <sup>a</sup>
Lightest	14.54 ± 0.15 <sup>c</sup>	12.01 ± 0.40 <sup>c</sup>	7.93 ± 0.18 <sup>c</sup>	6.07 ± 0.35 <sup>c</sup>

a,b,c Any two means in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$ . The data presented are the untransformed mean ± s.d. of three area meter readings.

standard object was significantly lighter than the actual calibration object, the appropriate conversion was applied to the photocopied plant area. Data from one photocopier calibration are in Table 4. For this photocopier, the setting was second from the lightest setting because the area of plants photocopied at this light intensity did not differ significantly from the actual plant area. Therefore, every time this particular photocopier was used, the second setting was used. This type of calibration was performed with every photocopier used during the course of this experiment. Due to photocopier repairs and maintenance schedules several photocopiers were used during the course of this study.

It can be difficult to determine the surface area or volume of plants with fine leaves, such as *Myriophyllum sibiricum*. The stiff wire calibration method utilised by Gerber *et al.* (1994) or the dye adsorption method recommended by Watala and Watala (1994) could be useful in determining the surface area and volume of individual leaves. However, these methods are more time consuming than the method of copying the whole plant as used in this study. The whole plant method may occasionally underestimate the total plant surface area but it is a less time consuming alternative.

#### 4.3.2.6 Chlorophyll/Carotenoid Analysis

##### 4.3.2.6.1 Introduction

Numerous extraction techniques, solvents and analytical methods have been utilised to determine pigment content from terrestrial plants (Arnon, 1949; Gaudillere, 1974; Moran and Porath, 1980), algae (Vollenweider, 1974; Wright and Shearer, 1984), and macrophytes (Westlake, 1974). Extraction techniques include various forms of maceration and centrifugation. Westlake (1974) recommended that the macrophyte tissue be ground or homogenised (manually or mechanically) to ensure thorough pigment extraction and centrifuged at 1 500 to 2 500 rpm for 3 to 15 min. Emergent macrophytes tested by Powell *et al.* (1996) were frozen with dry ice, ground with a blender, extracted with a solvent and then centrifuged. Solvents used by researchers include acetone (Inskeep and Bloom, 1985; Porra *et al.*, 1989; Westlake, 1974), ethanol (Richardson *et al.*, 1979; Wintermans and DeMots, 1965), methanol (Porra *et al.*, 1989; Wright and Shearer, 1984), N,N-dimethylformamide (DMF) (Greenberg *et al.*, 1992; Inskeep and Bloom, 1985; Madsen and Sand-Jensen, 1994; Madsen *et al.*, 1993; Moran, 1982; Moran and Porath, 1980; Porra *et al.*, 1989) and dimethyl sulfoxide

(DMSO) (Blanck *et al.*, 1984; Netherland and Getsinger, 1995a; 1995b; Netherland and Lembi, 1992; Powell *et al.*, 1996). Methods to quantify pigment content have included colourimetric, spectrophotometric, fluorimetric (Vollenweider, 1974; Wintermans and DeMots, 1965) and high pressure liquid chromatography (Braumann and Grimme, 1979; Khalyfa *et al.*, 1992; Minguez-Mosquera, 1992; Powell *et al.*, 1996; Wright and Shearer, 1984). Very little is known about pigment extraction from aquatic plants other than algae. The objective of the studies discussed in this chapter was to determine the best method of extracting chlorophyll *a*, chlorophyll *b* and carotenoids from *M. sibiricum* grown in axenic culture.

#### 4.3.2.6.2 Methods

##### 4.3.2.6.2.1 Extraction Technique

Solvents including ethanol, acetone and dimethyl sulfoxide were compared for their efficiency at extracting the photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoid) from *Myriophyllum sibiricum* apices. Grinding the plant tissue with a Brinkman® polytron and centrifuging at 2 000 rpm for 5 min was compared with leaving the whole apex in the solvent for several hours. Pigment content was determined using a Beckman DU® Series 60 Spectrophotometer with an unheated sipper accessory. Only samples extracted with ethanol could be quantified with the sipper accessory. The other solvents were processed using glass cuvettes. Samples were spectrophotometrically analysed at 470, 647 and 663 nm. When the sipper was used, the spectrophotometer was programmed to automatically calibrate with a blank 80% ethanol standard (Appendix 8.4.1) and to calculate the pigment content in mg/L (Appendix 8.4.2). The equations used were:

$$\text{Chlorophyll } a = 12.7 (A_{663}) - 2.69 (A_{647}) . \quad (2)$$

$$\text{Chlorophyll } b = 22.9 (A_{647}) - 4.68 (A_{663}) . \quad (3)$$

$$\text{Carotenoid} = 4.695 (A_{440}) - 0.268 (\text{Chloro } a + \text{Chloro } b) . \quad (4)$$

where A is the absorbance reading (MacKinney, 1940; von Wettstein, 1957). Once the pigment content had been determined in mg/L, this was converted to mg/g of fresh weight.

#### 4.3.2.6.2.2 Extraction Time

Once ethanol was chosen as the solvent that most effectively extracted the pigments, there were several other factors that needed to be examined. The length of time that the apices should soak in the solvent needed to be determined. In one experiment, pigment content was measured on the spectrophotometer at 1, 2.25, 4, 8, 16, 24.25, 36.25, 48.25 and 76 hours after the last apex was placed into the 10 mL of 80% ethanol. Pigment concentration was calculated on both a fresh and dry weight basis. The data were log transformed as required and a one-way ANOVA was conducted. The other comparison conducted involved measuring the pigment content at either 2 or 24 hours after placing the last apex in the 10 mL of 80% ethanol. This comparison was conducted 16 times. The timing of the first reading varied from 2 to 3½ hours after the last apex was placed in the 80% ethanol. Based on the spectrophotometer reading at 470, 647 and 663 nm, pigment content was calculated on a fresh weight basis. For every one of the 16 times, the chlorophyll *a*, chlorophyll *b* and carotenoid content were compared between the 2 and 24 hour values using the Mann-Whitney U-test. All data from the 2 hour sampling period were pooled and all the data from the 24 hour sampling period were pooled and one Mann-Whitney U-test was conducted.

#### 4.3.2.6.3 Results and Discussion

##### 4.3.2.6.3.1 Extraction Technique

Due to the higher human toxicity of DMSO when compared to ethanol and acetone, DMSO was deemed less safe for the experimenter. Ethanol was the most successful solvent at extracting the pigments from the *Myriophyllum sibiricum* plants (data not presented). N,N-dimethylformamide (DMF) was not tested in this toxicity test because of high human toxicity (The Merck Index, 1983) but this solvent might be appropriate for situations where a long storage period was required (Inskeep and Bloom, 1985; Moran and Porath, 1980) or when low concentrations of pigments were being extracted (Moran, 1982). Pigments stored in DMF, in the dark and at 4 °C did not degrade after 3 (Inskeep and Bloom, 1985) or 21 days (Moran and Porath, 1980).

Grinding the plant tissue was very time consuming and did not improve the pigment extraction. In fact, the amount of chlorophyll *b* extracted from the tissue was statistically less from ground than from whole tissue. There was no statistical difference

between grinding techniques for the other pigments (data not presented). Therefore, whole *Myriophyllum sibiricum* apices ( $50 \pm 2$  mg) were placed in 10 mL of ethanol for extraction of the pigments.

#### 4.3.2.6.3.2 Extraction Time

For the time series extraction experiment, Figure 6 shows the pigment concentration data for fresh weight while Figure 7 contains the dry weight pigment concentration data. The estimated pigment concentration after 1 h of extraction differed significantly from estimates based on all other extraction times when concentrations were calculated using the fresh weight (mg/g fresh weight) of the tissue (Figure 6). Similar effects of extraction time were observed when pigment concentrations were estimated on the basis of tissue dry weight (Figure 7). The one exception was that extraction times of 1 and 2.25 hours were not significantly different from each other for chlorophyll a and carotenoid content.

In the next series of experiments where the pigment concentration extracted

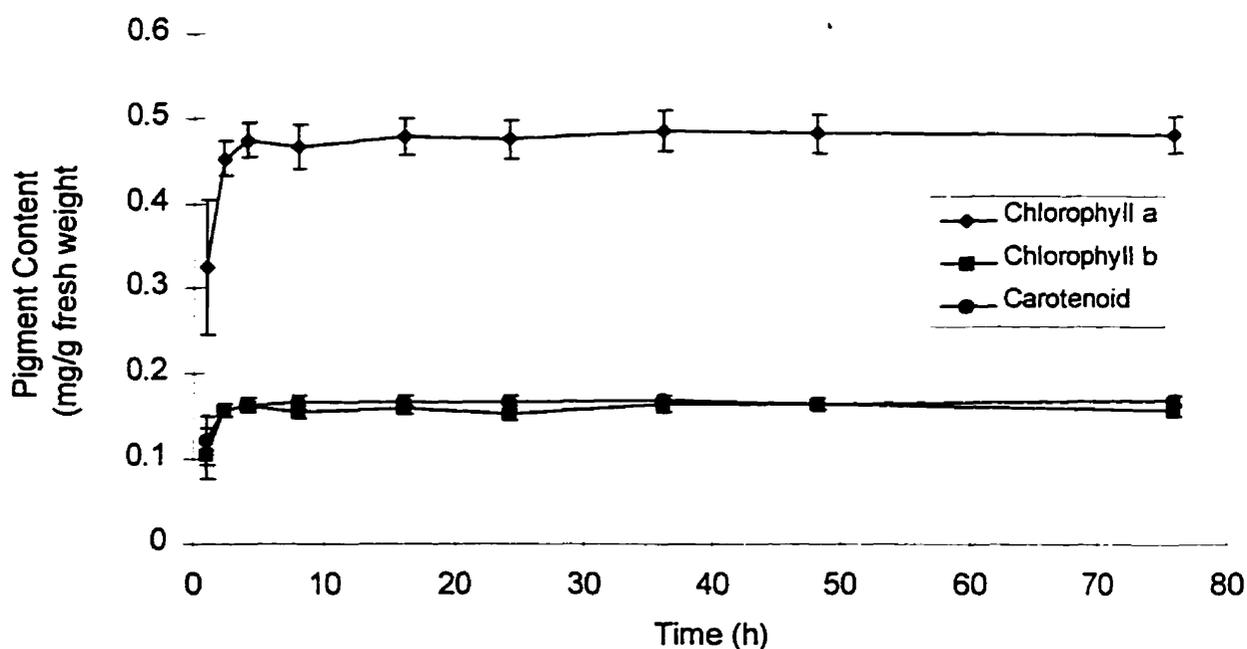


Figure 6: Pigment concentration, expressed as mg/g of fresh weight, for *Myriophyllum sibiricum* apices placed into 10 mL of 80% ethanol for 1 to 76 hours. Only the chlorophyll a, chlorophyll b and carotenoid content extracted after 1 hour differed significantly from the rest of the time periods ( $n = 5$ ;  $\alpha = 0.05$ ).

after 2 h was compared to the amount extracted after 24 h, the pigment concentration extracted per apex visually appeared greater after 24 hours of extraction than after 2 hours of extraction. When the values from individual experiments were compared, the amount of pigment extracted after 2 h did not differ significantly from the amount of pigment extracted over 24 h (Figure 8 and Figure 9). There were three exceptions for the chlorophyll *b* concentration (Figure 9). However, when all the 2 h data and all the 24 h data were combined into two populations, statistical analysis showed that the 2 hour extraction period extracted less chlorophyll *a* ( $p = 0.037$ ) (Figure 8), chlorophyll *b* ( $p = 0.004$ ) and carotenoids from the apices ( $p = 0.007$ ) (Figure 9) than the 24 h extraction period.

Most submersed aquatic leaves have a very thin cuticle (Arber, 1963; Bowes, 1985). The chloroplasts are abundant throughout the epidermal and mesophyll cells of

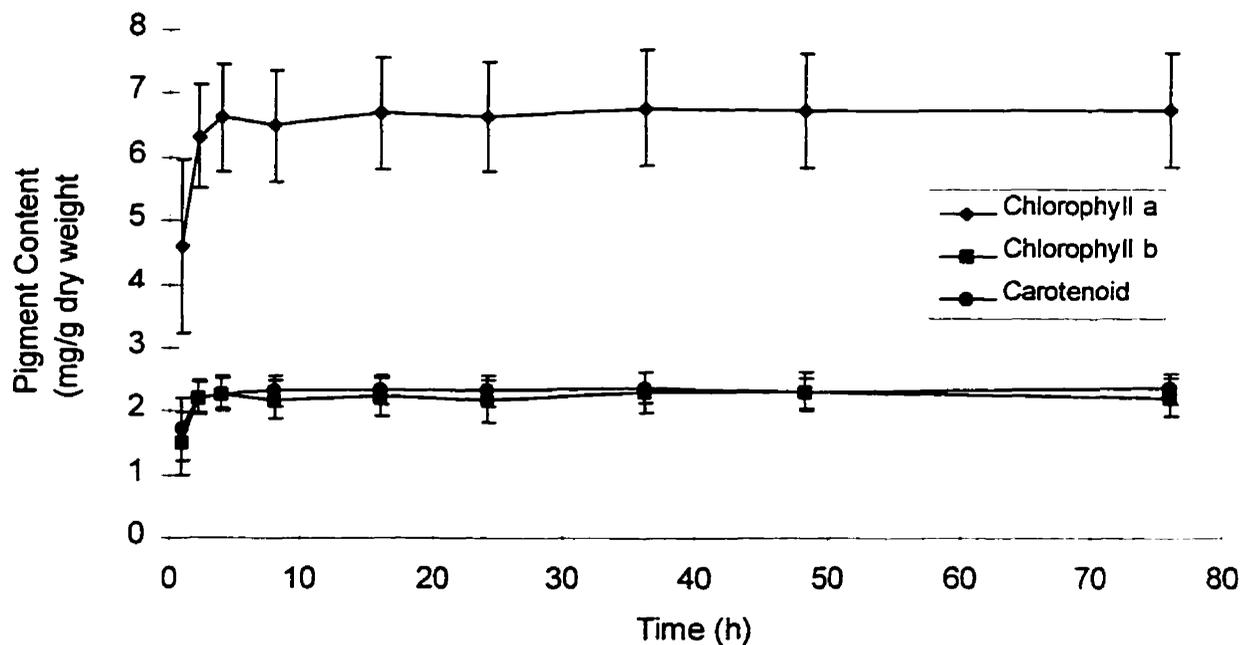


Figure 7: Pigment concentration extracted from *Myriophyllum sibiricum* apices over 1 to 76 h of soaking in 10 mL of 80% ethanol. Chlorophyll *a*, chlorophyll *b* and carotenoid concentration were expressed in mg/g of dry apical weight. For the chlorophyll *a* and carotenoid concentration, the amount of pigment extracted after 1 h did not differ from the amount extracted at 2.25 hours. All other time periods were significantly different from the amount extracted after 1 h but not from each other. The amount of chlorophyll *b* extracted after 1 h differed from all the other time periods ( $n = 5$ ;  $\alpha = 0.1$ ) but the other time periods did not differ.

submersed leaves (Arber, 1963; Sculthorpe, 1967). The leaves of parrot-feather (*Myriophyllum aquaticum* (Vell.) Verdc.) are covered by a thin epidermal layer of irregularly shaped cells (Sutton, 1985). If it can be assumed that the leaves of *M. sibiricum* are covered by epidermal cells containing numerous chloroplasts but without a thick waxy or cuticular layer, this would explain why it is easy to extract the chlorophyll from the apical segments without maceration or other rupturing of the cells. Emergent leaves have a thick waxy layer and epidermal cells that do not contain chloroplasts (Sculthorpe, 1967). Complete chlorophyll extraction in emergent species requires cellular disruption as well as extraction with a solvent, such as DMSO (Powell *et al.*, 1996; personal communication with R. Powell<sup>5</sup>).

This method of chlorophyll extraction for *Myriophyllum* is similar to that recommended for use with *Lemna gibba*. Chlorophyll is commonly extracted from *L. gibba* by allowing the whole plant to soak in the solvent for 24 h at 4°C in darkness (Greenberg *et al.*, 1992).

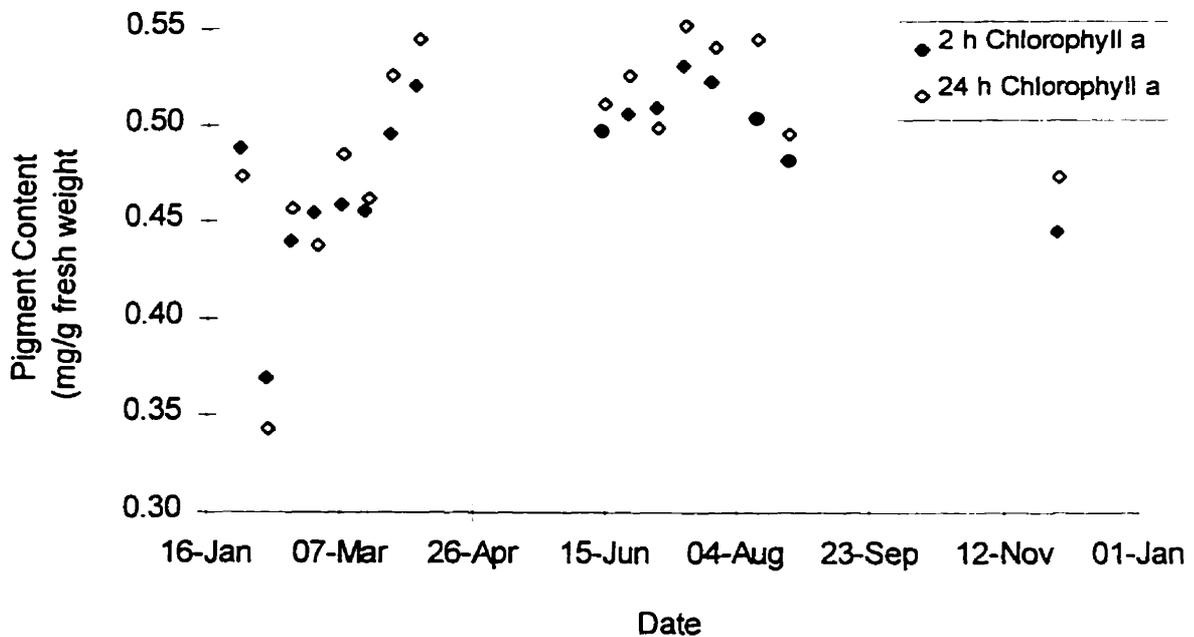


Figure 8: Chlorophyll a content in *Myriophyllum sibiricum* apices (~ 50 mg) after 2 and 24 hours of extraction in 80% ethanol. Data were collected from sixteen experiments conducted in 1994. On an individual experiment basis, chlorophyll a content extracted after 2 or 24 hours did not differ significantly from each other. When the data from all 16 experiments were combined, significantly more chlorophyll a was extracted after 24 hours of incubation than 2 hours.

Thus, on the basis of these results, extraction times between 24 and 76 h would be an acceptable method for pigment extraction from *M. sibiricum*. The choice would depend on the scheduling of other endpoint measurements, such as height and weight of plant tissue. For my procedures, combining the pigment measurements with conductivity measurements on the second day worked well.

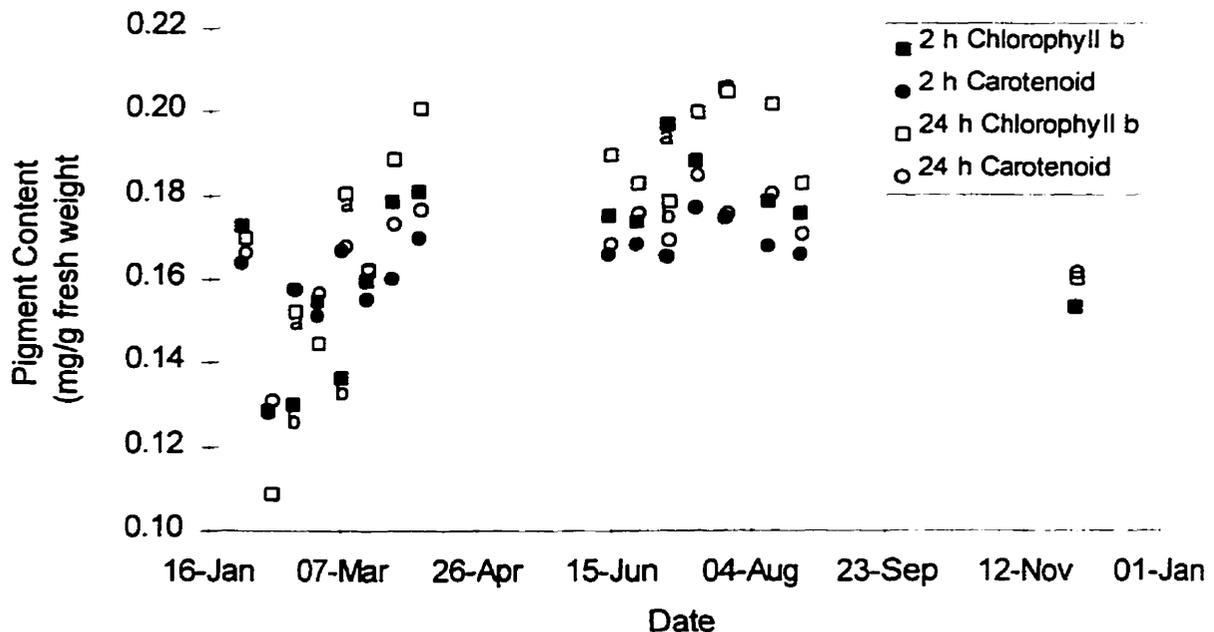


Figure 9: Chlorophyll *b* and carotenoid content extracted from *Myriophyllum sibiricum* apices placed into 80% ethanol for 2 or 24 hours. Data were collected from sixteen experiments conducted in 1994. On an individual experimental basis, only chlorophyll *b* showed significant differences between the 2 and 24 hour extraction period on three occasions. This difference was indicated by the symbols "a" and "b" on the graph. When the data from all sixteen experiments were combined, both chlorophyll *b* and carotenoid content extracted after 24 hours of incubation were significantly higher than pigment content extracted after 2 hours of incubation.

#### 4.3.2.7 Membrane Integrity

##### 4.3.2.7.1 Introduction

Plant cell membranes can be destroyed by numerous toxicants including air pollutants (Beckerson and Hofstra, 1980; Dijak and Ormrod, 1982; Spotts *et al.*, 1975), pesticides (Koch *et al.*, 1995; Ullrich *et al.*, 1990; Vanstone and Stobbe, 1977; Watson *et al.*, 1980), acetic acid (Spencer and Ksander, 1997), solvents (Koch *et al.*, 1995) and detergents (Koch *et al.*, 1995; Watson *et al.*, 1980). The effect of environmental

changes, such as drought, on membrane integrity can also be determined (Whitlow *et al.*, 1992). With severe membrane damage, plant tissue may become flaccid (Vanstone and Stobbe, 1977).

There are numerous methods of measuring changes in membrane integrity. Plants that have been exposed to toxicants are placed into deionized water and the specific conductivity measured. Leakage of electrolytes due to changes in membrane permeability can be measured by changes in electrical conductance (Koch *et al.*, 1995; O'Brien and Prendeville, 1978; 1979; Spencer and Ksander, 1997; Spotts *et al.*, 1975; Vanstone and Stobbe, 1977; Whitlow *et al.*, 1992; Yanase and Andoh, 1992). This change in conductivity can be a non-destructive method. Koch *et al.* (1995) described a method to determine the electrical conductivity of cellular leakage on a continual basis so that a time course of the damage could be determined (Koch *et al.*, 1995). The total capacity of the plant tissue to leak ions into solution can be determined by homogenising the tissue (Duke and Kenyon, 1992), by freezing the tissue (MacDonald *et al.*, 1993a; Spotts *et al.*, 1975) or by boiling the tissue (Spencer and Ksander, 1997). With cell cultures, the conductivity of the growth medium can be measured directly with a microelectrode after the cells have been incubated with the toxicants (Grossmann, 1992). Changes in membrane integrity and potential can be determined by monitoring specific ion changes (Na<sup>+</sup> and K<sup>+</sup>). For example, Ullrich *et al.* (1990) measured K<sup>+</sup> release from *Lemna gibba* exposed to glufosinate. Other authors have allowed plant roots to absorb radioactive compounds (e.g., <sup>32</sup>P) and then exposed the roots to the toxicant. The radioactivity that leaked from the cells was counted and quantified to determine the membrane damage (Mellis *et al.*, 1982). Leakage of radioactive sodium bicarbonate was measured from protoplasts exposed to herbicides (Watson *et al.*, 1980). Individual ion changes were not examined in the *Myriophyllum* toxicity test.

#### 4.3.2.7.2 Methods

Once the 14 day old experimental plants were measured for height, root length, plant area and fresh weight, the plants were segmented. The apical segment was used for pigment analysis. The next section along the plant stem was cut to 100 ± 5.0 mg. This section was triple rinsed in nanopure water to remove any external ions and placed into a flat bottomed test tube with 20 mL of nanopure water. In order to avoid excess cellular leakage, this 100 mg sample should consist of only one plant section. The test

tubes were capped and left for 24 hours. The conductivity of the water/plant solution in the flat bottomed tubes was measured with a portable conductivity meter (Corning® Checkmate 90 Field System). The flat bottomed tubes were carefully placed in an enamel container filled with a small amount of water and boiled for 20 min. The tubes were removed from the water and allowed to cool to room temperature. The conductivity of the solution was measured again. Membrane injury was determined as percentage of total electrolyte leakage:

$$\% \text{ Membrane Leakage} = \frac{\text{Conductivity before boiling}}{\text{Conductivity after boiling}} \cdot 100. \quad (5)$$

Leakage of ions through the membrane often increased as the toxicity increased. Thus, the results from this parameter differed from the other endpoints where the control was usually the greater value. For data manipulation, it was necessary to use a

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean} - \text{most toxic value}} \cdot 100. \quad (6)$$

modified percent inhibition formula:

#### 4.3.2.7.3 Results and Discussion

Membrane permeability was another valuable endpoint. Diquat and phenol caused severe damage to *Myriophyllum sibiricum* membranes. For calculations of membrane permeability inhibition, the most toxic value was 58.3%. This value was the largest amount of the cellular contents that leaked from the cells during the course of running this toxicity test. If the toxicant affected synthesis of cellular components, the membrane permeability value might be altered but not truly indicate a change in membrane integrity.

Problems that were encountered in determining membrane integrity included the lack of plant material. There were occasions where there was not enough plant material so that approximately 50 mg could be used for pigment analysis and approximately 100 mg could be used for membrane integrity. In the experiments conducted during this research project,  $50 \pm 2$  mg was always used for pigment analysis. If there was  $100 \pm 5$

mg left, that was used for membrane integrity. On occasion, some plants were lost during the boiling phase because the tube would tip over in the boiling water, the tube would crack with the change in temperature or the tube would be dropped upon removal from the boiling water bath. Freezing the plant tissue was also attempted in a preliminary experiment but more tubes were lost to breakage.

Changes in membrane permeability of plant cells have successfully detected air pollutant (Spotts *et al.*, 1975), herbicide (Ullrich *et al.*, 1990; Vanstone and Stobbe, 1977; Watson *et al.*, 1980) and acetic acid (Spencer and Ksander, 1997) damage. Membrane integrity was a viable endpoint for use with the *Myriophyllum sibiricum* toxicity test. Measurements of conductivity changes were much more rapid, less expensive and less time consuming than studies monitoring leakage of radioactive compounds (Watson *et al.*, 1980).

### 4.3.3 Physical Parameters

#### 4.3.3.1 Light Conditions

##### 4.3.3.1.1 Introduction

In laboratory testing with biological organisms, it is often necessary to supply artificial lighting. The light can interact with both the toxicant and the organisms, so it is important to ensure that the laboratory lighting mimics the natural lighting in the relevant environmental compartment (Greenberg *et al.*, 1996). Light fluence rate is defined as the flow rate of light or the amount of light per unit area per unit time. Occasionally, it is incorrectly referred to as light intensity but intensity refers to the amount of radiation in a unit angle. Photon fluence rate is expressed in units of  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (ASTM, 1995a; Greenberg *et al.*, 1996). Conversion to other units can be found in ASTM (1995a) and Greenberg *et al.* (1996). Experiments were conducted to determine the photon fluence rate that stimulates adequate *M. sibiricum* growth.

It is common practice in algal (ASTM, 1990; Environment Canada, 1992) and duckweed (ASTM, 1991b) toxicity testing to expose the test organisms to continuous light. Theoretically, this twenty-four hours of light will maximise the plant growth. In terrestrial plant testing, (e.g., seedling growth tests) a photoperiod of at least 14 hours is recommended (ASTM, 1994a). In emergent macrophyte toxicity testing, a photoperiod of 16 hours has been recommended (Powell *et al.*, 1996). This 16 hour light/8 hour dark

has been used with submersed macrophyte testing (Guilizzoni *et al.*, 1984). The appropriate photoperiod length for *Myriophyllum sibiricum* growth in axenic culture was determined by comparing a 24 hour continuous light to a 16 hour light/8 hour dark photoperiod.

Chlorophyll absorption of light has two maxima, at wavelengths of approximately 450 nm and 650 nm (Keeton, 1980; Salisbury and Ross, 1985). A supply of artificial light at these wavelengths might be important for the growth of plants under laboratory conditions. This artificial light may be supplied through many types of light bulbs. For low light fluence rates, fluorescent bulbs are often appropriate. The fluorescent bulbs can be supplemented with incandescent bulbs as long as the additional heat output of the incandescent bulbs is not detrimental. Other types of light bulbs can be added to the fluorescent light bulbs to increase the similarity with the full spectrum found in sunlight (ASTM, 1995a; Greenberg *et al.*, 1996). Fluorescent lighting is commonly used in aquatic macrophyte toxicity testing (Fairchild *et al.*, 1994b; Powell *et al.*, 1996). Various light sources were compared to determine their effect on *M. sibiricum* growth.

#### 4.3.3.1.2 Methods

##### 4.3.3.1.2.1 Light Fluence Range

The first light intensity experiment was conducted to determine the range of light intensity that was optimum for *Myriophyllum sibiricum* growth in axenic culture. All the plants were cultured in and transferred into half strength M & S medium. No rooting substrate was utilised. To create a dark treatment, five test tubes containing apices were covered in aluminium foil that was not removed during the 14 day incubation period. Plants were grown in different growth rooms with different light fluence rates provided by a mixture of fluorescent and incandescent light bulbs. The light fluence was measured using a LI-COR® Model LI-185A Quantum/Radiometer/Photometer with the quantum attachment. At the start of the experiment, the light sensor was placed under one of the translucent plastic test tube closures and intensities were established to approximate 30, 60, 90 and 150  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The test tube closure was used to determine whether the glass test tube and closure would allow the transmission of the correct spectral regions, as recommended by Greenberg *et al.* (1996). Light fluence was measured at the end of the experiment, with and without the test tube closure. Light fluences were also compared with the glass test tube on top of the sensor. On the

fourteenth day, the plants were harvested but only plant length, root number and length, total fresh weight and pigment content were determined. Pigment content was determined by grinding the plant tissue and using 80% ethanol as the extraction solvent. Pigment content was calculated using both the apical weight and the total plant weight. Other possible endpoint parameters were not determined because this experiment was conducted near the beginning of this research project and all the endpoint parameters had not been determined. Data were analysed using a one-way ANOVA followed by the Tukey multiple comparison.

#### 4.3.3.1.2.2 Light Duration

A comparison was made between plants exposed to 24 hours of light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and  $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and plants exposed to a 16 h light/8 h dark photoperiod with a fluence rate of  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The light fluence rate was measured at the start of the experiment with an uncovered sensor. The first comparison contained plants grown under the 16h/8h photoperiod while the second comparison utilised plants that had been exposed to continuous light. Both sets of experiments were conducted in modified Andrews' medium while the stock plants that were used to start the experiments had been cultured in half strength M & S medium with organics. Turface<sup>®12</sup> was used as an artificial rooting medium. Plants were incubated for 14 days and harvested with shoot height, root number and length, fresh weight, plant area, pigment content and membrane integrity being the parameters that were measured. Pigment content was determined by placing unmacerated plant tissue in 80% ethanol for 24 hours and analysing spectrophotometrically. Pigment content was calculated on the basis of apical and total plant fresh weight. The plant height data were graphed. One-way ANOVA was conducted to determine if the treatments were statistically different from each other. When differences were detected, the Tukey's multiple comparison was conducted.

#### 4.3.3.1.2.3 Influence of Light Quality

Another comparison was made between the mixture of Sylvania<sup>®</sup> GTE cool white high output fluorescent (60 W) and incandescent lights (60 W) with Duro-Test<sup>®</sup> very high output Vita-lite<sup>®</sup> bulbs. Part way through the current study, the Department of Environmental Biology changed lighting systems in the growth rooms from a mixture of fluorescent and incandescent bulbs to a combination bulb marketed by Duro-test<sup>®</sup>

Corporation under the trade name Vita-lite®. Based on a Duro-test® technical report, the Vita-lite® bulbs supplied more energy at light wavelengths (450 and 650 nm) that are very important for chlorophyll absorption. The combination of incandescent and fluorescent lights produced the maximum output around 650 nm (Anderson, 1992).

*Myriophyllum sibiricum* apices that had been cultured in modified Andrews' medium were transferred into test tubes containing the same type of medium. Five test tubes with apices were placed into either of two growth cabinets with either lighting system. Light fluence was measured at six points within each cabinet and compared between the two cabinets using the Mann-Whitney U-test. After 14 days of incubation, the endpoints were measured and compared using the nonparametric Mann-Whitney U-test.

#### 4.3.3.1.3 Results and Discussion

##### 4.3.3.1.3.1 Light Fluence Range

At the start of the experiment, the light fluence rates in the different treatments were  $32.0 \pm 0.71$ ,  $66.4 \pm 1.14$ ,  $94.8 \pm 0.84$ , and  $152.0 \pm 4.47 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . On day 14, the light fluence obtained with the translucent test tube closure on the sensor was  $30.4 \pm 0.89$ ,  $57.8 \pm 2.28$ ,  $179.0 \pm 4.18$ , and  $185.0 \pm 11.73 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Without the test tube closure on the light sensor, the light fluence rate was  $40.0 \pm 1.73$ ,  $79.0 \pm 3.61$ ,  $242.0 \pm 6.71$ , and  $250.0 \pm 15.0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Unfortunately near the end of the experiment, the last two treatments were exposed to an increase in light fluence because they were in a growth room where the light bulbs had been changed without this researcher's knowledge or consent. With the glass test tube on top of the sensor, the light fluence rate decreased on average by  $4.9 \pm 0.76 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Thus, the glass test tube did not intercept much light.

Visually, the plants from the dark treatment were severely stunted (Figure 10 and Figure 11). These dark grown plants were the smallest based on both height and weight. There was no root production (Table 5). When pigment production was based on the total plant fresh weight (Table 6), the plants grown in the dark contained the smallest amount of pigment ( $\alpha = 0.05$ ). Plants exposed to the two highest light fluence rates were intermediate in height (Figure 10, Figure 11 and Table 5). Plants that had

been exposed to  $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  appeared spindly when compared to plants exposed to  $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Figure 10 and Figure 11). The spindly plants at the lowest light intensity were hard to measure near the end of the 14 day period because they started to grow out of the liquid growth medium and curl around the inside of the tube. Shoot and internode elongation at low light intensity has been observed by other researchers (Barko *et al.*, 1982). Other than this height difference, it was difficult to differentiate between the treatments since there was no significant difference between the root number, fresh weight, and carotenoid content ( $\alpha = 0.05$ ). It appeared that any light fluence rate between 80 and  $130 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , as measured with an uncovered sensor, could be utilised as long as the height differences were not a problem. For these toxicity tests, a light fluence rate of approximately  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was chosen as a good light fluence rate to achieve adequate growth.

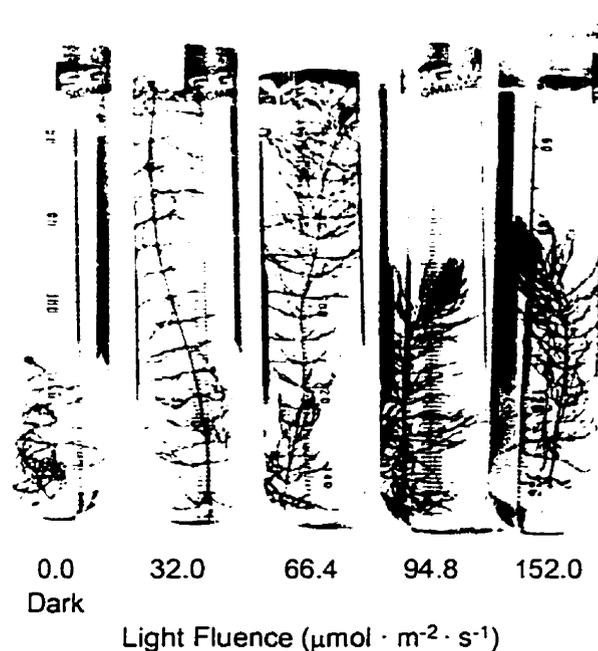


Figure 10: Representative *Myriophyllum sibiricum* plants exposed to five light fluence treatments.

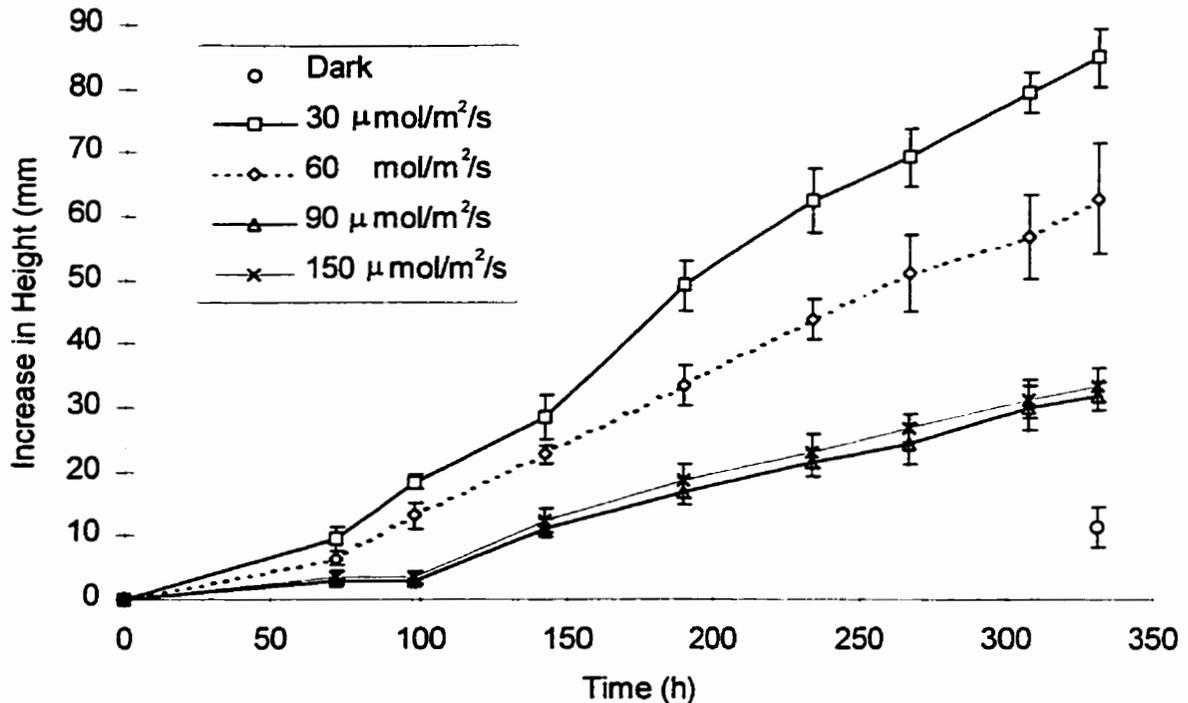


Figure 11: *Myriophyllum sibiricum* growth curves obtained for the different light fluence rates after 14 days of incubation in half strength M & S medium. Each data point was the average of five values with s.d. bars. There were only two data points for the plants in the dark treatment because the test tubes were covered in tin foil that was not removed during the course of the experiment.

The *Myriophyllum sibiricum* studied in the current research appeared to be similar in light requirements to other macrophytes reported in the literature. In submersed species, the submersed leaves have to supply all the photosynthate to the rest of the plant. There is a difference in the photosynthetic response of aerial and submersed leaves of *M. spicatum*. The light compensation point was  $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for submersed leaves and  $90 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for aerial leaves. Emergent leaves become light saturated at a photon flux density greater than  $1\ 200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  while the submerged leaves reach the light saturation point at about  $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Mitchell and Orr, 1985; Salvucci and Bowes, 1982; Sutton, 1985). One exception to the preferred light intensity was found for *M. spicatum* in aquarium culture where maximum shoot growth occurred with a light intensity of  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Hoffmann *et al.*, 1984).

For emergent macrophyte testing, Powell *et al.* (1996) recommended light fluences between  $115$  and  $160 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , as measured at the canopy level. Fairchild

*et al.* (1994b) used  $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for toxicity tests with algae, duckweed and submersed macrophytes.

#### 4.3.3.1.3.2 Light Duration

Growth curves between the experiments conducted using stock plants grown under the 16h/8h photoperiod and the continuous light were similar so only one graph was included (Figure 12). The minor difference between the two graphs was that several plants were lost to fungal contamination in the experiment conducted using plants cultured under continuous light so that the error bars were smaller. The twenty-four hour photoperiod with the high light fluence inhibited the growth of *Myriophyllum sibiricum* plants (Figure 12 and Table 7). Total fresh weight, plant area (Table 7), carotenoid content and chlorophyll *b* (when expressed on the basis of apical weight) (Table 8) were the endpoint parameters in which there was no significant difference between the three treatments. The use of the 16h/8h photoperiod significantly increased area under the growth curve and root number as compared with the other treatments (Table 7). There was no significant difference between the chlorophyll *a* content extracted from plants exposed to the low light fluence rate ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) with either photoperiod (Table 8). The data from the experiment conducted using stock plants that had been cultured under 24 hour illumination were not presented because they were very similar to those already presented. Only membrane integrity, area under the growth curve and chlorophyll *a* content showed slightly different trends. For these parameters, there was a significant difference between the high light fluence for 24 hours of exposure and both other treatments. Based on these results, a 16 hour light and 8 hour dark photoperiod is recommended. Continuous light at a low fluence rate could be used if it was necessary to conduct the *Myriophyllum sibiricum* toxicity test in the same growth cabinet as a *Lemna* or algal toxicity test. The use of a photoperiod is consistent with other macrophyte testing (Powell *et al.*, 1996).

**Table 5: Endpoint parameters demonstrating the effect of different light fluence rates upon the growth of *Myriophyllum sibiricum* after 14 days of incubation in M & S medium.**

Light Fluence Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)
Dark ( $0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$136.8 \pm 37.6^a$	$14.6 \pm 4.1^a$	$0.0 \pm 0.0^a$	$0.0 \pm 0.0^{a,c,d}$	$109.4 \pm 14.7^a$
$30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$13\ 206.4 \pm 864.2^b$	$90.0 \pm 2.1^b$	$2.0 \pm 1.2^b$	$11.0 \pm 7.9^{b,d}$	$227.3 \pm 41.5^b$
$60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$9\ 516.7 \pm 794.7^c$	$67.2 \pm 9.4^c$	$2.2 \pm 0.4^b$	$22.8 \pm 11.5^b$	$273.0 \pm 46.7^b$
$90 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$4\ 590.6 \pm 496.1^d$	$35.0 \pm 3.4^d$	$1.8 \pm 0.8^b$	$10.0 \pm 2.6^c$	$252.8 \pm 26.2^b$
$150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$4\ 991.4 \pm 508.3^d$	$36.2 \pm 3.8^d$	$1.4 \pm 0.5^b$	$10.8 \pm 4.1^{b,d}$	$278.0 \pm 68.6^b$

a,b,c,d Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . The data presented were the untransformed mean  $\pm$  s.d. of 5 replicates.

**Table 6: The effect of light fluence rate upon the pigment content of *Myriophyllum sibiricum* plants grown in axenic culture for 14 days in M & S medium.**

Light Fluence Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g total weight)	Chlorophyll b Content (mg/g total weight)	Carotenoid Content (mg/g total weight)
Dark ( $0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$0.22 \pm 0.05^a$	$0.09 \pm 0.01^a$	$0.06 \pm 0.02^a$	$0.02 \pm 0.007^a$	$0.009 \pm 0.002^a$	$0.006 \pm 0.003^a$
$30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$0.55 \pm 0.13^b$	$0.24 \pm 0.05^b$	$0.17 \pm 0.03^{b,d}$	$0.12 \pm 0.03^b$	$0.05 \pm 0.01^b$	$0.04 \pm 0.007^b$
$60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$0.32 \pm 0.08^a$	$0.14 \pm 0.03^c$	$0.11 \pm 0.02^c$	$0.09 \pm 0.03^c$	$0.04 \pm 0.01^c$	$0.03 \pm 0.008^{b,c}$
$90 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$0.28 \pm 0.02^a$	$0.13 \pm 0.03^{a,c}$	$0.13 \pm 0.01^{c,d}$	$0.07 \pm 0.009^{c,d}$	$0.03 \pm 0.005^c$	$0.03 \pm 0.003^{b,c}$
$150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$0.21 \pm 0.06^a$	$0.10 \pm 0.02^{a,c}$	$0.10 \pm 0.02^c$	$0.06 \pm 0.006^d$	$0.03 \pm 0.002^c$	$0.03 \pm 0.002^c$

a,b,c,d Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . Data = the untransformed mean  $\pm$  s.d. of 5 replicates.

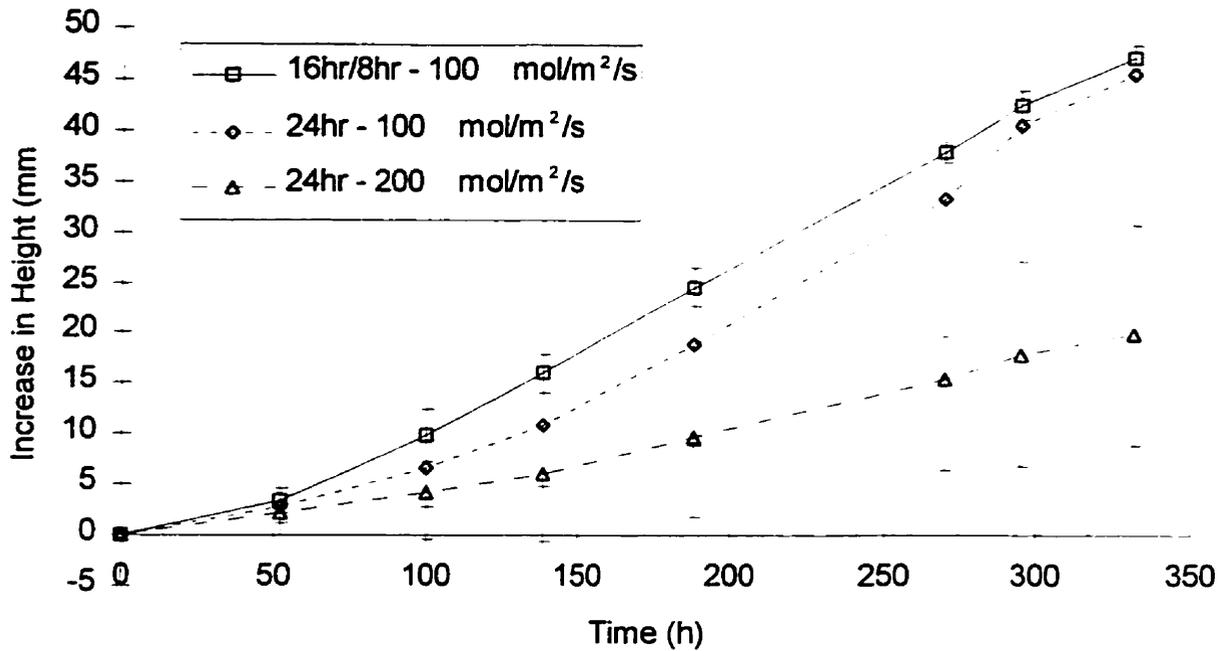


Figure 12: Growth curves for *Myriophyllum sibiricum* plants grown under different light regimes. Plants were cultured for 14 days in modified Andrews' medium. The stock plants utilised in this experiment had been cultured in a photoperiod consisting of 16 hour light and 8 hour dark. For clarification, unidirectional error bars were used to eliminate the overlap.

Table 7: Fourteen day endpoint parameter values for *Myriophyllum sibiricum* plants grown under three photoperiods. Plants were grown in modified Andrews' medium.

Photoperiod Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
16 hr/8 hr - 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	7 097.9 $\pm$ 369.5 <sup>a</sup>	47.5 $\pm$ 0.8 <sup>a</sup>	5.2 $\pm$ 0.8 <sup>a</sup>	313.2 $\pm$ 64.4 <sup>a</sup>	398.4 $\pm$ 77.9 <sup>a</sup>	10.3 $\pm$ 7.9 <sup>a</sup>	1.13 $\pm$ 1.00 <sup>a</sup>
24 hr - 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	5 983.5 $\pm$ 2502.9 <sup>b</sup>	49.6 $\pm$ 15.2 <sup>a</sup>	2.8 $\pm$ 1.5 <sup>b</sup>	185.2 $\pm$ 121.4 <sup>a</sup>	351.4 $\pm$ 157.5 <sup>a</sup>	15.9 $\pm$ 5.6 <sup>a,b</sup>	1.09 $\pm$ 0.96 <sup>a</sup>
24 hr - 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	2 910.4 $\pm$ 2115.4 <sup>b</sup>	21.3 $\pm$ 9.3 <sup>b</sup>	1.2 $\pm$ 0.4 <sup>b</sup>	31.4 $\pm$ 26.4 <sup>b</sup>	178.2 $\pm$ 148.0 <sup>a</sup>	27.0 $\pm$ 10.7 <sup>b</sup>	0.70 $\pm$ 0.75 <sup>a</sup>

a,b,c Any two means (untransformed mean of 5 values  $\pm$  s.d.) in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ .

Table 8: The effect of photoperiod on pigment content of *Myriophyllum sibiricum* plants cultured axenically for 14 days in modified Andrews' medium. Values are expressed on both apical fresh weight and total plant fresh weight bases.

Photoperiod Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g total weight)	Chlorophyll b Content (mg/g total weight)	Carotenoid Content (mg/g total weight)
16 hr/8 hr - 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	0.42 $\pm$ 0.08 <sup>a,b</sup>	0.14 $\pm$ 0.03 <sup>a,b</sup>	0.14 $\pm$ 0.03 <sup>a</sup>	0.16 $\pm$ 0.03 <sup>a,b</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	0.06 $\pm$ 0.01 <sup>a</sup>
24 hr - 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	0.50 $\pm$ 0.06 <sup>a</sup>	0.17 $\pm$ 0.03 <sup>a</sup>	0.18 $\pm$ 0.02 <sup>a</sup>	0.17 $\pm$ 0.08 <sup>a</sup>	0.06 $\pm$ 0.03 <sup>a</sup>	0.06 $\pm$ 0.03 <sup>ab</sup>
24 hr - 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	0.33 $\pm$ 0.06 <sup>b</sup>	0.13 $\pm$ 0.02 <sup>b</sup>	0.15 $\pm$ 0.03 <sup>a</sup>	0.06 $\pm$ 0.06 <sup>b</sup>	0.02 $\pm$ 0.02 <sup>a</sup>	0.03 $\pm$ 0.03 <sup>a</sup>

a,b Any two means (untransformed mean of 5 values  $\pm$  s.d.) in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ .

Table 9: The endpoint parameter values (untransformed mean  $\pm$  s.d.) obtained from growing the *Myriophyllum sibiricum* apices under two light regimes for 14 days in modified Andrews' medium.

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
Fluorescent/ Incandescent	9 323.8 $\pm$ 533.0 <sup>a</sup>	56.7 $\pm$ 2.3 <sup>a</sup>	7.3 $\pm$ 1.0 <sup>a</sup>	462.0 $\pm$ 123.2 <sup>a</sup>	58 $\pm$ 14.5 <sup>a</sup>	674.7 $\pm$ 76.2 <sup>a</sup>	10.4 $\pm$ 1.5 <sup>a</sup>	12.4 $\pm$ 1.4 <sup>a</sup>
Vita-lite®	8 013.7 $\pm$ 930.8 <sup>a</sup>	51.1 $\pm$ 2.0 <sup>b</sup>	6.5 $\pm$ 1.3 <sup>a</sup>	439.5 $\pm$ 66.3 <sup>a</sup>	71 $\pm$ 14.9 <sup>a</sup>	682.8 $\pm$ 64.3 <sup>a</sup>	8.9 $\pm$ 0.7 <sup>a</sup>	11.9 $\pm$ 2.2 <sup>a</sup>

a,b Any two means in the same column with the same superscript indicate that the values were not significantly different at  $\alpha = 0.05$ . n = 4.

Table 10: Pigment content of *Myriophyllum sibiricum* apices cultured under two types of light, expressed on a fresh and dry weight basis. Plants were cultured in test tubes with modified Andrews' medium for 14 days.

Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
Fluorescent/ Incandescent	0.57 $\pm$ 0.02 <sup>a</sup>	0.20 $\pm$ 0.02 <sup>a</sup>	0.19 $\pm$ 0.002 <sup>a</sup>	6.98 $\pm$ 1.06 <sup>a</sup>	2.44 $\pm$ 0.31 <sup>a</sup>	2.38 $\pm$ 0.41 <sup>a</sup>
Vita-lite®	0.57 $\pm$ 0.03 <sup>a</sup>	0.19 $\pm$ 0.02 <sup>a</sup>	0.20 $\pm$ 0.006 <sup>a</sup>	7.51 $\pm$ 0.66 <sup>a</sup>	2.44 $\pm$ 0.28 <sup>a</sup>	2.65 $\pm$ 0.26 <sup>a</sup>

a,b Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . Data are presented as the untransformed mean  $\pm$  s.d. of 4 values.

#### 4.3.3.1.3.3 Influence of Light Quality

The light fluence in the two cabinets was not significantly different at the  $\alpha = 0.05$  level. In the cabinet containing the mixture of fluorescent and incandescent light bulbs, the light fluence averaged  $118.0 \pm 25.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . In the cabinet containing the Vita-lite® bulbs, the fluence rate averaged  $97.8 \pm 10.6 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

For the lighting quality comparison, Table 9 gives the results for the endpoints examined except for pigment content, which can be found in Table 10. Table 10 lists the pigment content on both a fresh and dry weight basis. From both treatments, one plant was lost due to bacterial contamination and this lowered the number of replicates to four. There was no significant difference between the two lighting systems for all the parameters examined except for plant length ( $\alpha = 0.05$ ). The plants grown under the Vita-lite® bulbs were slightly smaller than the *M. sibiricum* plants grown under the mixture of fluorescent and incandescent lights. The growth parameters that might have been negatively influenced by light, such as pigment concentration, were not affected on either a fresh or dry weight basis. It appears that light quality and spectrum are not important environmental factors for this species of submersed macrophyte.

It was decided to stay with the mixture of incandescent and fluorescent light bulbs rather than switch to another lighting system mid-way through this study. Other researchers may use which ever system is available to them as long as they realise that the plants they culture might be slightly smaller than the plants discussed in the current research. No attempt was made to simulate sunlight and determine the effect of a full light spectrum upon *M. sibiricum* growth. If a spectrum that more closely mimics natural sunlight is required, modifications such as those recommended by Greenberg *et al.* (1996) and ASTM (1995a) could be followed.

#### 4.3.3.2 Rooting Substrate Selection

Roots of submersed aquatic plants are important for mechanical support as is demonstrated by the cork-screw shaped roots in *Myriophyllum* species (Arber, 1963). It has been reported that roots of aquatic species grew better when they penetrated a substrate (Bristow, 1975). Since the roots have an innate anchorage function, providing them with a substrate is an important component of creating a realistic aquatic plant toxicity test. In the axenic cultures of *Myriophyllum sibiricum*, it was observed that without the substrate, the roots pushed the plant away from the bottom of the culture

tube so that the apex eventually extended beyond the surface of the liquid growth medium. For this toxicity test, it was important to choose a standard rooting substrate that is easily obtainable and chemically consistent.

#### 4.3.3.2.1 Toxicity Test Comparison

##### 4.3.3.2.1.1 Introduction

In order to be used successfully in this toxicity test, any substrate used would have to be sterile. Sterilisation of substrates can be conducted by autoclaving or gamma irradiation. Gamma irradiation with 25 megarads has been recommended for bacterial elimination (Breach, 1968). A dose between 5 and 12 kGy successfully sterilised marine sediments with minimal chemical changes (Östlund *et al.*, 1989). It has been suggested that autoclaving substrates can increase the redox potential, decrease pH (Tratnyek and Wolf, 1993), mobilise organic matter, change the surface area and shape of clay and silt particles, change the amount of water soluble nutrients (Wolf *et al.*, 1989), increase penetrability, decrease permeability or decrease shear strength. Autoclaving did not release phytotoxic compounds from formulated sediment (Hoffmann *et al.*, 1984). Gamma irradiation can also solubilize extra carbon, alter the redox-buffering capacity of the pore water (Östlund *et al.*, 1989), and change the extractable concentration of some microelements (Wolf *et al.*, 1989). Microwaving can be used to sterilise soil. Ferris (1984) determined that microwaving released fewer nutrients than autoclaving for one hour on each of two consecutive days (Ferriss, 1984). Sediment manipulations, such as autoclaving, were found to be detrimental to the survival of some invertebrates (ASTM, 1994b; Day *et al.*, 1995). ASTM (1990) has recommended microwaving as an acceptable alternative to autoclaving for algal toxicity testing. Microwaving was not examined in the current study but it might be a useful alternative.

Numerous substrates have been used in toxicity tests with aquatic plants. Natural sediments covered with washed sand in aquaria have been used by Cassidy and Rodgers (1989) to culture and conduct toxicity tests with *Hydrilla verticillata*. Sand has been used for the culture and toxicity testing of *Elodea canadensis* (Forney and Davis, 1981). Nelson and Fairchild (1994) used a soil extract to test four species of submersed aquatic plants. For the culturing of *Myriophyllum spicatum*, Hoffmann *et al.* (1984) developed an artificial substrate that resembled a natural marl and could be readily sterilised. Natural sediments and synthetic sediments formulated with washed

quartz sand, silt, clay and organic matter were used for toxicity testing with *Echinochloa crusgalli* (L.) Palisot de Beauvois (Walsh *et al.*, 1991a; 1991b) and *Spartina alterniflora* Loisel. (salt-water cord-grass) (Walsh *et al.*, 1991b). The standardised aquatic microcosm uses an artificial sediment composed of 200 g of sand and 0.5 g each of chitin and cellulose (ASTM, 1991a). If formulated sediments are used in toxicity testing, it is important to ensure that they represent of a variety of natural sediments in terms of particle and pore sizes, chemical and nutrient composition and organic content (Walsh *et al.*, 1991b). Formulated sediments have been used successfully with invertebrate toxicity testing (ASTM, 1995b).

#### 4.3.3.2.1.2 Methods

##### 4.3.3.2.1.2.1 Rooting Substrate Comparison

The growth of *Myriophyllum sibiricum* apices was compared in different substrates with different methods of sterilisation. In the first experiment, the substrates (glass beads, silica sand, mineral soil, mineral soil covered with silica sand, mineral soil covered with glass beads, organic soil covered with mineral soil, organic soil covered with silica sand and organic soil covered with glass beads) were compared with no substrate. The organic soil was always covered with another substrate because this soil floated when the liquid medium was added. For each treatment, the substrates were weighed into five empty test tubes and autoclaved once for 20 min at 121 °C and  $1.31 \cdot 10^5$  Pa with no exhaust cycle. Forty mL of half strength M & S medium with minimal organics and 30 g/L sucrose were aseptically pipetted into each test tube. Three centimetre long apical segments were placed into each tube along with a 15 cm section of a 1 mL disposable pipette marked in 1/100 mL increments. Each 0.01 mL mark corresponded to 1.6 mm. Plant height was measured on the day of transfer and then 10 times during the 2 week toxicity test. The plant height as measured in the test tube was converted into length in millimetres before area under the growth curve was calculated. When the plants were harvested, shoot height, number and length of roots, fresh weight and dry weight were measured. The data were tested for normality. One-way ANOVAs were conducted on the normal data. If significant differences were detected, multiple comparison tests determined which treatments differed. For endpoint parameters that were not normal and could not be transformed, the nonparametric one-

way Kruskal-Wallis test was used to determine if there were differences between the treatments.

#### 4.3.3.2.1.2.2 Type of Sterilisation

The second set of experiments compared substrates (glass beads, silica sand, vermiculite, Turface<sup>®</sup>, mineral soil and organic soil) that had been either autoclaved or gamma irradiated. All substrates were pre-moistened with nanopure water, allowed to saturate overnight and drained. For the autoclave treatment, the test tubes containing the substrates were autoclaved twice. The first cycle was for 45 min at 121 °C and  $1.31 \cdot 10^5$  Pa with a slow exhaust cycle. Ninety-six hours later, these tubes were autoclaved again with a 20 min cycle at 121 °C,  $1.31 \cdot 10^5$  Pa and no exhaust period. The gamma irradiation was conducted in the Physics Department at the University of Guelph. The substrates were weighed into 20 mL glass scintillation vials so that the samples could fit into the 6 by 8 inch gamma irradiation cell. The Co<sup>60</sup> source in the  $\gamma$ -irradiator was emitting 35 554.49 rads/h two days previous to its first use in these experiments<sup>13</sup>. The substrates were gamma irradiated for 28 hours for the first toxicity test and for 60 hours for the second toxicity test. The test tubes and closures for use in the  $\gamma$ -irradiation treatment were autoclaved. All transfers occurred under the laminar flow hood. After surface disinfecting the scintillation vials with 95% ethanol, the  $\gamma$ -irradiated substrates were scraped into the appropriate test tubes using a sterile metal spatula. The vials were rinsed out with 10 to 15 mL of full strength M & S medium and the rinsate added to the appropriate test tubes. A total of 40 mL of the full strength M & S medium was pipetted into all the test tubes. Three cm apical segments were transferred into all the tubes, a measuring rod with millimetre markings (Westergren Blood Sedimentation Tube) was added, the tubes parafilm<sup>®</sup>ed shut and the initial plant height measured. The test tubes were randomised in test tube racks and placed in a growth cabinet. Plants were incubated for 14 days during which time the plant height was measured seven times. When the plants were harvested, plant length, root number and length, fresh weight, membrane integrity, plant area and pigment content were measured. Experiments were conducted twice with gamma irradiated substrates. The

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data were tested for normality. One-way ANOVA was conducted on normal data. If differences were detected, multiple comparison tests were used to determine which treatment differed. If the data for a specific endpoint could not be normalised, the nonparametric one-way Kruskal-Wallis test was used to determine if the treatments differed. If treatment differences were indicated, multiple comparison tests were used to determine which substrate treatments differed.

#### 4.3.3.2.1.2.3 Rooting Substrate Autoclaving Time

A comparison was made to determine the number of times the substrates should be autoclaved to maximise their sterility. The substrates utilised were vermiculite, Turface<sup>®</sup>, silica sand and glass beads. Test tubes were autoclaved once, twice or three times for one hour at 121 °C and  $1.31 \cdot 10^5$  Pa with no exhaust cycle. Plants were aseptically transferred into test tubes containing 40 mL of sterile full strength Hoagland's solution with 3% sucrose. Measuring rods were added, the tubes were parafilmmed shut and the initial plant height was measured. The test tubes were randomised in test tube racks and placed into a growth cabinet. Plant height was measured an additional seven times during the course of the 14 day toxicity test. During the plant harvest, shoot height, root number and total length, fresh weight, membrane integrity, plant area and pigment content were measured. The data were checked for normality and the root number, root length and membrane permeability data required transformation. A two-way ANOVA with unequal sample sizes was conducted to determine if there were differences between the treatments. If differences were detected, pairwise comparisons between the means were conducted.

#### 4.3.3.2.1.2.4 Natural Sediments

The growth of *Myriophyllum sibiricum* plants was compared between plants grown with 3 g of Turface<sup>®</sup> (as the control) and sediment from two field sites. Sediment from the Canadian Wildlife Bird Sanctuary, Long Point, Ontario has been used as a control sediment for invertebrate toxicity tests by researchers at the National Water Research Institute (NWRI), Burlington (Day *et al.*, 1994; 1995) and the University of Guelph, Guelph. The sediment was wet sieved through a 500 µm mesh screen (ASTM, 1994b). After sieving, this sediment contains 1.05% sand, 66.33% silt and 32.62% clay (Day *et al.*, 1995). Based on 12% solids in the Long Point sediment, approximately 8 g of wet sediment was weighed into five replicate test tubes. The test tubes containing

the Long Point sediment and the Turface® were autoclaved three times (121 °C and  $1.31 \cdot 10^5$  Pa for 20 min with no exhaust cycle). The other sediment was collected from a marsh near Lake Erie. The sediment and water in this marsh were determined to be relatively free from organic contaminants<sup>14</sup>, such as pesticides. This sediment was gamma irradiated (30 kGy) in glass scintillation vials at NWRI<sup>14</sup>. Using aseptic technique, the sediment was transferred into test tubes and the scintillation vials were rinsed out with 15 mL of sterile modified Andrews' medium. A total of 40 mL of sterile modified Andrews' medium was transferred into all the test tubes. Three centimetre apical *M. sibiricum* segments were transferred randomly into the test tubes. Measuring rods were added and the test tube closures were secured to the test tubes with laboratory sealant film. The plants were incubated for 14 days during which time the plant height was assessed every second day. During the plant harvest, the dissolved oxygen in the liquid medium in each test tube was measured and each experimental plant was measured for total shoot height, root number and total root length, fresh weight, membrane integrity, plant area, and pigment content based on total plant weight. The data were tested for normality and only membrane integrity and plant area required transformation. Data were compared using a one-way ANOVA and if differences were detected between the treatments, a Tukey-Kramer multiple comparison was conducted.

#### 4.3.3.2.1.2.5 Formulated Sediments

The last toxicity test conducted in this series of substrate experiments involved a formulated sediment. The formulated sediment developed by Hoffmann *et al.* (1984) for culturing *M. spicatum* contained 1 part vermiculite clay, 3 parts peat humus, 1 part quartz sand and 5 parts calcium carbonate. The vermiculite and peat were ground in a blender and all the sediment components were combined. A 2.0 mM aqueous solution of  $(\text{NH}_4)_2\text{HPO}_4$  was added to the dry component to achieve a 1:10 ratio (w/w) of sediment to solution. This mixture was covered and agitated on a rotary shaker at 120 rpm for 24 hours. This mixture was filtered through Whatman No. 1 filter paper. Three grams of the formulated sediment mixture and three millilitres of the filtrate were added to each test tube. Turface® was used as an artificial rooting medium in 5 test tubes. After the test tubes and sediments had been autoclaved, 40 mL of sterile modified

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Andrews' medium with 3% sucrose were added to the test tubes containing Turface® and 5 replicate test tubes containing the formulated sediment. To the other 5 test tubes containing formulated sediment, 40 mL of modified Andrews' medium without sucrose, was pipetted. Apices were aseptically transferred into randomly selected tubes, measuring rods were added and the test tube closures were fastened to the test tubes with laboratory sealant film. Plants were placed into a growth cabinet and allowed to incubate for 14 days during which time plant height was measured. At the end of the 14 day growth period, dissolved oxygen, shoot height, root number and root length, fresh weight, plant area, membrane integrity, and pigment content (chlorophyll *a*, chlorophyll *b* and carotenoids) were measured. The data were tested for normality. Only the fresh weight and the total plant chlorophyll *a* (log transformed) and total plant carotenoid content (log transformed) were normal. One-way ANOVA followed by a Tukey-Kramer multiple comparison test was conducted on these three parameters. All other parameters were compared using the nonparametric one-way Kruskal-Wallis test that was followed by the appropriate multiple comparison test (Conover, 1980).

#### 4.3.3.2.1.3 Results and Discussion

##### 4.3.3.2.1.3.1 Rooting Substrate Comparison

The organic soil in the test tubes tended to float once the medium was added. Unsuccessful preliminary trials were conducted with overnight shaking to allow the organic soil to saturate. During the 14 day toxicity test, the organic soil did not saturate and sink. The mineral soil tended to coat the sides of the test tubes and stayed suspended in the medium. This inhibited the amount of light reaching the plants. In the treatments with mineral and organic soil, numerous plants and media became contaminated by bacteria. No plants remained uncontaminated in the mineral soil covered with silica sand, only one plant was uncontaminated in the mineral soil treatment and only two plants remained in the treatments containing mineral soil covered with glass beads and organic soil covered with mineral soil. Visually, the roots of plants grown with glass beads and silica sand contained chlorophyll.

Growth curves were not shown for these sediment trials because there was no significant difference between any of the treatments ( $\alpha = 0.05$ ) for area under the growth curve and plant height on Day 14 (Table 11). There were no significant differences detected between the effect of substrate type upon root number ( $\alpha = 0.05$ ). The fresh

weight of plants grown in the organic soil covered by glass beads and silica sand was significantly greater than those plants grown in medium without a substrate, mineral soil and mineral soil covered by glass beads. The roots produced by plants grown in the organic soil were significantly longer than those of the other plants (Table 11). Bristow (1975) reported that the roots of *Myriophyllum exalbescens* grew well in both organic mud and gravel.

#### 4.3.3.2.1.3.2 Type of Sterilisation

Neither the 28 nor 60 hours of gamma irradiation successfully sterilised the substrates. In test tubes where the substrates had been gamma irradiated, all but four plants were lost from the first experiment and all but three plants were lost from the second experiment due to bacterial and fungal contamination. It is impossible to determine whether these microbes survived the gamma irradiation or whether they arose during the transfer process from scintillation vials into the test tubes. Other researchers have successfully used Co<sup>60</sup> irradiation to eliminate microbial populations from soil samples (Wolf *et al.*, 1989). The detrimental effects of bacteria in the toxicity test to plant growth outweigh any effects of sterilisation. Autoclaving was recommended as the sterilisation procedure to be used for the substrates and was used to sterilise the substrate for the remaining toxicity experiments.

The results presented were from the first experiment only and they compare the *M. sibiricum* endpoints for plants that were grown in tubes containing autoclaved substrates. There was no significant difference between the substrate treatments for area under the growth curve (Table 12). Therefore, the growth curves were not displayed. For total root length, carotenoid content and membrane integrity there was no difference between the substrate treatments (Table 12). Plants in the mineral soil and organic soil had the smallest plant area and final shoot height. Based on these results and those presented in the previous section (4.3.3.2.1.3.1), it was decided that the mineral soil and the organic soil were not acceptable for use in the axenic *Myriophyllum sibiricum* toxicity test. The Turface<sup>®</sup> and silica sand treatments produced plants with the greatest plant fresh weight and amount of chlorophyll *a* and chlorophyll *b* on a total plant basis (Table 12). The visible disadvantage to using silica sand and glass beads was that these substrates allowed light to penetrate into the rooting zone and the roots produced chlorophyll. This corresponded to the report of Bristow (1975) that some

aquatic plant roots grew better in the dark. Pieces of vermiculite occasionally floated in the medium and this substrate was hard to remove from the plant roots to accurately obtain fresh weight values.

**Table 11: *Myriophyllum sibiricum* toxicity test endpoints for plants grown in half strength M & S medium with different substrates.**

Treatment	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)
No Substrate	13 102.9 ± 2 512.8 <sup>a</sup>	93.8 ± 8.8 <sup>a</sup>	2.4 ± 0.5 <sup>a</sup>	27.2 ± 13.5 <sup>a,c</sup>	142.5 ± 35.8 <sup>a</sup>
Glass Beads	12 440.9 ± 1 505.1 <sup>a</sup>	107.0 ± 7.5 <sup>a</sup>	3.6 ± 1.1 <sup>a</sup>	40.8 ± 20.2 <sup>a,c</sup>	176.6 ± 28.8 <sup>a,b</sup>
Silica Sand	12 466 ± 2 548.0 <sup>a</sup>	95.0 ± 9.5 <sup>a</sup>	2.8 ± 0.4 <sup>a</sup>	34.2 ± 14.9 <sup>a,c</sup>	179.2 ± 40.8 <sup>a,b</sup>
Mineral Soil	10 122	87.0	2.0	4.0	40
Mineral Soil/Glass Beads	11 695.8 ± 1 441.6	80.5 ± 4.9	1.5 ± 0.7	2.0 ± 1.4	102.4 ± 22.0
Organic Soil/Mineral Soil	11 259.2 ± 3 398.6	101.5 ± 23.3	2.0 ± 0.0	123.0 ± 56.6	159.1 ± 107.1
Organic Soil/Silica Sand	11 288.8 ± 2 723.6 <sup>a</sup>	94.4 ± 15.0 <sup>a</sup>	2.2 ± 0.4 <sup>a</sup>	67.8 ± 8.8 <sup>c</sup>	265.5 ± 125.0 <sup>b</sup>
Organic Soil/Glass Beads	12 741.7 ± 2 782.3 <sup>a</sup>	110.8 ± 20.2 <sup>a</sup>	2.8 ± 1.0 <sup>a</sup>	97.3 ± 18.7 <sup>b</sup>	248.4 ± 82.9 <sup>b</sup>

a,b,c Any two means ( $\pm$  s.d.) in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . One-way ANOVA were conducted for area under the growth curve, plant length, root length and weight. If differences were detected, multiple comparisons were conducted. The root number was analysed using a one-way Kruskal-Wallis test.  $n = 5$  for no substrate, glass beads, silica sand and organic soil/silica sand combination;  $n = 4$  for organic soil covered with glass beads;  $n = 2$  for mineral soil/glass beads and organic soil/mineral soil;  $n = 1$  for mineral soil. Because of the low number of replicates, statistical significance could not accurately be determined for the mineral soil/glass beads, organic soil/mineral soil and for the mineral soil treatments.

**Table 12: The growth and development parameters for *Myriophyllum sibiricum* plants grown in full strength M & S medium with different substrates that had been autoclaved prior to use. Plants were incubated for 14 days.**

Treatment	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g total weight)	Chlorophyll b Content (mg/g total weight)	Carotenoid Content (mg/g total weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
No Substrate	11 610.1	88.0 ±	2.8 ±	43.6 ±	286.4 ±	0.14 ±	0.05 ±	0.05 ±	29.6 ± 4.9 <sup>a</sup>	8.7 ±
	± 1 362.3 <sup>a</sup>	4.8 <sup>a</sup>	0.5 <sup>a,b</sup>	17.9 <sup>a</sup>	19.6 <sup>a,b,c</sup>	0.02 <sup>a,b</sup>	0.007 <sup>a,b</sup>	0.005 <sup>a</sup>		0.2 <sup>a</sup>
Glass Beads	9 345.8 ±	78.3 ±	1.5 ±	36.3 ±	197.9 ±	0.12 ±	0.04 ±	0.04 ±	18.9 ± 3.8 <sup>a</sup>	5.8 ±
	1 848.0 <sup>a</sup>	16.7 <sup>a,b</sup>	0.7 <sup>b,c</sup>	43.9 <sup>a</sup>	85.3 <sup>a,c,d</sup>	0.04 <sup>a,b</sup>	0.01 <sup>a,b</sup>	0.01 <sup>a</sup>		2.0 <sup>a,b</sup>
Silica Sand	6 363.3 ±	87.8 ±	3.0 ±	56.3 ±	310.2 ±	0.16 ±	0.05 ±	0.05 ±	29.0 ± 5.8 <sup>a</sup>	8.4 ±
	626.1 <sup>a</sup>	8.1 <sup>a</sup>	0.7 <sup>a</sup>	14.2 <sup>a</sup>	42.3 <sup>b,e,f</sup>	0.03 <sup>a</sup>	0.01 <sup>a</sup>	0.01 <sup>a</sup>		1.7 <sup>a</sup>
Vermiculite	9 397.3 ±	85.1 ±	3.0 ±	79.4 ±	275.7 ±	0.14 ±	0.05 ±	0.05 ±	22.3 ± 0.9 <sup>a</sup>	8.3 ±
	719.3 <sup>a</sup>	3.9 <sup>a</sup>	1.0 <sup>a</sup>	2.6 <sup>a</sup>	6.9 <sup>a,c,i,g</sup>	0.004 <sup>a,b</sup>	0.002 <sup>a,b</sup>	0.0007 <sup>a</sup>		0.9 <sup>a</sup>
Turface®	10 148.4	84.5 ±	3.0 ±	58.5 ±	298.6 ±	0.17 ±	0.06 ±	0.05 ±	26.6 ± 2.6 <sup>a</sup>	8.8 ±
	± 1 116.7 <sup>a</sup>	15.8 <sup>a</sup>	1.0 <sup>a</sup>	48.7 <sup>a</sup>	151.2 <sup>c,o</sup>	0.08 <sup>a</sup>	0.03 <sup>a</sup>	0.03 <sup>a</sup>		3.8 <sup>a</sup>
Mineral Soil	8 827 ±	57.3 ±	0.8 ±	8.7 ±	86.4 ±	0.04 ±	0.02 ±	0.01 ±	29.4 <sup>a</sup>	2.9 ±
	1 505.2 <sup>a</sup>	8.5 <sup>b</sup>	0.5 <sup>c,d</sup>	16.1 <sup>a</sup>	27.7 <sup>d</sup>	0.02 <sup>b</sup>	0.008 <sup>b</sup>	0.007 <sup>a</sup>		1.0 <sup>b</sup>
Organic Soil	8 047.6 ±	57.0 ±	1.7 ±	39.8 ±	168.7 ±	0.11 ±	0.04 ±	0.04 ±	20.3 ± 11.2 <sup>a</sup>	5.2 ±
	2 634.4 <sup>a</sup>	19.9 <sup>b</sup>	0.6 <sup>b,d</sup>	27.5 <sup>a</sup>	95.4 <sup>a,d,g</sup>	0.07 <sup>a,b</sup>	0.02 <sup>a,b</sup>	0.02 <sup>a</sup>		2.1 <sup>a,b</sup>

a,b,c,d,e,f,g All the means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . Data presented are the untransformed means  $\pm$  s.d. Normal data (area under the growth curve, root length, chlorophyll a, chlorophyll b, carotenoid and plant area) were analysed with a one-way ANOVA followed by multiple comparison tests. Data that could not be normalised was analysed using the nonparametric Kruskal-Wallis test and compared using multiple comparisons. n = 5 for the treatments containing silica sand and Turface® (except membrane integrity where one sample was lost); n = 4 for test tubes without a substrate and mineral soil (except for membrane integrity where there was only enough plant material for this measurement for one replicate); n = 3 for vermiculite and peat soil; n = 2 for the glass bead treatment.

#### 4.3.3.2.1.3.3 Rooting Substrate Autoclaving Time

Plants were lost to bacterial and fungal contamination even if the substrates were autoclaved once, twice or three times on consecutive days. There was no statistically significant difference between the number of surviving plants with the different number of autoclave treatments. These results differ from those reported in the literature. Wolf *et al.* (1989) found that a single autoclave treatment of 2 hours reduced bacterial and fungal populations but autoclaving the soil two or three times resulted in complete destruction of all microbial populations (Wolf *et al.*, 1989).

Based on the results from the two-way ANOVA, there were no interactions between sediment type and number of autoclave treatments for all endpoints (p values between 0.054 and 0.691). When comparing the results for the surviving plants, varying the autoclave time resulted in no significant difference ( $\alpha = 0.05$ ) for any of the endpoint parameters except total root length. Root length showed both a substrate and autoclaving effect (Table 13). Roots produced by plants grown with glass beads and silica sand were not significantly different from each other. Regardless of autoclave time, the root length of plants grown with Turface® and vermiculite were not significantly different from each other. Based on root length, either one of these substrates would be acceptable for use in the toxicity test. Vermiculite often floated in the medium so Turface® was selected for use in the pesticide toxicity tests. Double or triple autoclaving may not be absolutely necessary but it did ensure that any resulting contamination will not arise from the substrate.

Autoclave Periods	Substrate			
	Glass Beads	Silica Sand	Turface®	Vermiculite
One	19.7 ± 12.4 <sup>a</sup>	68.4 ± 46.1 <sup>a,b</sup>	144.4 ± 33.6 <sup>b,c</sup>	150.2 ± 4.2 <sup>b,c</sup>
Two	37.1 ± 24.2 <sup>a</sup>	54.0 ± 16.0 <sup>a,d</sup>	176.2 ± 55.0 <sup>c,e</sup>	259.1 ± 48.7 <sup>e</sup>
Three	43.8 ± 14.2 <sup>a</sup>	42.8 ± 19.6 <sup>a</sup>	138.7 ± 24.7 <sup>b,c,d</sup>	160.3 ± 30.6 <sup>c</sup>

a,b,c,d,e Any two means ( $\pm$  s.d.) with the same superscript were not significantly different at  $\alpha = 0.05$ . n = 2 for vermiculite autoclaved once and twice; n = 3 for silica sand autoclaved once and twice, for Turface® autoclaved one, two or three times, for glass beads autoclaved three times, for vermiculite autoclaved three times; n = 4 for glass beads autoclaved once and twice, for silica sand autoclaved three times.

Substrate differences were also observed for some of the other endpoint parameters. Substrate type did not affect shoot length (Table 14). Plants grown with the Turface® as a substrate had a significantly greater fresh weight than plants grown in the other three substrates. For plant area, the plants grown with the Turface® were significantly larger than plants grown with either the glass beads or the silica sand (Table 14). Based on this data, Turface® was used as the control substrate in the remaining pesticide toxicity tests.

Table 14: Endpoint parameters showing the effect of substrate upon *Myriophyllum sibiricum* growth. For these parameters, the two-way ANOVA demonstrated that there was no interaction and no autoclaving effect. The results could be combined and analysed in terms of substrate treatment.

Substrate	Area under the Growth Curve	Shoot Length (mm)	Root #	Total Fresh Weight (mg)	Chloro a Content (mg/g)	Chloro b Content (mg/g)	Carotenoid Content (mg/g)	Plant Area (cm <sup>2</sup> )
Glass Beads	13 224.7 ± 2 018.5 <sup>a</sup>	123.8 ± 10.4 <sup>a</sup>	3.2 ± 1.5 <sup>a</sup>	450.5 ± 95.4 <sup>a</sup>	0.04 ± 0.02 <sup>a</sup>	0.016 ± 0.006 <sup>a</sup>	0.014 ± 0.006 <sup>a</sup>	11.8 ± 2.6 <sup>a</sup>
Silica Sand	10 565.8 ± 2 147.6 <sup>b</sup>	116.5 ± 10.5 <sup>a</sup>	4.7 ± 1.5 <sup>b</sup>	401.6 ± 79.7 <sup>a</sup>	0.06 ± 0.02 <sup>b</sup>	0.024 ± 0.007 <sup>b</sup>	0.021 ± 0.007 <sup>b</sup>	11.2 ± 1.5 <sup>a</sup>
Turface®	11 328.9 ± 1 989.8 <sup>b,c</sup>	118.6 ± 21.7 <sup>a</sup>	4.0 ± 0.9 <sup>a,b</sup>	732.3 ± 83.0 <sup>b</sup>	0.03 ± 0.01 <sup>a</sup>	0.013 ± 0.003 <sup>a</sup>	0.012 ± 0.003 <sup>a</sup>	16.3 ± 2.1 <sup>b</sup>
Vermiculite	12 347.1 ± 1 447.9 <sup>a,c</sup>	127.7 ± 7.3 <sup>a</sup>	4.4 ± 1.4 <sup>a,b</sup>	593.1 ± 88.6 <sup>c</sup>	0.05 ± 0.01 <sup>a,b</sup>	0.019 ± 0.004 <sup>a,b</sup>	0.016 ± 0.004 <sup>a,b</sup>	13.3 ± 1.9 <sup>a,b</sup>

a,b Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . Values are untransformed means  $\pm$  s.d. n = 7 for vermiculite; n = 9 for Turface®; n = 10 for silica sand; n = 11 for glass beads.

#### 4.3.3.2.1.3.4 Natural Sediments

In the *Myriophyllum sibiricum* toxicity test, the use of natural sediment from Long Point increased plant height when compared to plants grown in natural sediment from a marsh near Lake Erie or with Turface® (Figure 13, Figure 14 and Table 15). However, this increase in height did not correlate with an increase in root length, dissolved oxygen, weight, membrane integrity, plant area or pigment content (Table 15). Plants grown with the marsh sediment differed from the plants grown with the Turface® for root length, dissolved oxygen, fresh weight, plant area, chlorophyll a and carotenoid content (Table 15). The increase in shoot height and decrease in other toxicity test parameters may be caused by interference with the light intensity because of suspended particulate matter or sediment particles adhering to the test tubes (Figure 14). The reduction in

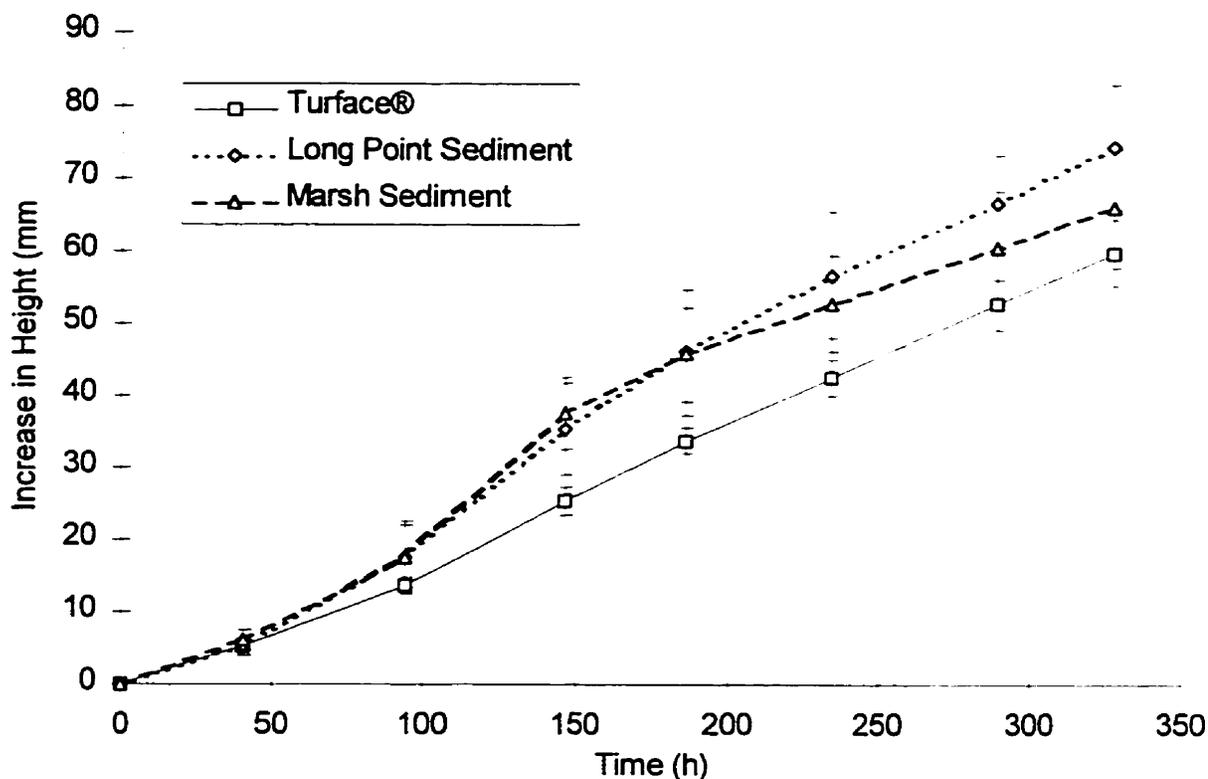


Figure 13: Growth curves for *Myriophyllum sibiricum* plants grown in test tube culture with natural sediments or Turface® as the substrate. Plants cultured with Long Point or Marsh sediment were slightly elongated because particulate matter suspended in the growth medium reduced light penetration.

root length, fresh weight and pigment content were sufficient to negate the recommendation of natural sediments in this axenic *M. sibiricum* toxicity test. If the use of natural sediment is part of an experimental plan, the reduction in these parameters should be noted. This time, microbial populations were not detected in either the autoclaved or gamma irradiated sediments. If natural sediments are used in toxicity testing, collection, storage and manipulation of the sediments should follow the procedures outlined by ASTM (1994b).

Other researchers have also noted problems with using natural sediments for toxicity testing. These problems included the presence of seeds from native flora, unidentified toxicants present in the sediment (Walsh *et al.*, 1991b), pH decreases over time and variable nutrient content (Tratnyek and Wolf, 1993; Walsh *et al.*, 1991b). The sediment properties could be measured before and after autoclaving and experimentation to monitor any changes in physical and chemical characteristics

Table 15: Effect of two natural sediments compared with the artificial rooting medium, Turface®, upon the growth and development of *Myriophyllum sibiricum* grown in axenic test tube culture.

Treatment	Area under the growth curve	Shoot Length (mm)	Root #	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g total weight)	Chlorophyll b Content (mg/g total weight)	Carotenoid Content (mg/g total weight)
Turface®	9 441.2 ± 461.0 <sup>a</sup>	61.7 ± 5.3 <sup>a</sup>	6.0 ± 0.8 <sup>a</sup>	361.1 ± 55.9 <sup>a</sup>	61.8 ± 5.6 <sup>a</sup>	474.2 ± 47.0 <sup>a</sup>	8.3 ± 1.5 <sup>a</sup>	8.9 ± 0.7 <sup>a</sup>	0.21 ± 0.01 <sup>a</sup>	0.07 ± 0.002 <sup>a</sup>	0.07 ± 0.004 <sup>a</sup>
Long Point Sediment	12 308.3 ± 1 774.0 <sup>b</sup>	83.1 ± 12.7 <sup>b</sup>	4.0 ± 1.0 <sup>a</sup>	141.5 ± 101.2 <sup>b</sup>	18.4 ± 10.8 <sup>b</sup>	188.2 ± 47.6 <sup>b</sup>	12.1 ± 3.3 <sup>b</sup>	3.6 ± 1.2 <sup>b</sup>	0.12 ± 0.04 <sup>b</sup>	0.05 ± 0.01 <sup>b</sup>	0.04 ± 0.01 <sup>b</sup>
Marsh Sediment	11 777.9 ± 1 602.0 <sup>a,b</sup>	72.0 ± 9.7 <sup>a,b</sup>	4.8 ± 2.2 <sup>a</sup>	124.8 ± 112.5 <sup>b</sup>	21.5 ± 8.9 <sup>b</sup>	205.5 ± 44.0 <sup>b</sup>	11.3 ± 0.9 <sup>a,b</sup>	4.1 ± 0.5 <sup>b</sup>	0.14 ± 0.05 <sup>b</sup>	0.05 ± 0.02 <sup>a,b</sup>	0.04 ± 0.02 <sup>b</sup>

a,b Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as determined using a one-way ANOVA. Data presented are the untransformed mean  $\pm$  s.d.  $n = 4$  for the Turface® and marsh sediment treatment,  $n = 5$  for the Long Point sediment.



Turface® Long Point Marsh  
Sediment Sediment  
**Substrate Type**

Figure 14: Visual assessment of Turface®, Long Point sediment and sediment from a marsh near Lake Erie for effects upon the growth of *M. sibiricum* after 14 days.



Turface® Formulated Formulated  
Sediment Sediment  
with Sucrose without  
Sucrose  
**Substrate Type**

Figure 15: *Myriophyllum sibiricum* plants grown with Turface® or a formulated sediment. The medium in the formulated sediment test tubes contained either sucrose or no carbon source.

(Tratnyek and Wolf, 1993). When Nelson and Fairchild (1994) used a soil extract for submersed macrophyte testing, no EDTA was used in the liquid growth medium. Changes to the modified Andrews' medium were not compared in the current study but modifications to the growth medium could be tested before using sediments. The axenic *Myriophyllum sibiricum* toxicity test could be used to determine whether or not there are toxic compounds in a natural sediment and what effects these compounds might have on aquatic plants. A control sediment would be required for experiments with natural sediment. An uncontaminated sediment, such as Long Point sediment, would have to be characterised to ensure that it was uncontaminated and similar in composition to the test sediment. For sediment toxicity testing situations, one advantage of this toxicity test over the algal or *Lemna* tests was that the *M. sibiricum* produced roots that were in direct contact with the substrate. Further research should be conducted on the appropriate liquid growth medium to use when sediments are being tested in the *M. sibiricum* toxicity test.

#### 4.3.3.2.1.3.5 Formulated Sediments

For plant height, area under the growth curve, root number or root length, there was no significant difference between plants grown with Turface® or with the formulated sediment (Hoffmann *et al.*, 1984) and 3% sucrose combination (Figure 15, Figure 16 and Table 16). Plants grown with the formulated sediment differed significantly from the Turface® control plants for other endpoint parameters, such as the amount of oxygen dissolved in the medium, fresh weight, plant area and pigment content (Table 16). Formulated sediments, such as the one designed by Hoffmann *et al.* (1984), could be used in the axenic *Myriophyllum sibiricum* toxicity test so long as the researcher recognises that the plants may have a decreased fresh weight, plant area and pigment content as compared to cultures grown with Turface®.

Plants grown with the formulated sediment without the sucrose in the medium were significantly smaller than plants grown with Turface®. All endpoints for plants grown in the formulated sediment without sucrose were significantly different from the control plants grown with Turface® as an artificial rooting medium (Figure 15, Figure 16 and Table 16). These results reinforce that the *Myriophyllum sibiricum* apices will not grow under these artificial conditions without an additional carbon source, such as sucrose. As was noted with the natural sediments, the medium in the tubes with the

formulated sediment contained suspended particulate matter that could reduce the light intensity reaching the plants (Figure 15). In toxicity testing, the effect of herbicides upon growth of aquatic plant seedlings was significantly greater in synthetic sediments than in natural sediments (Walsh *et al.*, 1991b). Therefore, if synthetic sediments are used for toxicity testing, it is important to be aware that toxicity might be enhanced. Formulated sediments other than the one developed by Hoffmann *et al.* (1984) could be tested in the *M. sibiricum* toxicity test. These synthetic sediments include those developed for aquatic macrophyte testing (Walsh *et al.*, 1991a; 1991b) and for invertebrate testing (Suedel and Rodgers, 1994).

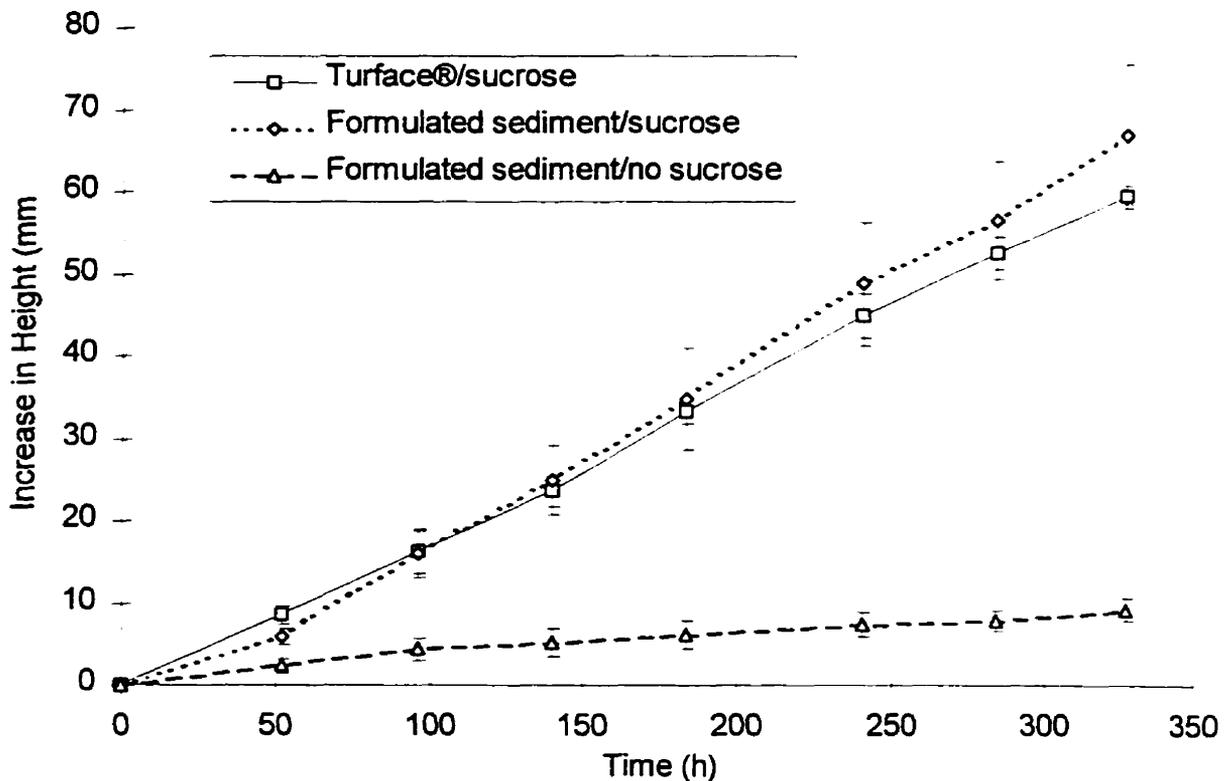


Figure 16: Fourteen day growth curves of *Myriophyllum sibiricum* plants grown with Turface® or a formulated sediment (Hoffmann *et al.*, 1984). In the tubes with the formulated sediment, the modified Andrews' medium contained either 3% sucrose or no sucrose. Sucrose was present in the liquid medium in the tubes with the Turface®.

Table 16: The effect of a formulated sediment (Hoffmann *et al.*, 1984) with and without sucrose added to the liquid modified Andrews' medium upon the growth and development of *Myriophyllum sibiricum*. Plants were cultured for 14 days in test tubes.

Treatment	Area under the growth curve	Shoot Length (mm)	Root #	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g total weight)	Chlorophyll b Content (mg/g total weight)	Carotenoid Content (mg/g total weight)
Turface <sup>®</sup> with Sucrose	9 648.3 ± 471.2 <sup>a</sup>	63.5 ± 3.0 <sup>a</sup>	8.4 ± 1.5 <sup>a</sup>	404.5 ± 68.5 <sup>a</sup>	60.8 ± 6.6 <sup>a</sup>	491.6 ± 51.0 <sup>a</sup>	6.8 ± 0.3 <sup>a</sup>	10.5 ± 1.5 <sup>a</sup>	0.26 ± 0.03 <sup>a</sup>	0.09 ± 0.01 <sup>a</sup>	0.09 ± 0.01 <sup>a</sup>
Formulated Sediment with Sucrose	10 173.8 ± 1 478.6 <sup>a</sup>	74.0 ± 11.0 <sup>a</sup>	7.2 ± 0.8 <sup>a</sup>	380.4 ± 75.9 <sup>a</sup>	39.2 ± 2.5 <sup>b</sup>	322.7 ± 73.4 <sup>b</sup>	7.3 ± 0.9 <sup>a</sup>	6.1 ± 2.3 <sup>b</sup>	0.10 ± 0.02 <sup>b</sup>	0.03 ± 0.005 <sup>b</sup>	0.04 ± 0.009 <sup>b</sup>
Formulated Sediment without Sucrose	1 764.0 ± 386.6 <sup>b</sup>	11.0 ± 2.3 <sup>b</sup>	1.2 ± 0.4 <sup>b</sup>	25.8 ± 11.8 <sup>b</sup>	68.6 ± 2.2 <sup>c</sup>	84.1 ± 16.8 <sup>c</sup>	42.1 ± 7.2 <sup>b</sup>	2.2 ± 0.7 <sup>b</sup>	0.05 ± 0.015 <sup>c</sup>	0.02 ± 0.005 <sup>c</sup>	0.02 ± 0.004 <sup>c</sup>

a,b Any two means in the same column with the same superscript were significantly different at  $\alpha = 0.05$  as determined using either a one-way ANOVA (weight, chlorophyll a and carotenoid content) or one-way Kruskal-Wallis test. Values are means of five replicates  $\pm$  s.d. For membrane integrity of plants grown with the formulated sediment without the sucrose, there were only 4 replicates.

#### 4.3.4 Growth Media Selection

##### 4.3.4.1 Introduction

This chapter has been published (Roshon *et al.*, 1996) and summarises the results from two experiments comparing *Myriophyllum sibiricum* growth in different media. Concern exists that nutrient supplements used in toxicity tests can interfere with toxicity results. Therefore, Wang (1991) recommended seed germination tests. Unfortunately, seed germination tests examine only the effect of a toxicant to a higher plant during a small portion of the possible time that the plant would be exposed in the environment. Plant seeds contain food reserves that benefit the germinating embryo and might influence the test results. Medium composition affected the toxicity of several metals to the growth of *Lemna paucicostata* Hegelm. ex Engelm. (= *L. perpusilla* Torrey). There was a ten-fold difference in the toxicity of some metals depending on the medium composition (Nasu and Kugimoto, 1981).

There are numerous media used for the laboratory culture of aquatic macrophytes. Five commonly used liquid culture media (Murashige and Skoog (M & S), Hoagland's, Gaudet's, modified Andrews', and Hard Water (HW) media) were compared. M & S medium (Murashige and Skoog, 1962) was originally designed for tobacco tissue culturing. It has been successfully used in tissue culture or solution culture experiments with aquatic plants, including other species of *Myriophyllum*. Some of these experiments examined the effect of herbicides upon plant growth (Bird, 1993; Christopher and Bird, 1992), while other experiments examined the effects of growth hormones (Kane and Albert, 1987; 1989b; Kane and Gilman, 1991; Kane *et al.*, 1988b). Fleming *et al.* (1991) used Murashige Shoot Multiplication Medium B for propagating and testing *Potamogeton pectinatus*. Hoagland's (Hoagland and Arnon, 1938) medium, the most commonly used medium for plant culture, has been used extensively for both terrestrial and aquatic plant culturing in its original (Hinman and Klaine, 1992) and modified forms (ASTM, 1991b). Powell (1993) recommended half strength Hoagland's for the culture and toxicity testing of emergent macrophytes. Nelson<sup>6</sup>, Hughes<sup>7</sup> and Alexander<sup>8</sup> (personal communication) have recommended a 10% Hoagland's solution for culturing *Ceratophyllum demersum* and *Myriophyllum heterophyllum*. They had experimented with different concentrations of Hoagland's medium (1%, 10% and 25%) and 1 x and 10 x the medium recommended by ASTM (1991b) for *Lemna* testing. The medium was

made using well water (Nelson and Fairchild, 1994). Sutton *et al.* (1969) cultured *M. brasiliense* in full strength Hoagland's solution but then transferred the young plants to half strength Hoagland's medium for testing. *M. aquaticum* plants cultured in one-tenth Hoagland's solution had a lower dry weight than plants cultured in one-half or stronger strength Hoagland's medium. This suggested that high concentrations of nutrients are required for good growth of *M. aquaticum* (Sutton, 1985). Gaudet's medium was developed to examine the mechanism which forced an aquatic macrophyte to switch between the aquatic and land forms (Gaudet, 1963) but has subsequently been utilised to study aquatic plant nutrition (Bristow and Whitcombe, 1971). For the culture of *M. spicatum*, Gerloff's (Gerloff and Krombholz, 1966) medium was modified by Andrews (Selim *et al.*, 1989). Guilizzoni *et al.* (1984) combined the medium recommended by Gerloff and Krombholz (1966) and Andrews (1980, as cited in Guilizzoni *et al.*, 1984) for experiments with *M. spicatum*. A bicarbonate-containing medium was developed specifically for aquatic plants normally found growing in hard water lakes (Smith, 1993) and has been used to study plant/pathogen interactions. The majority of studies on aquatic plant culturing have compared few media and have examined a limited number of endpoints. This study was undertaken to find a liquid medium which produced rapid and consistent development in axenically cultured *M. sibiricum*. Five different growth media and nine different morphological and physiological endpoints were evaluated.

#### 4.3.4.2 Methods

Using an axenic culture of *M. sibiricum*, stems were cut into segments, transferred into sterile culture tubes (150 x 25 mm) containing 45 mLs of half strength Murashige and Skoog Basal Salts with minimal organics (Sigma Chemical Company, St. Louis, MI, U.S.A.) and 3% sucrose (Kane and Gilman, 1991). After ten days, one 3 cm long *M. sibiricum* axillary bud from each clonal stock plant was transferred into culture tubes containing 40 mL of sterile test medium and 3 g of sterile Turface<sup>®</sup>. A 15 cm length of Westergren Blood Sedimentation Tube, marked in mm, was placed in the centre of the test tube and held in place by a 3 cm piece of Tygon<sup>®</sup> tubing glued into the cap. Each treatment was replicated five times. The results from two experiments are reported here. The first was a comparison of the five full strength media, for which the chemical composition can be found in Table 17. All stock solutions and final media were made with nanopure water (Barnstead Nanopure II) and each medium was supplemented with 3%

sucrose. Preliminary experiments demonstrated that this species was unable to grow substantially in culture without the addition of an external carbon source, especially sucrose. The second experiment further evaluated modified Andrews' and Hoagland's media. Racks of randomized plants were maintained in a growth cabinet set at  $25 \pm 2^\circ\text{C}$ , a 16 hour day/8 hour night and a light intensity of  $100 \pm 5 \mu\text{E m}^{-2} \text{s}^{-1}$ . Growth curves were established by visually measuring the plant length every second day and the area under the growth curve was determined.

Table 17: Elemental breakdown of the media tested in the *Myriophyllum* toxicity test.

Element	Final Concentrations of Elements in Media (mmol/L)				
	M+S With Organics	Hoagland's	Gaudet's	Modified Andrews'	Hard Water
C	1061	1057	1057	1057	5457
N	60.2	16.0	3.4	2.6	2.4
K	16.0	6.0	1.7	1.0	1.0
P	2.5	1.0	0.8	0.2	0.2
Ca	3.4	1.7	1.3	0.8	1.2
S	25.0	20.0	5.0	5.0	0.4
Mg	9.1	6.0	3.0	3.0	1.0
Cl	0.30	0.01	0.03	0.01	1.2
B	0.10	0.025	0.0243	0.0025	0.0025
Cu	1e-04	5e-04	1.6e-04	5e-05	5e-05
Co	9e-05	0	0	0	0
Fe	0.10	0.02	0.01	0.01	0.01
Mn	0.0999	0.002	0.0201	0.001	0.001
Mo	0.001	0.00055	3.3e-05	2e-05	2e-05
Zn	0.0299	0.002	0.0104	0.0004	0.0004

At the end of two weeks, shoot and total root length, plant area, chlorophyll and carotenoid content, fresh weight and membrane integrity were determined. These morphological and physiological parameters were selected because they should cover most of the possible effects caused by toxicants during pesticide testing. Dry weight on the remaining stem segments was also determined but is not presented here. Pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) were extracted into 10 mL of 80% ethanol

from each 50 mg apical segment (Lichtenthaler and Wellburn, 1983) and measured on a spectrophotometer (Beckman Du<sup>®</sup>-65) at 470, 647 and 663 nm. Modifying the method of Beckerson and Hofstra (1980), membrane integrity was determined by placing a 100 mg stem segment into 20 mL of nanopure water for 24 hours. The conductivity of the solution was measured using a portable conductivity meter (Corning<sup>®</sup> Checkmate 90 Field System), the tubes were placed into boiling water for 20 min and allowed to cool. The conductivity of the solution was measured again to determine the conductivity after complete membrane disruption. Membrane integrity was determined as percentage of total electrolyte leakage.

For the experiment comparing the five media, the data were tested for normality, log transformed (except for root number which was square root transformed) and analyzed using a single classification ANOVA. The Tukey-Kramer test was used to determine which means differed. The results from the second experiment were compared using the nonparametric Mann-Whitney U-test (Sokal and Rohlf, 1981).

#### 4.3.4.3 Results and Discussion

The media selection process was an intensive study of the five media but only selected results are discussed here.

##### 4.3.4.3.1 Comparison of All Five Media

The increase in shoot length over the two week experiment for *M. sibiricum* grown in the five media is shown in Figure 17. The media separated into three groups; with the Gaudet's producing the smallest increase in height, plants grown in Hoagland's and M & S produced the greatest increase in shoot height, and the remaining two media were intermediate in shoot height (Table 18). The area under the growth curve compares not only the final height but also the rate at which the plants grew (Boutin *et al.*, 1993). From Figure 17 and Table 18, it is evident that the media divided into the same three categories. It is noteworthy that even though plants grown in HW medium had a final height slightly greater than in modified Andrews' medium, they had a slightly smaller area under the growth curve (not statistically significant at  $p = 0.05$ ). This may be due to their slower initial growth rate.

Roots are important to aquatic plant growth because they absorb ions from the sediment (Bristow and Whitcombe, 1971; Mantai and Newton, 1982). The M & S medium (Table 18) produced the lowest number of roots and the shortest total root length, while

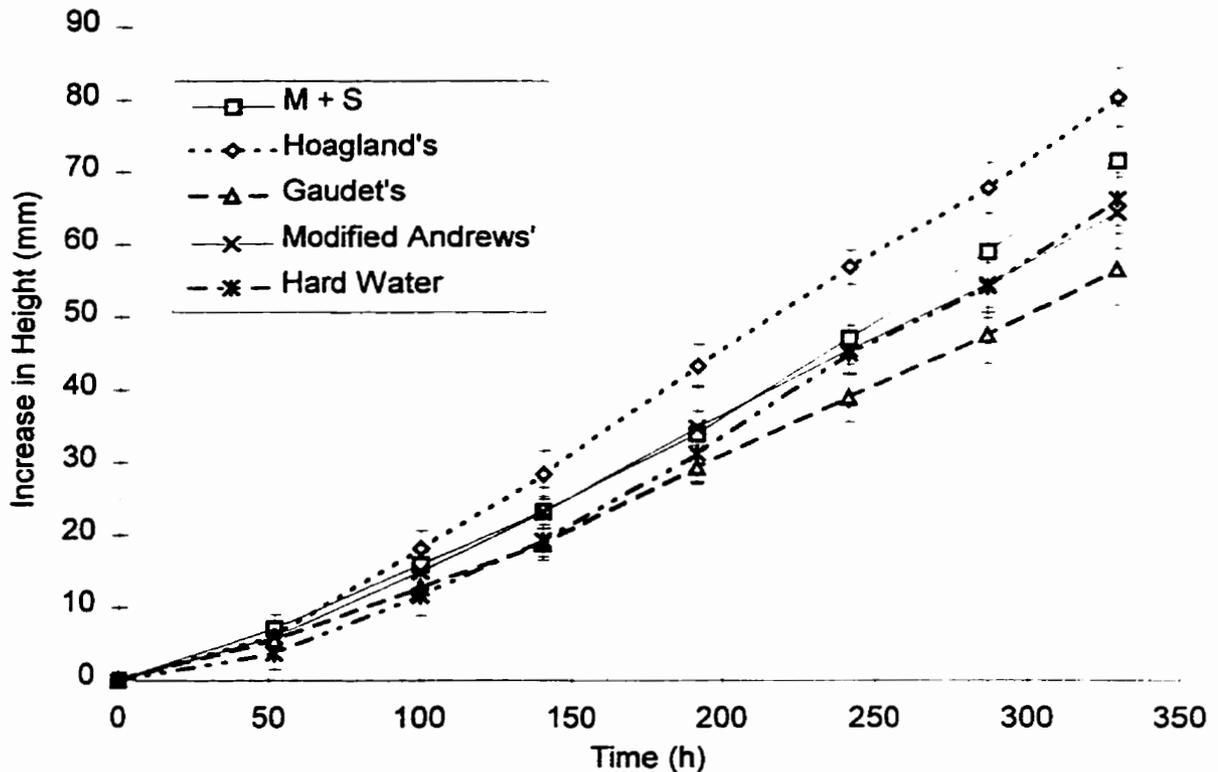


Figure 17: *Myriophyllum sibiricum* growth in Murashige & Skoog, Hoagland's, Gaudet's, modified Andrews' and Hard Water media (mean  $\pm$  s.d.) (n = 5, except for HW medium where n = 4).

there was no statistically significant difference between the roots of plants grown in the other media ( $\alpha = 0.05$ ). Fleming *et al.* (1991) found that with Murashige Shoot Multiplication Medium B as the testing medium, *Potamogeton pectinatus* plants produced few roots or none. If roots were produced, they were often short (Fleming *et al.*, 1991).

The pigment content of apices grown in M & S was significantly higher than for apices grown in the other media. Chlorophyll *b* and carotenoid content of apices grown in the other four media were not significantly different.

Changes in membrane permeability are indicated by cellular leakage. The high membrane permeability value obtained with the M & S medium possibly indicates that this medium disrupts cell membrane function. The lower percentage leakage from plants grown in the other four media indicate normal membrane function (Beckerson and Hofstra, 1980; Dijak and Ormrod, 1982).

Hoagland's and modified Andrews' media produced plants with the greatest area and weight. The modified Andrews' medium was the only medium which induced the formation of branches. The formation of branches is an important component of this toxicity test because this species has a branched growth form in natural environments (Aiken, 1981; Sculthorpe, 1971).

Based upon this experiment, Gaudet's medium was eliminated from further consideration due to the small increase in shoot length of plants grown in it. Due to the small plant area and the low fresh weight of plants grown in M & S medium, it was dropped from consideration. HW medium was not considered an acceptable medium because of it caused a slow initial growth rate (Figure 17). Another problem observed with the HW medium, designed to examine plant-microbe interactions (Smith, 1993), was accidental colonization by bacteria and fungi. In this experiment one sample was lost due to bacterial contamination.

There was no significant difference between the Hoagland's and modified Andrews' medium for root number and length, total fresh weight, chlorophyll *b* and carotenoid content, membrane integrity and plant area.

#### 4.3.4.3.2 Comparison of Two Media

Based on the above study, the Hoagland's medium was further compared with the modified Andrews' medium. Figure 18 displays the growth curve for this experiment. For

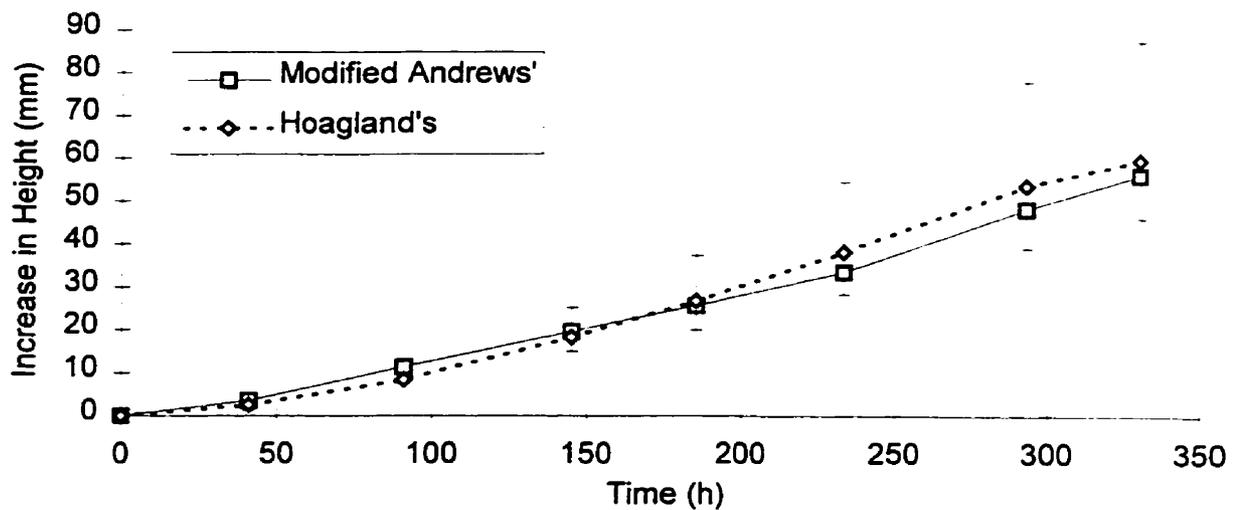


Figure 18: Growth curves for *Myriophyllum sibiricum* from the repeated experiment of the comparison between Hoagland's and modified Andrews' media (mean  $\pm$  s.d.).

Table 18: *Myriophyllum sibiricum* growth in five media used in aquatic plant culture (mean  $\pm$  s.d.).

	Area under the growth curve	Increase in Shoot Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant area (cm <sup>2</sup> )
M & S	10 290.4 $\pm$ 1 568.9 <sup>a,b</sup>	81.6 $\pm$ 9.8 <sup>a</sup>	3.8 $\pm$ 1.6 <sup>a</sup>	130.2 $\pm$ 54.7 <sup>a</sup>	439.2 $\pm$ 116.4 <sup>a</sup>	0.60 $\pm$ 0.04 <sup>a</sup>	0.22 $\pm$ 0.02 <sup>a</sup>	0.20 $\pm$ 0.017 <sup>a</sup>	35.6 $\pm$ 9.5 <sup>a</sup>	10.6 $\pm$ 4.5 <sup>a</sup>
Hoagland's	12 091.9 $\pm$ 724.5 <sup>a</sup>	89.1 $\pm$ 3.6 <sup>a</sup>	9.2 $\pm$ 0.8 <sup>b</sup>	402.0 $\pm$ 58.7 <sup>b</sup>	741.6 $\pm$ 72.6 <sup>c</sup>	0.59 $\pm$ 0.04 <sup>a,b</sup>	0.21 $\pm$ 0.02 <sup>a,b</sup>	0.19 $\pm$ 0.011 <sup>a,b</sup>	12.4 $\pm$ 0.5 <sup>b</sup>	18.3 $\pm$ 1.8 <sup>b</sup>
Gaudet's	8 394.6 $\pm$ 639.2 <sup>c</sup>	61.4 $\pm$ 6.6 <sup>c</sup>	7.8 $\pm$ 1.3 <sup>b</sup>	482.9 $\pm$ 138.5 <sup>b</sup>	466.8 $\pm$ 63.9 <sup>a,b</sup>	0.52 $\pm$ 0.03 <sup>b,c</sup>	0.20 $\pm$ 0.02 <sup>a,b</sup>	0.18 $\pm$ 0.008 <sup>b</sup>	9.4 $\pm$ 0.8 <sup>b,c</sup>	10.4 $\pm$ 2.0 <sup>a</sup>
Modified Andrews'	9 781.8 $\pm$ 782.2 <sup>b,c</sup>	68.1 $\pm$ 3.9 <sup>b,c</sup>	9.6 $\pm$ 0.9 <sup>b</sup>	420.5 $\pm$ 37.1 <sup>b</sup>	560.2 $\pm$ 30.3 <sup>b,c</sup>	0.51 $\pm$ 0.01 <sup>c</sup>	0.18 $\pm$ 0.01 <sup>b</sup>	0.17 $\pm$ 0.003 <sup>b</sup>	9.4 $\pm$ 1.6 <sup>b,c</sup>	13.1 $\pm$ 1.2 <sup>a,b</sup>
Hard Water	9 162.9 $\pm$ 631.4 <sup>b,c</sup>	78.5 $\pm$ 5.1 <sup>a,b</sup>	9.0 $\pm$ 0.8 <sup>b</sup>	566.7 $\pm$ 45.1 <sup>b</sup>	485.1 $\pm$ 49.7 <sup>a,b</sup>	0.50 $\pm$ 0.03 <sup>c</sup>	0.17 $\pm$ 0.02 <sup>b</sup>	0.17 $\pm$ 0.008 <sup>b</sup>	7.5 $\pm$ 0.5 <sup>c</sup>	10.3 $\pm$ 0.3 <sup>a</sup>

a,b,c Any two values in the same column with the same superscript are not significantly different at  $\alpha = 0.05$ . n = 5, except for HW medium where n = 4.

plants grown in these two media, the two parameters which differed statistically ( $\alpha = 0.05$ ) were total root length and area under the growth curve. Because a larger standard deviation was obtained with the Hoagland's medium, the modified Andrews' medium was adopted for subsequent pesticide experiments.

From the existing media cited in the literature, there does not appear to be a "perfect" medium for the axenic culture of *M. sibiricum*. However, modified Andrews' medium at a pH of 5.8 and without the addition of a buffer was selected for further use in pesticide experiments. Plants with large total root length, root number, total fresh weight and plant area could be produced consistently and variation in growth parameters between replicates was acceptably low.

#### 4.3.5 Carbon Source Comparison

##### 4.3.5.1 Introduction

Aquatic plants, like terrestrial plants, absorb and utilise carbon by different methods. The method of utilisation may be either C<sub>3</sub>, C<sub>4</sub> or CAM. Plants utilising these various pathways differ in biochemical, physiological and anatomical features (Bowes, 1985). The Calvin cycle, which is also termed the photosynthetic carbon reduction cycle or C<sub>3</sub> photosynthesis, is the most common method of incorporating CO<sub>2</sub> and is found in all plant species. The first product of C<sub>3</sub> photosynthesis is a three-carbon compound (glyceraldehyde 3-phosphate). Some plant species use CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> to produce compounds containing four carbons in the process termed C<sub>4</sub> photosynthesis. Oxaloacetate, the first four carbon compound, is reduced to malate or aspartate. These plants often have chloroplasts in their bundle sheath cells in an arrangement termed Kranz anatomy. Crassulacean Acid Metabolism (CAM) involves fixing CO<sub>2</sub> in the absence of light to form malic acid. More details regarding the different types of carbon dioxide fixation may be found in most general biology and plant physiology textbooks (e.g., Keeton, 1980; Lea and Leegood, 1993; Raven *et al.*, 1986; Salisbury and Ross, 1985) and the review article by Raven (1970).

Environmental factors, such as pH (Adams, 1985; Keeley and Sandquist, 1992; Madsen and Sand-Jensen, 1991; Miller, 1985; Sand-Jensen, 1983; Spence and Maberly, 1985), alkalinity (Allen and Spence, 1981; Madsen and Sand-Jensen, 1991; Peñuelas and Menéndez, 1990; Spence and Maberly, 1985), turbulence (Keeley and Sandquist, 1992), light and temperature (Allen and Spence, 1981; Raven, 1970; Spence

and Maberly, 1985) may affect the ratio of  $\text{CO}_2/\text{HCO}_3^-$  and the method of carbon assimilation. The ratio of  $\text{CO}_2$  to  $\text{HCO}_3^-$  varies seasonally and diurnally and thus influences carbon assimilation (Bowes, 1985; Keeley and Sandquist, 1992). Between freshwater ecosystems, there is a 20 fold difference in levels of dissolved inorganic carbon (Adams, 1985).

Different macrophyte species within the same genus may differ in their ability to assimilate  $\text{HCO}_3^-$  (Prins *et al.*, 1982). Within an aquatic plant species, there is a lot of phenotypic plasticity in the ability to utilise  $\text{HCO}_3^-$  (Bowes, 1985; Keeley and Sandquist, 1992; Madsen and Sand-Jensen, 1991; 1994) and this ability is often induced at low  $\text{CO}_2$  levels and suppressed at high ambient  $\text{CO}_2$  concentrations (Madsen and Sand-Jensen, 1994). Different plant tissues can vary in their ability to utilise  $\text{HCO}_3^-$  as a carbon source. The stems, turions, aerial and young leaves of some submersed macrophytes are not able to utilise  $\text{HCO}_3^-$  even though mature leaves are able to employ  $\text{HCO}_3^-$  (Mitchell and Orr, 1985; Prins *et al.*, 1982; Salvucci and Bowes, 1982; Spence and Maberly, 1985). The age of the macrophyte also influences the rate of carbon uptake (Nalewajko and Godmaire, 1993).

Among aquatic macrophytes there are species of plants that utilise different methods of carbon fixation. For numerous aquatic plants, the type of carbon fixation is summarised in the scientific literature (Keeley and Sandquist, 1992; Madsen and Sand-Jensen, 1991; Spence and Maberly, 1985). All aquatic plants are able to use  $\text{CO}_2$  to some extent. Anatomical adaptations, such as finely dissected leaves, thin cuticle and epidermal chloroplasts, allow some species of submersed aquatic plants to extract  $\text{CO}_2$  from the water (Bowes, 1985; Madsen and Sand-Jensen, 1991). Other aquatic macrophytes are able to utilise atmospheric  $\text{CO}_2$  or can extract  $\text{CO}_2$  from the sediment (Bristow, 1975; Madsen and Sand-Jensen, 1991; Spence and Maberly, 1985). Obligate  $\text{C}_3$  plants include *Callitriche cophocarpa* Sendtner (Madsen and Sand-Jensen, 1994), *C. stagnalis* Scop., *Sparganium simplex* Huds. (Sand-Jensen, 1983), *Nuphar lutea* (= *N. microphyllum* (Pers.) Fern.), *Ludwigia natans* Ell., *Echinodorus tenellus* (Mart.) Buchenau. (burhead), *E. paniculatus* P. Micheli. (Amazon sword plant), *Riccia fluitans* L. (crystal wort) (Prins *et al.*, 1982), *Fontinalis antipyretica* Hedw., *Potamogeton polygonifolius* Pourret (Allen and Spence, 1981; Black *et al.*, 1991), *Utricularia purpurea* Walt. (bladderwort) (Moeller, 1978), *Myriophyllum hippuroides* Nutt. (Prins *et al.*, 1982),

*M. brasiliensis* (Adams, 1985; Keeley and Sandquist, 1992; Prins *et al.*, 1982) and *M. verticillatum* (Keeley and Sandquist, 1992; Prins *et al.*, 1982).

There is a high concentration of bicarbonate in most freshwater and marine systems (Madsen and Sand-Jensen, 1991). In alkaline water, the  $\text{HCO}_3^-$  concentration is quite high and  $\text{CO}_2$  may be limited so  $\text{HCO}_3^-$  becomes an alternative carbon source for many macrophyte species (Prins *et al.*, 1982). The ability to utilise the bicarbonate ion for photosynthesis allows completely submersed plants to grow in waters with a pH greater than 8.3 (Mitchell and Orr, 1985). More details describing the chemical mechanisms involved in diffusion of gases in aqueous solution can be found in the literature (Madsen and Sand-Jensen, 1991; Miller, 1985; Smith, 1985; Spence and Maberly, 1985). Within the plants that can use bicarbonate, there appear to be two methods of bicarbonate usage. The bicarbonate ion may be absorbed by the lower leaf surface or converted into  $\text{CO}_2$  by acidification of the cell wall space. Hydroxyl ions are excreted from the upper leaf surface in a polarised transport process (Mitchell and Orr, 1985; Prins *et al.*, 1982; Prins and Helder, 1985). This process is accompanied by transport of cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and/or  $\text{Na}^+$ ) from the solution in contact with the lower leaf surface to the upper surface (Madsen and Sand-Jensen, 1991; Prins *et al.*, 1982). Other bicarbonate utilising plant species have a non-polar uptake mechanism (Prins *et al.*, 1982), in which exposure to light initiates the carbon uptake process and the pH at both leaf surfaces increases (Madsen and Sand-Jensen, 1991). Based on a species bicarbonate compensation point, there is a range in the amount of bicarbonate used (Allen and Spence, 1981; Peñuelas and Menéndez, 1990; Spence and Maberly, 1985). Fifty percent of the species tested have the ability to use bicarbonate (Madsen and Sand-Jensen, 1991) and the majority of these are monocotyledonous angiosperms (Madsen and Sand-Jensen, 1991; Spence and Maberly, 1985). *Elodea canadensis* (Allen and Spence, 1981; Madsen and Sand-Jensen, 1994), *Myriophyllum spicatum* (Keeley and Sandquist, 1992; Mitchell and Orr, 1985; Owttrim and Colman, 1989; Prins *et al.*, 1982; Spence and Maberly, 1985), *Ranunculus aquatilis* L. (Keeley and Sandquist, 1992), *Egeria densa*, *Vallisneria spiralis* L., *Hydrilla verticillata*, *Ceratophyllum demersum*, *Potamogeton lucens* (= *Potamogeton illinoensis* Morong.), *P. perfoliatus* (Prins *et al.*, 1982), *P. crispus* (Allen and Spence, 1981; Sand-Jensen, 1983), *P. pectinatus* (Sand-Jensen, 1983) and *Ruppia cirrhosa* (Peñuelas and Menéndez, 1990) use both  $\text{CO}_2$  and  $\text{HCO}_3^-$ . In aquatic plants, the use of  $\text{HCO}_3^-$  does not

necessarily indicate C<sub>4</sub> physiology (Raven, 1985). Aquatic C<sub>4</sub> plants differ from terrestrial C<sub>4</sub> plants because they lack true Kranz anatomy (Bowes, 1985; Madsen and Sand-Jensen, 1991; Smith, 1985).

Some aquatic plants can fix CO<sub>2</sub> in the dark and exhibit the crassulacean acid metabolic (CAM) pathway. These plants are mainly isoetids (Madsen and Sand-Jensen, 1991), including *Isoetes howellii* Engelm. (Keeley and Sandquist, 1991; Keeley and Sandquist, 1992), *I. lacustris* L., *I. orcuttii* A.A. Eaton, *I. bolanderi* Engelm. (Keeley and Sandquist, 1992), plus other species, such as *Littorella* spp. and *Crassula* spp. (Bowes, 1985). In *Littorella uniflora* (L.) Asch. and *Isoetes macrospora* Dur., CAM contributed 40 to 50% of the annual carbon gain (Adams, 1985). CAM plants inhabit slightly acidic soft waters (Madsen and Sand-Jensen, 1991).

The introduction of CO<sub>2</sub> or air into an aquatic laboratory system has been shown to be a successful method of providing carbon for aquatic macrophytes. High and low carbon levels were obtained by bubbling air or CO<sub>2</sub> enriched air into aquaria (Hoffmann *et al.*, 1984), culture flasks (Huebert *et al.*, 1990) or jars (Ailstock *et al.*, 1991).

*Myriophyllum spicatum* cultures, used in herbicide testing, were continuously aerated with 0.5% CO<sub>2</sub> enriched air (Netherland and Lembi, 1992). Air enriched with 0.5 to 1% CO<sub>2</sub> was continuously injected into modified Andrews' medium containing *M. spicatum* segments (Selim *et al.*, 1989). After 6 weeks of culturing, aeration of Hoagland's nutrient solution (full and one-tenth strength) did not influence the dry weight of *M. aquaticum* (Sutton, 1985). In a study by Guilizzoni *et al.* (1984), filtered air was continuously bubbled into flasks containing liquid growth medium and *Myriophyllum spicatum* segments.

Other toxicity tests with aquatic plants have used sucrose in the liquid growth medium. Christopher and Bird (1992) tested the effect of herbicides and leaf defoliants on the development of axillary buds, leaves, roots and branches of *Myriophyllum spicatum* in tissue culture. The medium utilised was M & S with 3% sucrose (Christopher and Bird, 1992). Medium for culturing and testing *Lemna paucicostata* was supplemented with 1% sucrose before autoclaving (Nasu and Kugimoto, 1981). ASTM (1991b) recommended that the liquid growth medium contain sucrose for culturing *L. gibba* but that the sucrose be removed for toxicological experimentation (ASTM, 1991b). Tissue culturing medium is often supplemented with 2% (20 g/L) (Dixon, 1985; Kakkar and Ram, 1986; Kane and Albert, 1989a; Morris *et al.*, 1985; Rao and Ram, 1981;

Wainwright and Marsh, 1986) or 3% (30 g/L) sucrose (Chang and Hsing, 1978; Dixon, 1985; Kane and Albert, 1977; 1989b; Kane and Gilman, 1991; Kane *et al.*, 1988a; 1988b; 1991; Morris *et al.*, 1985). Glucose has also been used as a organic carbon source for culturing aquatic plants (Huebert *et al.*, 1990).

Mannitol, singly or in combination with sucrose, has been used as a carbon source for some tissue cultures (Bolwell, 1985; Power and Chapman, 1985). Power and Chapman (1985) recommended 9% mannitol for protoplast fusion and 3% sucrose combined with 9% mannitol for protoplast culture. A combination of 10 mg/L sucrose and 36 mg/L mannitol were used for culturing *Zinnia elegans* Jacq. (common zinnia) mesophyll cells (Bolwell, 1985).

Herbicide toxicity tests with *Myriophyllum spicatum* and *Hydrilla verticillata* used filtered NaHCO<sub>3</sub> added to either Gerloff's or Hoagland's liquid growth media after autoclaving (Netherland and Lembi, 1992). Static toxicity tests with either *Ceratophyllum demersum* or *Myriophyllum heterophyllum* were conducted with plants grown in a 10% Hoagland's solution (personal communication with M. Nelson<sup>6</sup>) buffered with 200 mg/L NaHCO<sub>3</sub> (Byl and Klaine, 1991). Bicarbonate was added to liquid growth medium as a carbon source in studies with *Lemna trisulca* L. (Huebert *et al.*, 1990), *M. spicatum* (Guilizzoni *et al.*, 1984) and *Potamogeton pectinatus* (Ailstock *et al.*, 1991).

The enormous variety of carbon sources used in laboratory culture of aquatic macrophytes indicated a need to determine an appropriate source of carbon for *Myriophyllum sibiricum*. Numerous experiments were initiated to determine the best source of carbon for use in the axenic *M. sibiricum* toxicity test.

#### 4.3.5.2 Methods

##### 4.3.5.2.1 Aeration

Two slightly different test tube aeration systems were designed and compared to determine if aeration could improve *M. sibiricum* growth in this system. The first system consisted of an Elite<sup>®</sup> 802 air pump that forced air through a main polyethylene line. Using a 2-way splitter, this main air line was subdivided into 2 sublines, each of which entered an Ultra air control manifold that subdivided the system into five smaller lines. In the middle of each of the ten small air lines, a 3.0 µm Brinkman<sup>®</sup> autoclavable membrane filter (replacement for the Brinkman<sup>®</sup> Pipet Helper) was installed to filter the ambient air. The end of each small air line was attached to a glass Pasteur pipette (23

mm long) that entered the test tube through a small opening in the translucent test tube closure. The narrow end of the Pasteur pipette was in contact with the Turface<sup>®</sup>, while the end of the pipette attached to the tubing was secured to the test tube closure using laboratory sealant film. In the second air delivery system (Figure 19), an Elite<sup>®</sup> 802 air pump delivered air through a main polyethylene line. The end of the main air line was clamped shut. At regular intervals along the main air line, small needles (hypodermic replacement needles, O.D. = 0.64 mm, 25 mm long) were inserted. Each needle was attached to a 3.0 µm Brinkman<sup>®</sup> autoclavable membrane filter, which was attached to an individual section of polyethylene tubing. Each of these individual lines entered a test tube through a small opening in the test tube closure with the help of a stainless steel nitrogen-evaporator needle (17 gauge, 89 mm long). The narrow opening of the needle was positioned above the surface of the Turface<sup>®</sup>. The upper end of the N-evap needle was secured to the hose and the test tube closure with fine wire and ducktape.

In both aeration experiments, three types of media were tested: full strength modified Andrews' medium with 30 g/L of sucrose, full strength modified Andrews' medium without sucrose and Hard Water medium without sucrose. Using nanopure water, the media were prepared based on the composition listed in Table 17. For the first experiment, the media were autoclaved at 121 °C for 20 min followed by a slow exhaust cool down segment. In the second experiment, all three media were filter sterilised through a 0.2 µm Supor<sup>®</sup> filter. The equipment used in both experiments (test tubes with 3 g of Turface<sup>®</sup>, test tube closures, the air injection equipment, forceps, Petri plates) was autoclaved at 121 °C for 20 min with a final fast exhaust segment. Under the Laminar airflow hood, 40 mL of each type of media were aseptically pipetted into five replicate test tubes. Axenic *M. sibiricum* segments (3 cm), which had been growing in half strength M & S medium, were randomly transferred into the test tubes. Westergren measuring rods were placed into each test tube. Test tube closures were fixed on top of each test tube and the appropriate injection needle was inserted through the closure into the medium. In order to determine if the aeration system was an effective alternative to supplementing the medium with sucrose in a non-aerated system, only the two media lacking sucrose were aerated. Initial plant height was determined. Once the test tubes were randomised within the growth cabinet, the air pump was started and the air flow regulated. The plants were allowed to incubate for fourteen days, after which the plants were harvested.

#### 4.3.5.2.2 Different Concentrations of Sucrose

Experiments were conducted with full strength Hoagland's and modified Andrews' media with various amounts of sucrose. Appropriate amounts of sucrose were added to 250 mL of medium to obtain 0.0, 0.5, 1.0, 5.0, 10.0, 15.0 and 30.0 g/L of sucrose. The medium was autoclaved after the addition of the sucrose. Apical segments (3 cm) of *Myriophyllum sibiricum*, which had been cultured in half strength M & S medium, were transferred into test tubes containing 40 mL of the modified Andrews' medium containing the various concentrations of sucrose. Each tube also contained 3 g of sterile Turface® and a 15 cm measuring rod. Every treatment was replicated five times. Initial plant height was measured and all the test tubes were randomised in test tube racks. During the 14 day growth period, plant height was measured seven times. After the incubation period, the plants were harvested and measured for total shoot height, root number and length, dissolved oxygen in the medium, fresh weight, plant area, membrane integrity and the amount of chlorophyll *a*, chlorophyll *b* and carotenoid pigment in the apex. The data were tested for normality. Normal data were analysed with a one-way ANOVA followed by multiple comparisons if differences were detected. The Kruskal-Wallis test and multiple comparisons were used to detect differences in nonparametric data.

#### 4.3.5.2.3 Mannitol versus Sucrose Comparison

In two separate experiments, full strength modified Andrews' or Hard Water media was prepared and 250 mL were measured into seven 500 mL Erlenmeyer flasks. The appropriate amount of sucrose and mannitol was added to each flask to maintain a constant carbon content. The flasks containing the medium plus the additional carbon were autoclaved. Forty mL of medium were pipetted into five replicate test tubes containing sterile Turface®. Three centimetre *Myriophyllum sibiricum* apical segments, which had been cultured in half strength M & S medium supplemented with 30 g/L sucrose, were utilised in this experiment. A measuring rod was added to each tube. The plants were incubated for 14 days and plant height was determined every second day. Plants were harvested and the following parameters were measured: plant height, dissolved oxygen in the medium, root number, root length, total fresh weight, plant area, membrane integrity and pigment content (chlorophyll *a*, chlorophyll *b* and carotenoid). The data were tested for normality. If the data were normal, a one-way ANOVA was

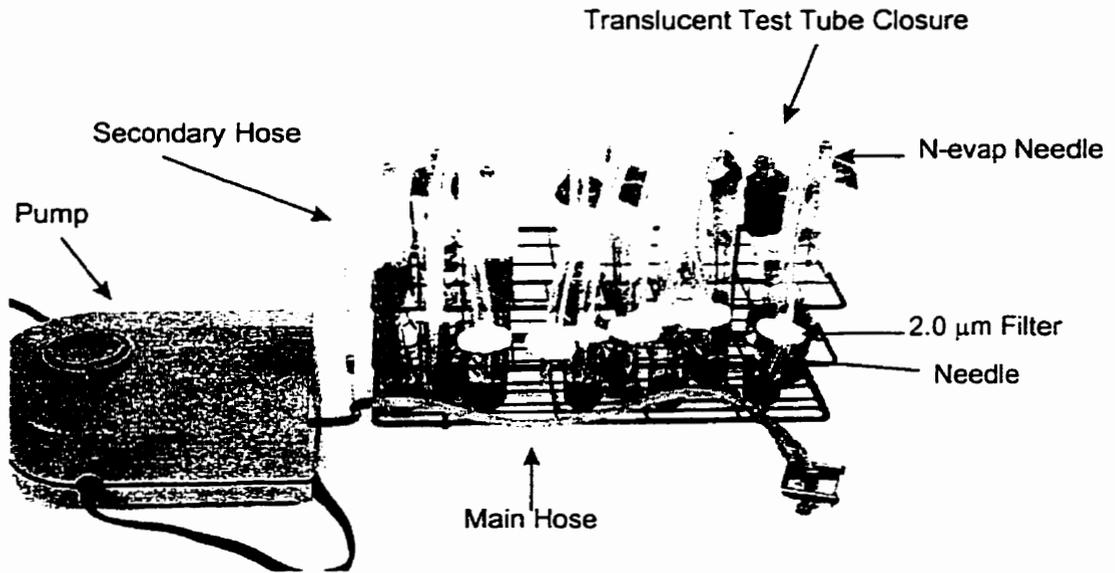


Figure 19: An example of an air delivery system tested with the axenic *Myriophyllum sibiricum* toxicity test.

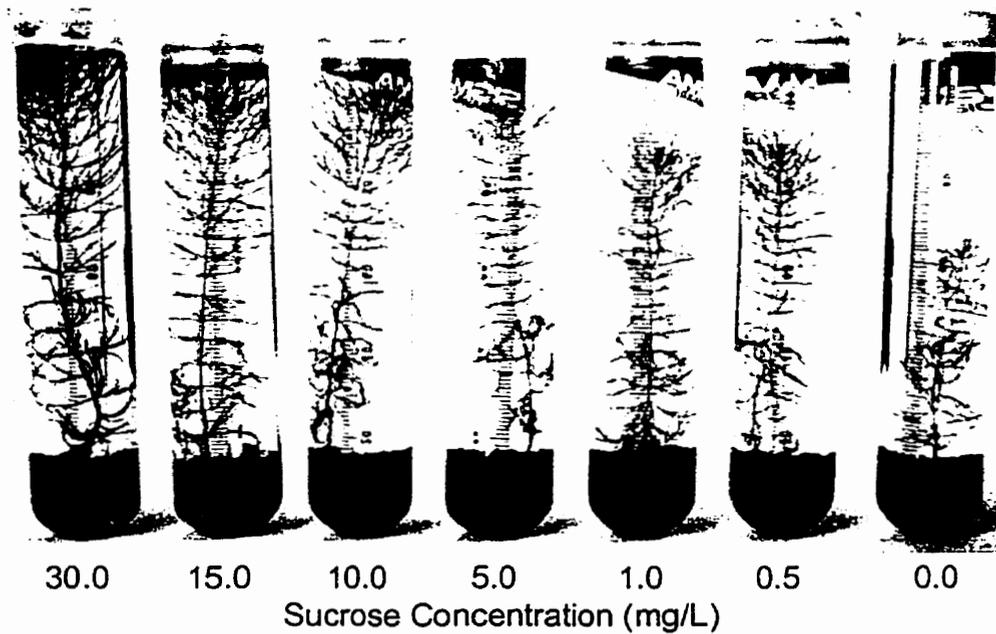


Figure 20: The effect of different concentrations of sucrose on the growth of *Myriophyllum sibiricum* in sterile modified Andrews' medium. This photograph was taken after the plants were incubated for 14 days.

conducted followed by multiple comparisons. Nonparametric data were analysed using the Kruskal-Wallis test and if significant differences were detected, multiple comparisons were conducted.

#### 4.3.5.2.4 Bicarbonate Addition

Sodium bicarbonate ( $\text{NaHCO}_3$ ) was filter sterilised prior to addition to autoclaved liquid growth medium. Concentrations of sodium bicarbonate tested in one experiment ranged between  $1 \cdot 10^{-3}$  to  $5 \cdot 10^{-3}$  M and the range was expanded to include doses between  $1 \cdot 10^{-1}$  to  $1 \cdot 10^{-3}$  M. These two sodium bicarbonate ranges were tested in both full and half strength modified Andrews' and Hoagland's media. Prior to autoclaving, 3% sucrose was added to the control growth medium. The following procedures were conducted in the sterile environment of the Laminar airflow hood. Forty-five mL of the appropriate medium were pipetted into five replicate test tubes per treatment. No rooting substrate was used in these experiments. Axenic stock plants were cultured in half strength M & S medium prior to experimentation. Three centimetre apical segments were placed into each test tube along with a measuring rod. Translucent test tube closures were placed on top of each test tube and secured with laboratory sealant film. Initial plant height was determined. The test tubes were randomised in test tube racks and placed in a growth cabinet with a 16 hour photoperiod. Plant height was measured an additional 7 times during the 14 day incubation period. When the plants were harvested, total plant height, root number and length, fresh weight and plant area were measured. The apices were placed in 80% ethanol and the pigments were extracted for 24 hours. Chlorophyll *a*, chlorophyll *b* and carotenoid content were spectrophotometrically measured at 470, 647 and 663 nm on a Beckman DU® Series 60 Spectrophotometer. Dissolved oxygen was not measured in these experiments. For most of the replicates without sucrose, there was insufficient plant material for determination of membrane integrity. The data were tested for normality and analysed with a one-way ANOVA followed by Tukey-Kramer multiple comparisons. Nonparametric data were analysed with the Kruskal-Wallis test followed by multiple comparisons.

### 4.3.5.3 Results and Discussion

#### 4.3.5.3.1 Aeration

From both aeration experiments, numerous plants were removed because of fungal and bacterial contamination or extreme evaporation of the liquid growth media. During the first experiment, all five replicates were lost from the full strength modified Andrews' medium without sucrose treatment and two replicates were eliminated from the treatment with full strength Hard Water medium without sucrose. One replicate was lost from both treatments lacking sucrose in the second aeration experiment. Even in aerated treatments that were not eliminated from the experiment, there was a loss of medium due to evaporation. This indicated that the parafilm seal was not impermeable to the passage of gases. In both air delivery systems, it was difficult to adequately regulate the flow of air. The plants that survived were very variable in height and weight. Due to the above problems with survival, the data from these two experiments are not presented. Godmaire (1987) found that aeration did not improve the growth of *M. spicatum* in hydroponic axenic culture. However, other researchers have successfully aerated cultures of aquatic macrophytes (Hoffmann *et al.*, 1984; Madsen and Sand-Jensen, 1994; Netherland and Lembi, 1992; Selim *et al.*, 1989; Sutton, 1985) but it appeared unsuccessful in this instance. Culture aeration would increase the equipment costs plus the time and space requirements. In order to maintain this toxicity test as a rapid and repeatable screening test, aeration was determined not to be a worthwhile option for axenic toxicity tests with *M. sibiricum*.

#### 4.3.5.3.2 Different Concentrations of Sucrose

The effect of various amounts of sucrose in modified Andrews' medium on the growth of *Myriophyllum sibiricum* in axenic culture may be visually assessed in Figure 20. Visually, the plants with additional sucrose were healthier than plants grown in the absence of sucrose. There was also a trend towards a healthier appearance with increases in sucrose (Figure 20). Statistically, this was not supported because of the wide standard deviations in the data (Table 19). The plants did not greatly increase in height or area under the growth curve in the absence of sucrose (Figure 21 and Table 19). The membrane of plants grown in culture without any sucrose lacked integrity when statistically compared to plants from all the other treatments. Total root length of plants grown in 0.0 and 0.5 g/L of sucrose was statistically shorter compared to the

other treatments. Root number and fresh weight were statistically greater for plants cultured with high amounts of sucrose. Carotenoid content was not affected by sucrose content (Table 19). The results from the sucrose additions to Hoagland's medium are not presented because they were similar to the results obtained from the experiment in modified Andrews' medium.

The presence of sucrose in the growth medium can be advantageous. Supplementing the stock medium with sucrose quickly reveals the presence of contaminating microorganisms (Greenberg *et al.*, 1992). The toxicity of heavy metals was clearly exhibited in medium with 1% sucrose (Nasu and Kugimoto, 1981). Fleming *et al.* (1991) used 1% sucrose in studies with *Potamogeton pectinatus*. The lack of sucrose has been found to be disadvantageous. Without sucrose in the medium, the number of fronds produced by *L. paucicostata* in seven days was reduced two to three times.

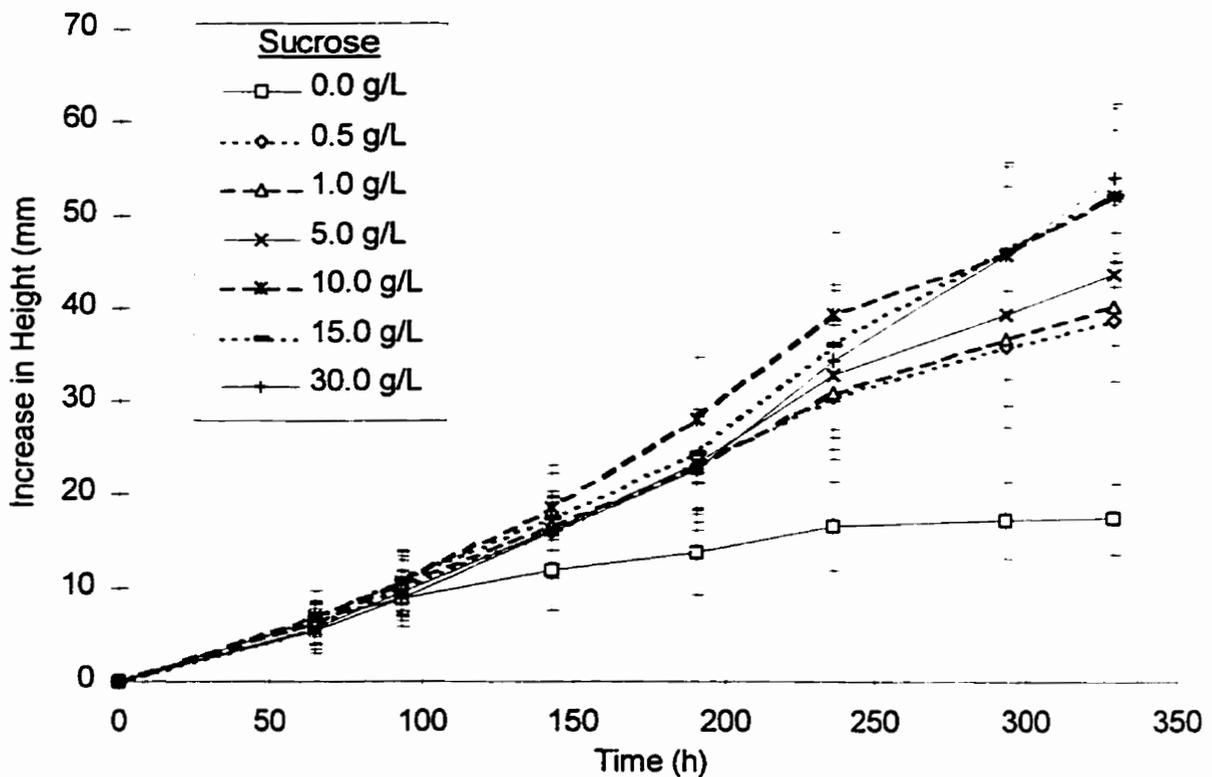


Figure 21: Increase in shoot height of axenic *Myriophyllum sibiricum* over 14 days in modified Andrews' medium containing different concentrations of sucrose. Plant growth was reduced in the absence of sucrose.

Table 19: The effect of different concentrations of sucrose added to the modified Andrews' medium on the growth and development of *Myriophyllum sibiricum* plants grown in axenic culture for two weeks. The stock plants used in this experiment were cultured in half strength M & S medium.

Sucrose (g/L)	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)
0.0	3 825.8 ± 1 117.2 <sup>a</sup>	18.2 ± 3.9 <sup>a</sup>	1.6 ± 0.5 <sup>a,b</sup>	54.3 ± 6.4 <sup>a</sup>	87 ± 4.5 <sup>a</sup>	80.3 ± 16.9 <sup>a</sup>	23.1 ± 4.7 <sup>a</sup>	0.7 ± 0.2 <sup>a</sup>	0.41 ± 0.09 <sup>a</sup>	0.18 ± 0.04 <sup>a,b</sup>	0.14 ± 0.03 <sup>a</sup>
0.5	6 445.9 ± 1 339.1 <sup>b</sup>	40.1 ± 6.0 <sup>b</sup>	1.4 ± 0.5 <sup>a</sup>	111.6 ± 40.2 <sup>a</sup>	87.2 ± 3.1 <sup>a</sup>	174.3 ± 41.5 <sup>b</sup>	11.7 ± 3.4 <sup>b</sup>	1.7 ± 0.7 <sup>b</sup>	0.53 ± 0.04 <sup>b</sup>	0.21 ± 0.01 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>
1.0	6 608.1 ± 1 700.6 <sup>b</sup>	42.8 ± 7.9 <sup>b</sup>	2.2 ± 0.4 <sup>b,c</sup>	164.1 ± 34.9 <sup>b</sup>	87.8 ± 3.6 <sup>a</sup>	205.3 ± 58.4 <sup>b,c</sup>	12.3 ± 3.3 <sup>b</sup>	1.6 ± 0.9 <sup>b</sup>	0.53 ± 0.04 <sup>b</sup>	0.20 ± 0.02 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>
5.0	6 816.0 ± 1 297.1 <sup>b</sup>	47.1 ± 5.7 <sup>b</sup>	2.4 ± 0.5 <sup>c,d</sup>	180.3 ± 25.6 <sup>b</sup>	85.6 ± 8.7 <sup>a</sup>	215.8 ± 64.1 <sup>b,c</sup>	12.1 ± 2.0 <sup>b</sup>	1.7 ± 0.5 <sup>b</sup>	0.52 ± 0.10 <sup>b</sup>	0.20 ± 0.04 <sup>c</sup>	0.16 ± 0.03 <sup>a</sup>
10.0	8 048.3 ± 1 750.7 <sup>b</sup>	55.6 ± 12.6 <sup>b</sup>	3.4 ± 1.1 <sup>d,e</sup>	185.8 ± 91.2 <sup>b</sup>	77.2 ± 11.1 <sup>a,b</sup>	279.5 ± 90.1 <sup>c,d</sup>	8.5 ± 3.4 <sup>b</sup>	1.9 ± 0.8 <sup>b</sup>	0.46 ± 0.06 <sup>a,b</sup>	0.17 ± 0.02 <sup>b,c</sup>	0.15 ± 0.02 <sup>a</sup>
15.0	7 673.1 ± 2 011.2 <sup>b</sup>	52.5 ± 10.6 <sup>b</sup>	4.3 ± 1.0 <sup>e,f</sup>	195.8 ± 52.2 <sup>b</sup>	69.8 ± 7.5 <sup>b,c</sup>	278.3 ± 101.8 <sup>c,d</sup>	8.9 ± 5.4 <sup>b</sup>	2.1 ± 1.0 <sup>b</sup>	0.40 ± 0.02 <sup>a</sup>	0.14 ± 0.01 <sup>b</sup>	0.14 ± 0.01 <sup>a</sup>
30.0	7 306.5 ± 1 497.8 <sup>b</sup>	55.3 ± 8.8 <sup>b</sup>	5.4 ± 1.8 <sup>f</sup>	225.9 ± 82.7 <sup>b</sup>	51.6 ± 4.0 <sup>c</sup>	310.1 ± 99.5 <sup>d</sup>	8.5 ± 2.6 <sup>b</sup>	2.0 ± 1.2 <sup>a,b</sup>	0.43 ± 0.05 <sup>a</sup>	0.15 ± 0.02 <sup>b</sup>	0.15 ± 0.02 <sup>a</sup>

a,b,c,d,e,f Means ± s.d. within the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to a one-way ANOVA followed by multiple comparisons. Only root number and dissolved oxygen were nonparametric and the data were analysed using the Kruskal-Wallis test. There were five replicates for all treatments, except the 15.0 mg/L sucrose treatment that lost one plant due to stem damage during the transfer process. For membrane permeability at 0.0 mg/L sucrose, there were only three replicates because the plants did not weigh enough for both pigment and membrane analysis.

#### 4.3.5.3.3 Mannitol versus Sucrose Comparison

The results from the two experiments utilising different concentrations of mannitol and sucrose added to modified Andrews' or Hard Water media were similar so only the results from the experiment with modified Andrews' medium are discussed. Figure 23 visually shows the effects of various combinations of sucrose with mannitol on the growth of *Myriophyllum sibiricum*. With increasing levels of sucrose and decreasing levels of mannitol, plant growth improved ( Figure 22, Figure 23 and Table 20). Due to fungal contamination, two plants were lost from the 30.0 g/L sucrose and 0.0 g/L mannitol treatment. For all endpoints, there was no significant difference between the values for the three highest concentrations of sucrose : lowest concentrations of mannitol (30.0 : 0.0, 25.0 : 2.66 and 20.0 g/L : 5.32 g/L). Fresh weight and plant length were significantly reduced by sucrose concentrations less than or equal to 5 g/L and mannitol concentrations greater than or equal to 13.3 g/L. Sucrose levels equal to or less than 15.0 g/L and mannitol levels equal to or greater than 7.98 g/L significantly decreased root number compared to the higher sucrose : lower mannitol levels (Table 20). Even though combinations of sucrose and mannitol were able to support cell (Bolwell, 1985) and protoplast (Power and Chapman, 1985) cultures, the combination of these carbon sources was not adequate to support maximum growth of *Myriophyllum sibiricum* in axenic culture. Another reason that mannitol might not be a suitable source of carbon for this toxicity test is that it has been shown to induce the submersed shoots of *Callitriche heterophylla* Pursh. to produce land-form leaves (Raven *et al.*, 1986).

Table 20: The effect of various amounts of sucrose and mannitol in modified Andrews' medium on the growth and development of *Myriophyllum sibiricum* plants grown in axenic culture for two weeks. The stock plants used in this experiment were cultured in half strength M & S medium.

Sucrose : Mannitol (g/L)	Area under the Growth Curve	Plant Length (mm)	Root #	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)
30.0 : 0.0	7 875.7 ± 1 131.1 <sup>a</sup>	54.4 ± 5.5 <sup>a</sup>	6.0 ± 1.0 <sup>a</sup>	262.3 ± 47.0 <sup>a</sup>	45.7 ± 11.6 <sup>a</sup>	474.0 ± 47.1 <sup>a</sup>	8.4 ± 0.4 <sup>a</sup>	7.2 ± 0.8 <sup>a</sup>	0.42 ± 0.06 <sup>a</sup>	0.15 ± 0.02 <sup>a</sup>	0.14 ± 0.02 <sup>a</sup>
25.0 : 2.66	7 880.5 ± 1 732.2 <sup>a</sup>	52.4 ± 7.0 <sup>a</sup>	5.6 ± 0.9 <sup>a</sup>	272.7 ± 59.9 <sup>a</sup>	44.8 ± 2.9 <sup>a</sup>	410.0 ± 118.5 <sup>a</sup>	9.4 ± 4.0 <sup>a</sup>	8.3 ± 3.3 <sup>a</sup>	0.50 ± 0.06 <sup>a,b</sup>	0.18 ± 0.03 <sup>a</sup>	0.17 ± 0.02 <sup>a,b</sup>
20.0 : 5.32	8 190.0 ± 1 875.2 <sup>a</sup>	50.4 ± 8.0 <sup>a</sup>	5.0 ± 1.0 <sup>a,b</sup>	289.3 ± 27.0 <sup>a</sup>	44.6 ± 7.9 <sup>a</sup>	400.2 ± 90.1 <sup>a</sup>	8.3 ± 1.1 <sup>a</sup>	7.4 ± 2.1 <sup>a</sup>	0.47 ± 0.07 <sup>a,b</sup>	0.16 ± 0.03 <sup>a</sup>	0.16 ± 0.02 <sup>a,b</sup>
15.0 : 7.98	6 832.2 ± 1 052.8 <sup>a</sup>	45.8 ± 4.4 <sup>a</sup>	3.8 ± 0.8 <sup>b,c</sup>	193.4 ± 28.0 <sup>a,b</sup>	48.8 ± 3.6 <sup>a</sup>	321.6 ± 73.6 <sup>a,b</sup>	9.9 ± 3.9 <sup>a</sup>	7.1 ± 2.4 <sup>a</sup>	0.53 ± 0.04 <sup>a,b</sup>	0.17 ± 0.01 <sup>a</sup>	0.18 ± 0.01 <sup>a,b</sup>
10.0 : 10.64	6 757.3 ± 1 878.6 <sup>a</sup>	41.8 ± 8.2 <sup>a,b</sup>	3.8 ± 0.4 <sup>c</sup>	153.2 ± 49.0 <sup>b</sup>	55.2 ± 5.0 <sup>a,b</sup>	295.2 ± 104.7 <sup>a,b</sup>	9.8 ± 2.2 <sup>a</sup>	5.8 ± 1.5 <sup>a,b</sup>	0.54 ± 0.07 <sup>a,b</sup>	0.18 ± 0.02 <sup>a</sup>	0.18 ± 0.02 <sup>a,b</sup>
5.0 : 13.3	5 240.3 ± 1 849.6 <sup>a</sup>	31.1 ± 7.0 <sup>b</sup>	3.6 ± 0.9 <sup>c</sup>	115.2 ± 43.3 <sup>b</sup>	60.2 ± 3.8 <sup>b</sup>	224.0 ± 67.6 <sup>b,c</sup>	10.7 ± 4.9 <sup>a</sup>	5.5 ± 2.6 <sup>a,b</sup>	0.59 ± 0.07 <sup>b</sup>	0.20 ± 0.03 <sup>a</sup>	0.20 ± 0.02 <sup>b</sup>
0.0 : 15.96	1 604.2 ± 746.6 <sup>b</sup>	9.1 ± 2.3 <sup>c</sup>	1.4 ± 0.9 <sup>d</sup>	26.4 ± 15.4 <sup>c</sup>	72.6 ± 1.9 <sup>c</sup>	114.5 ± 20.4 <sup>c</sup>	21.6 ± 12.5 <sup>b</sup>	3.3 ± 1.0 <sup>b</sup>	0.51 ± 0.10 <sup>a,b</sup>	0.19 ± 0.04 <sup>a</sup>	0.19 ± 0.03 <sup>a,b</sup>

a,b,c Any two untransformed means (± s.d.) in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to a one-way ANOVA followed by post hoc comparison. Membrane integrity was not normal so it was analysed using a Kruskal-Wallis test followed by multiple comparisons. Two replicates were lost to fungal contamination from the 30.0 : 0.0 mg/L sucrose : mannitol treatment (n = 3) but there were 5 replicates for all other treatments.

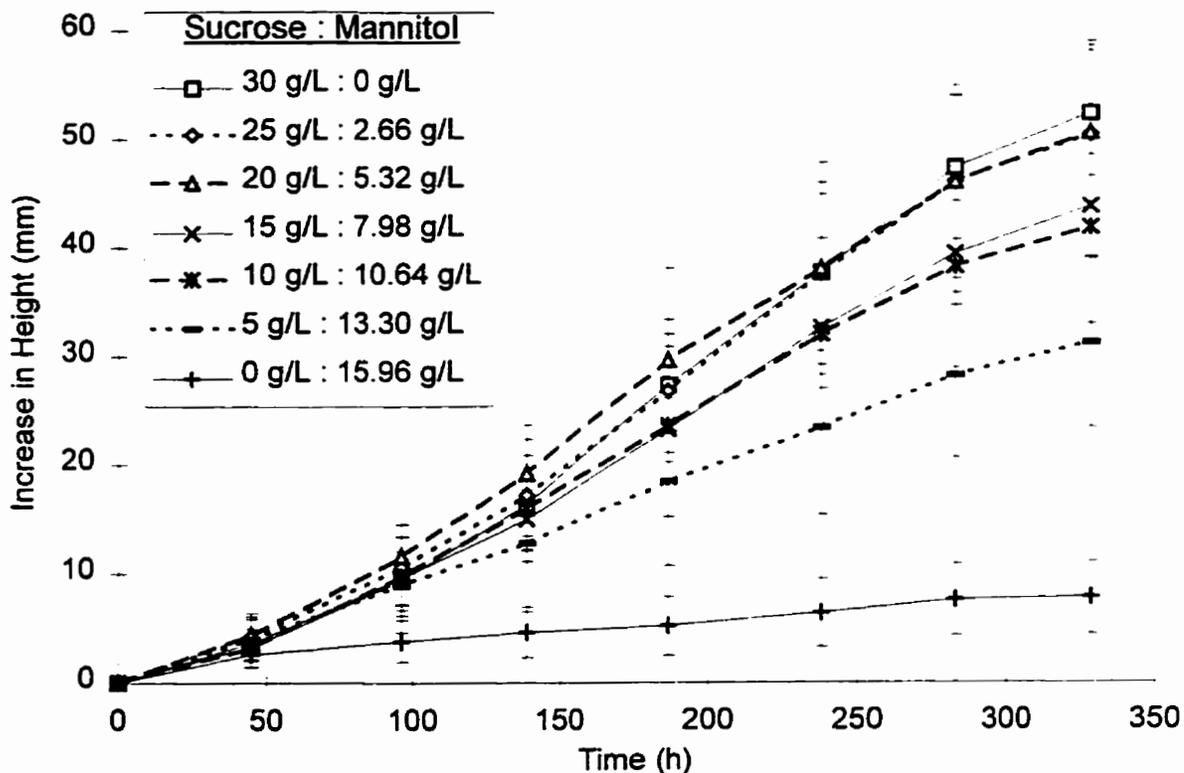


Figure 22: The effect of combinations of sucrose and mannitol in modified Andrews' medium on the growth and development of *Myriophyllum sibiricum* over a 14 day experimental period.

#### 4.3.5.3.4 Bicarbonate Addition

In the experiments using full and half strength media (Hoagland's and modified Andrews'), none of the concentrations of bicarbonate added to the liquid growth medium were able to support the same rapid growth of *M. sibiricum* as those cultured in medium supplemented with sucrose. Therefore, only the results of sodium bicarbonate ( $1 \cdot 10^{-1}$  to  $1 \cdot 10^{-3}$  M) added to full strength modified Andrews' medium are presented in this section. Figure 24 visually depicts the inadequate growth of this aquatic plant species in the presence of  $\text{NaHCO}_3$ . Even the best plant growth in the presence of bicarbonate was about half of the plant growth in the presence of sucrose (Figure 25 and Table 21). Area under the growth curve, increase in shoot height, fresh weight and plant area were significantly greater for plants cultured with sucrose than for plants cultured in any concentration of sodium bicarbonate (Table 21). Thus, sodium bicarbonate does not promote acceptable growth of *M. sibiricum*. However, other

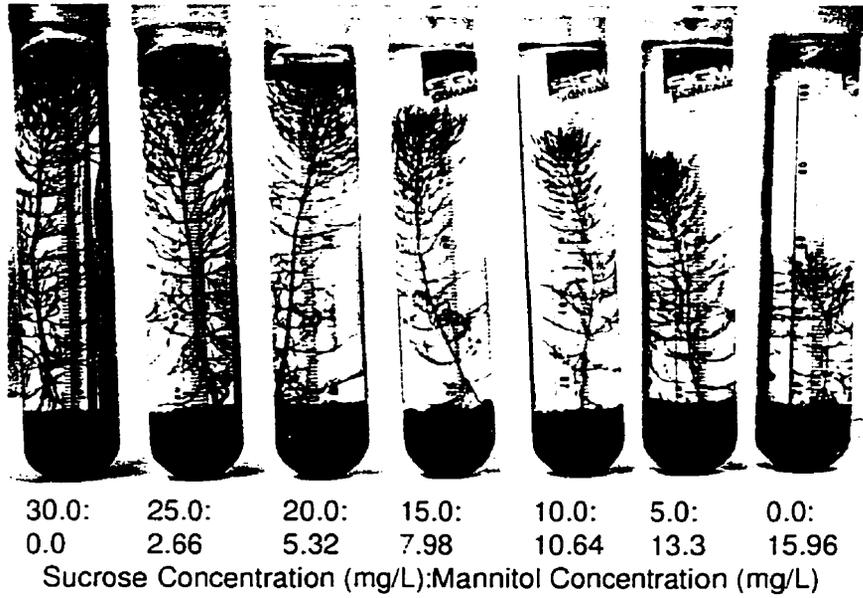


Figure 23: The effect of sucrose and mannitol in modified Andrews' medium on the growth and development of *M. sibiricum* in axenic culture after 14 days of incubation.

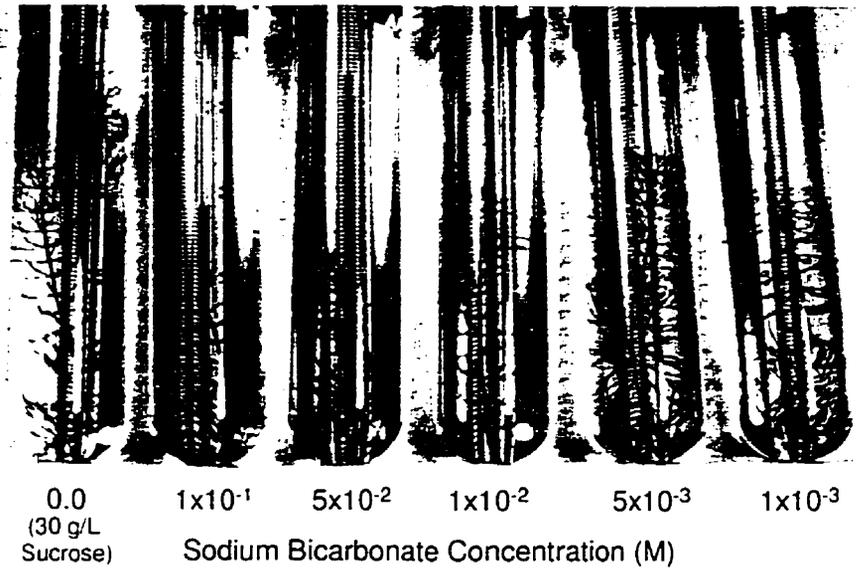


Figure 24: *Myriophyllum sibiricum* cultured with various amounts of sodium bicarbonate ( $\text{NaHCO}_3$ ).

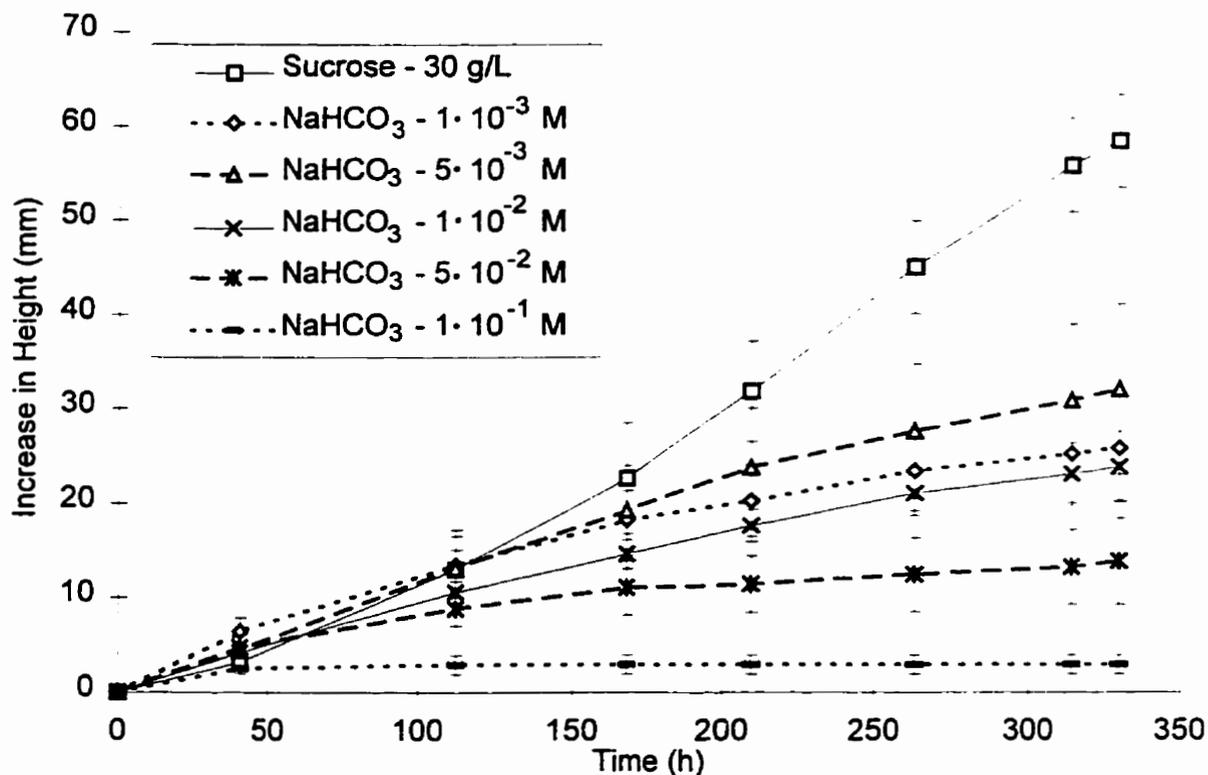


Figure 25: Various concentrations of sodium bicarbonate added to sterile full strength modified Andrews' liquid growth medium affected the growth of *Myriophyllum sibiricum* over the 14 day incubation period.

concentrations of sodium bicarbonate or other bicarbonate sources, such as  $\text{KHCO}_3$ , might be able to support adequate *Myriophyllum sibiricum* growth in axenic culture.

Bicarbonate has been successfully used by other researchers to culture some aquatic plants. For macrophyte testing, Nelson and Fairchild (1994) used Hoagland's medium with EDTA but no additional carbon source. Byl and Klaine (1991) utilised 200 mg/L  $\text{NaHCO}_3$  to buffer a culturing system for *Hydrilla verticillata*. Concentrations of  $\text{NaHCO}_3$  ranging from 0 to 5 mM were tested on segments of *Elodea canadensis* in liquid culture. The amount of  $\text{NaHCO}_3$  required for optimum oxygen evolution was 1 mmol/L at pH 6.7 (Brown and Rattigan, 1979).

Pre-experimental conditions, such as temperature, may affect  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake kinetics (Allen and Spence, 1981; Bowes, 1985). Furthermore, Godmaire and Nalewajko (1989) observed that the maximum rate of bicarbonate uptake was greater in non-axenic *Myriophyllum spicatum* than it was in axenic *M. spicatum* plants

Table 21: The effect of various amounts of sodium bicarbonate (NaHCO<sub>3</sub>) added to full strength modified Andrews' medium on the growth and development of *Myriophyllum sibiricum* plants grown in axenic culture for two weeks. The stock plants used in this experiment were cultured in half strength M & S medium.

NaHCO <sub>3</sub> (M)	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)
0.0 (Sucrose - 30 g/L)	8 307.6 ± 1119.9 <sup>a</sup>	57.0 ± 5.2 <sup>a</sup>	3.8 ± 0.4 <sup>a</sup>	46.7 ± 16.1 <sup>a,b</sup>	265.1 ± 39.5 <sup>a</sup>	6.0 ± 1.0 <sup>a</sup>	0.47 ± 0.02 <sup>a</sup>	0.16 ± 0.01 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>
1·10 <sup>-3</sup>	5 330.4 ± 926.5 <sup>b</sup>	25.9 ± 4.0 <sup>b</sup>	2.4 ± 0.9 <sup>b</sup>	54.8 ± 26.8 <sup>a,c</sup>	88.0 ± 23.5 <sup>b,c</sup>	1.7 ± 0.5 <sup>b</sup>	0.49 ± 0.06 <sup>a</sup>	0.20 ± 0.03 <sup>b</sup>	0.16 ± 0.02 <sup>a,b</sup>
5·10 <sup>-3</sup>	5 878.9 ± 1 508.6 <sup>b</sup>	31.2 ± 9.3 <sup>b</sup>	2.8 ± 0.8 <sup>a,b</sup>	98.0 ± 43.0 <sup>c,d</sup>	118.4 ± 50.7 <sup>b</sup>	2.7 ± 1.7 <sup>b</sup>	0.46 ± 0.13 <sup>a</sup>	0.18 ± 0.05 <sup>a,b</sup>	0.15 ± 0.04 <sup>a,b</sup>
1·10 <sup>-2</sup>	4 515.2 ± 369.6 <sup>b,c</sup>	24.7 ± 3.3 <sup>b</sup>	1.8 ± 0.4 <sup>b,c</sup>	67.1 ± 22.3 <sup>a,d</sup>	84.0 ± 14.8 <sup>b,c,d</sup>	2.6 ± 0.4 <sup>b</sup>	0.42 ± 0.06 <sup>a</sup>	0.17 ± 0.03 <sup>a</sup>	0.14 ± 0.02 <sup>b</sup>
5·10 <sup>-2</sup>	3 113.7 ± 790.5 <sup>c,d</sup>	13.9 ± 5.8 <sup>c</sup>	0.8 ± 0.4 <sup>c,d</sup>	4.8 ± 5.8 <sup>b,e</sup>	63.7 ± 23.0 <sup>c,d</sup>	2.3 ± 0.6 <sup>b</sup>	0.16 ± 0.05 <sup>b</sup>	0.06 ± 0.02 <sup>c</sup>	0.05 ± 0.02 <sup>c</sup>
1·10 <sup>-1</sup>	894.4 ± 298.6 <sup>d</sup>	2.2 ± 1.7 <sup>d</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>e</sup>	46.9 ± 8.1 <sup>d</sup>	1.5 ± 0.3 <sup>b</sup>	0.10 ± 0.02 <sup>b</sup>	0.05 ± 0.01 <sup>c</sup>	0.02 ± 0.01 <sup>c</sup>

a,b,c Any two untransformed means (± s.d.) in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to a one-way ANOVA followed by post hoc comparison. Pigment content was not normal so that data were analysed using a Kruskal-Wallis test followed by multiple comparisons. There were 5 replicates for all treatments.

(Godmaire and Nalewajko, 1989). This might indicate that axenic *M. sibiricum* might not utilise bicarbonate as efficiently as a population of non-axenic plants. There is also evidence that certain epiphytes promote aquatic plant growth (Andrews *et al.*, 1982; Underwood and Baker, 1991) by changing the chemical and physical conditions at the macrophyte leaf and water interface or by modifying nutrients for easier adsorption by the macrophyte.

Peñuelas and Menéndez (1990) and Allen and Spence (1981) examined the interaction of pH and  $\text{HCO}_3^-$  uptake. Allen and Spence (1981) concluded that most macrophytes will not use  $\text{HCO}_3^-$  as their main source of carbon until after the pH exceeds 9.0. The photosynthetic rate will be only about 10% of its potential at saturating  $\text{CO}_2$  concentrations (Allen and Spence, 1981). With the axenic *M. sibiricum* toxicity test, the interaction of pH and carbon uptake was not examined.

The finely dissected leaf structure of *Myriophyllum* species is an anatomical adaptation that increases their photosynthetic surface area to volume ratio (Mitchell and Orr, 1985) and allows the plants to photosynthesise and respire in the presence of very slow gas diffusion rates (Ceska and Ceska, 1985). Different species within the *Myriophyllum* genus have different carbon utilisation mechanisms. *M. spicatum* has been shown to metabolise bicarbonate ions (Keeley and Sandquist, 1992; Mitchell and Orr, 1985; Prins *et al.*, 1982; Spence and Maberly, 1985). The submerged leaves of *M. hippuroides* (Prins *et al.*, 1982) and *M. brasiliensis* and all the leaves of *M. verticillatum* use only  $\text{CO}_2$  for photosynthesis (Keeley and Sandquist, 1992; Prins *et al.*, 1982). It appears that *M. sibiricum* may be similarly unable to metabolise bicarbonate. The data collected with the axenic *M. sibiricum* toxicity test do not conclusively prove that this species cannot use the bicarbonate ion as a carbon source. Further study would be required to conclusively support this hypothesis. To conclusively determine the carbon utilisation method of *M. sibiricum*, possible techniques might include the measurement of alkalinity (Peñuelas and Menéndez, 1990; Spence and Maberly, 1985), pH (Allen and Spence, 1981; Moeller, 1978; Nielsen and Sand-Jensen, 1991; Prins *et al.*, 1982; Prins and Helder, 1985; Raven, 1970; Sand-Jensen, 1983; Smith, 1985; Spence and Maberly, 1985),  $\text{K}^+$  (Prins *et al.*, 1982; Prins and Helder, 1985),  $\text{CO}_2$  and  $\text{HCO}_3^-$  concentration in the unstirred layer near the leaf surface (Prins *et al.*, 1982; Sand-Jensen, 1983), total inorganic carbon (Moeller, 1978), photosynthetic rate (Allen and Spence, 1981; Madsen *et al.*, 1993; Peñuelas and Menéndez, 1990; Spence and Maberly, 1985; Van *et al.*,

1976), CO<sub>2</sub> compensation point (Lloyd *et al.*, 1977; Owttrim and Colman, 1989; Salvucci and Bowes, 1982; Sand-Jensen, 1983; Spence and Maberly, 1985; Van *et al.*, 1976), ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) content (Keeley and Sandquist, 1991; Madsen *et al.*, 1993; Owttrim and Colman, 1989; Salvucci and Bowes, 1982) or phosphoenolpyruvate carboxylase (Owttrim and Colman, 1989; Salvucci and Bowes, 1982). Measurement of the trans-leaf potential difference has successfully verified bicarbonate use in other species (Prins *et al.*, 1985; Prins and Helder, 1985). Radiolabelled carbon sources (Black *et al.*, 1981; Keeley and Sandquist, 1991; Miller, 1985; Nalewajko and Godmaire, 1993; Owttrim and Colman, 1989; Raven, 1970) might also be used to elucidate the photosynthetic pathways of *M. sibiricum*.

#### 4.3.5.4 Conclusions

There is a widely known but invisible trend among researchers to publish only positive results (Sterling *et al.*, 1995). This chapter dealt with numerous inconclusive and negative results. However, discussing them here could prevent other researchers from spending time trying to replace the sucrose with another carbon source. It has been suggested that supplementing the medium with sucrose might artificially enhance plant growth and interfere with the toxicological results (Gentner and Hilton, 1960).

In the axenic toxicity test, it appeared that *M. sibiricum* was unable to utilise bicarbonate. Aeration systems were ineffective at maintaining consistent plant growth. Mannitol was not a viable substitute for sucrose. The only carbon source that supported adequate plant growth in this toxicity test was sucrose.

#### 4.3.6 Buffer Comparison

##### 4.3.6.1 Introduction

Buffers may be added to aqueous media to prevent pH shifts during toxicity tests (Cooney, 1995; Versteeg, 1990). The buffering capacity of an experimental solution may affect the photosynthetic rate by influencing the pH at the leaf surface (Prins *et al.*, 1982). In experiments examining the transport of ions in aquatic plants, buffered medium has been advantageous in removing localised pH differences (Smith, 1985). In this study, the effect of buffers upon the growth and development of *Myriophyllum sibiricum* was examined in axenic culture.

#### 4.3.6.2 Methods

Buffers tested in the axenic *Myriophyllum sibiricum* toxicity test included  $\text{H}_2\text{CO}_3/\text{NaHCO}_3$  ( $1 \cdot 10^{-1}$  to  $1 \cdot 10^{-3}$  M), citric acid/sodium citrate ( $1 \cdot 10^{-1}$  to  $1 \cdot 10^{-3}$  M), sodium citrate ( $1 \cdot 10^{-1}$  to  $5 \cdot 10^{-4}$  M) and citric acid/sodium hypophosphate buffer ( $1 \cdot 10^{-2}$  to  $5 \cdot 10^{-4}$  M). The appropriate amount of each buffer was added to full strength modified Andrews' medium prior to autoclaving. In the medium without any buffer, 30 g/L of sucrose was added. Forty mL of medium from each treatment was pipetted into 5 replicate test tubes containing 3 g of Turface®. Plants, which had been cultured in half strength M & S medium with sucrose, were segmented into 3 cm apical tips and placed into test tubes. Measuring rods were added and each tube was covered with a translucent cap and sealed with laboratory sealant film. The test tubes were randomised within test tube racks and incubated for 14 days. Plant height was measured eight times including the initial measurement on the day the experiment was started. On the day the plants were harvested, a photograph was taken of one representative plant per treatment. Final plant height, root number, total root length, total plant fresh weight and plant area were determined. The apices ( $50 \pm 2$  mg) were placed into 80% ethanol and the pigments were extracted over a 24 hour period. Pigment content was determined spectrophotometrically at 470, 647 and 663 nm on a Beckman DU® Series 60 Spectrophotometer. Membrane integrity was determined by placing an 100 mg stem segment in nanopure water and allowing the ions to diffuse into the surrounding water over 24 hours. The conductivity of this solution was measured prior to and after boiling for 20 mins. Percent membrane disruption was determined. The data were examined for normality. Normal data were analysed using one-way ANOVA and non-normal data were analysed with the Kruskal-Wallis test. When differences were detected between treatments, each test was followed by multiple comparisons.

#### 4.3.6.3 Results and Discussion

Only the results from the citric acid/sodium hypophosphate buffer ( $1 \cdot 10^{-2}$  to  $5 \cdot 10^{-4}$  M) are reported in this section because the results from the other buffers were very similar. At the concentrations tested, all the buffers severely inhibited the growth of *Myriophyllum sibiricum* in axenic culture. This was also evident from the data collected from the citric acid/sodium hypophosphate buffer experiment (Figure 26, Figure 27 and

Table 22). Area under the growth curve, increase in shoot height, root number, root length and plant area were significantly greater for plants cultured with 30 g/L sucrose than plants grown in all concentrations of citric acid/sodium hypophosphate (Table 22). Pigment content was significantly reduced in plants grown with sucrose as compared with most of the buffer treatments (Table 22). Pigment content was only calculated on a fresh weight basis so the reduction in pigment content might have been an anomaly caused by differences in fresh/dry weight ratios. In another study, Prins and Helder (1985) showed that Tris-Tes, K-phosphate and MES buffers reduced oxygen evolution in *Potamogeton lucens* and *Elodea canadensis* (Prins and Helder, 1985).

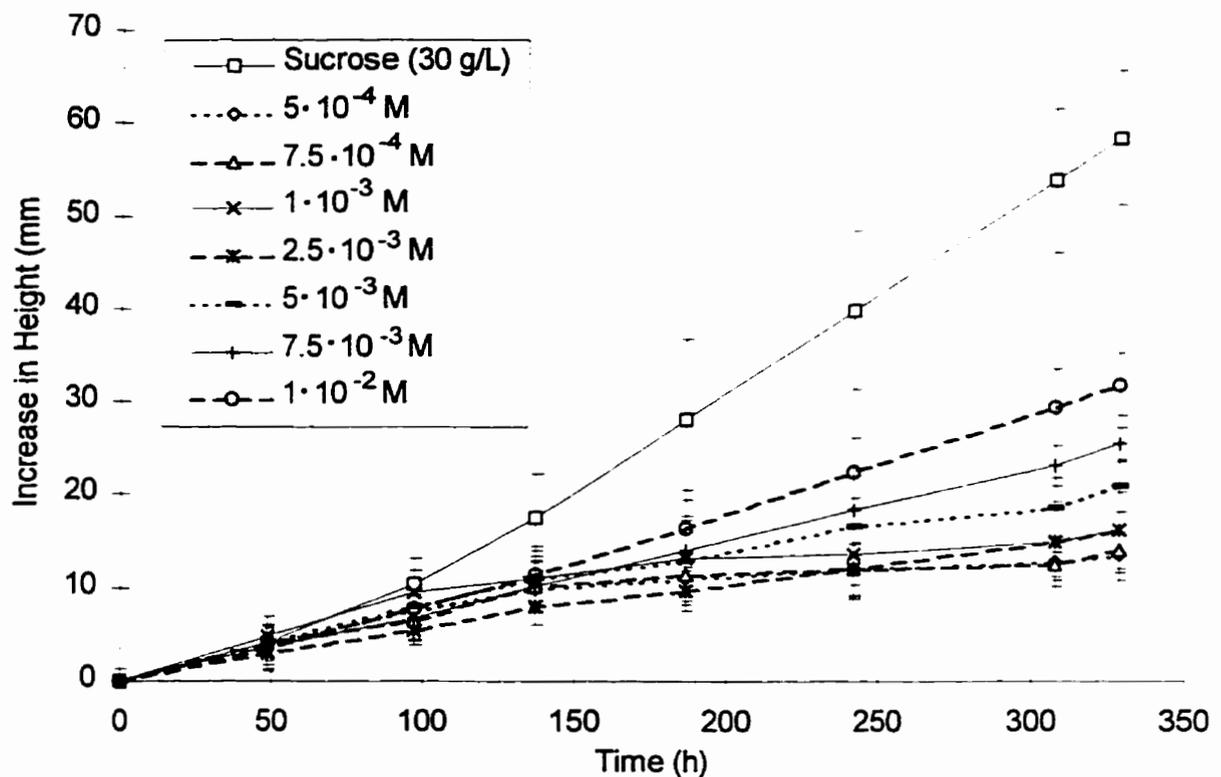


Figure 26: Fourteen day growth curves for *Myriophyllum sibiricum* plants axenically cultured with different concentrations of citric acid/ sodium hypophosphate buffer added to modified Andrews' medium.

Table 22: Added to full strength modified Andrews' medium, different concentrations of a citric acid/sodium hypophosphate buffer affected the growth and development of axenic *Myriophyllum sibiricum* plants over a two week incubation period. The stock plants used in this experiment were cultured in half strength M & S medium.

Citric Acid/ Sodium Hypophosphate Buffer (M)	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)
0.0 (Sucrose)	8 277.6 ± 1 671.3 <sup>a</sup>	65.9 ± 11.4 <sup>a</sup>	5.4 ± 1.8 <sup>a</sup>	335.0 ± 87.3 <sup>a</sup>	468.8 ± 185.7 <sup>a</sup>	10.7 ± 4.1 <sup>a</sup>	10.0 ± 3.5 <sup>a</sup>	0.45 ± 0.06 <sup>a</sup>	0.17 ± 0.02 <sup>a</sup>	0.16 ± 0.02 <sup>a</sup>
5·10 <sup>-4</sup>	2 971.8 ± 753.8 <sup>b</sup>	14.9 ± 3.4 <sup>b</sup>	1.0 ± 0.0 <sup>b</sup>	32.1 ± 8.1 <sup>b,c</sup>	121.9 ± 14.9 <sup>b,d</sup>	31.4 ± 5.7 <sup>b,c,d</sup>	3.5 ± 0.2 <sup>b</sup>	0.56 ± 0.05 <sup>b</sup>	0.22 ± 0.02 <sup>b</sup>	0.19 ± 0.02 <sup>b,c</sup>
7.5·10 <sup>-4</sup>	2 961.3 ± 425.0 <sup>b</sup>	16.7 ± 3.2 <sup>b,c</sup>	0.8 ± 0.5 <sup>b,c</sup>	19.8 ± 14.6 <sup>b</sup>	88.9 ± 25.5 <sup>c</sup>	38.6 ± 12.0 <sup>b,c,d</sup>	3.0 ± 1.0 <sup>b</sup>	0.63 ± 0.05 <sup>b</sup>	0.24 ± 0.02 <sup>b</sup>	0.21 ± 0.01 <sup>b</sup>
1·10 <sup>-3</sup>	3 480.2 ± 1 054.1 <sup>b,c</sup>	19.1 ± 4.8 <sup>b,c,d</sup>	1.0 ± 0.0 <sup>b,c</sup>	27.0 ± 16.6 <sup>b,c</sup>	123.6 ± 39.8 <sup>b,c,d</sup>	33.7 ± 4.2 <sup>b,d</sup>	3.6 ± 0.7 <sup>b</sup>	0.58 ± 0.08 <sup>a</sup>	0.22 ± 0.03 <sup>b</sup>	0.20 ± 0.03 <sup>b,d</sup>
2.5·10 <sup>-3</sup>	2 798.0 ± 655.7 <sup>b</sup>	17.2 ± 5.0 <sup>b,c</sup>	1.0 ± 0.0 <sup>b,c</sup>	37.1 ± 4.8 <sup>c</sup>	93.5 ± 26.8 <sup>b,c</sup>	29.0 ± 6.3 <sup>b,c,d</sup>	2.9 ± 0.9 <sup>b</sup>	0.52 ± 0.10 <sup>a</sup>	0.20 ± 0.04 <sup>a,b</sup>	0.17 ± 0.03 <sup>a,c,d</sup>
5·10 <sup>-3</sup>	3 758.1 ± 751.2 <sup>b,c</sup>	22.9 ± 3.7 <sup>c,d,e</sup>	1.4 ± 0.5 <sup>c</sup>	63.6 ± 10.1 <sup>d</sup>	134.9 ± 32.7 <sup>d</sup>	20.6 ± 4.6 <sup>a,c</sup>	3.6 ± 1.1 <sup>b</sup>	0.49 ± 0.05 <sup>a</sup>	0.19 ± 0.02 <sup>a,b</sup>	0.17 ± 0.02 <sup>a,c</sup>
7.5·10 <sup>-3</sup>	4 049.0 ± 774.8 <sup>b,c</sup>	27.1 ± 1.7 <sup>d,e</sup>	1.2 ± 0.4 <sup>b,c</sup>	73.9 ± 5.1 <sup>d</sup>	114.8 ± 21.1 <sup>b,c,d</sup>	23.9 ± 5.1 <sup>a,c</sup>	3.3 ± 0.6 <sup>b</sup>	0.64 ± 0.05 <sup>b</sup>	0.24 ± 0.02 <sup>b</sup>	0.20 ± 0.02 <sup>b,d</sup>
1·10 <sup>-2</sup>	4 851.7 ± 930.9 <sup>c</sup>	32.8 ± 4.8 <sup>e</sup>	1.2 ± 0.4 <sup>b,c</sup>	100.2 ± 14.9 <sup>e</sup>	138.2 ± 30.8 <sup>d</sup>	21.0 ± 6.2 <sup>a,d</sup>	3.9 ± 1.0 <sup>b</sup>	0.61 ± 0.05 <sup>b</sup>	0.24 ± 0.02 <sup>b</sup>	0.21 ± 0.01 <sup>b</sup>

a,b,c,d,e Any two untransformed means (± s.d.) in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to a one-way ANOVA or Kruskal-Wallis test followed by post hoc comparisons. There were 5 replicates for all treatments except for the 7.5·10<sup>-4</sup> M treatment where one tube was broken during the experimental transfer.

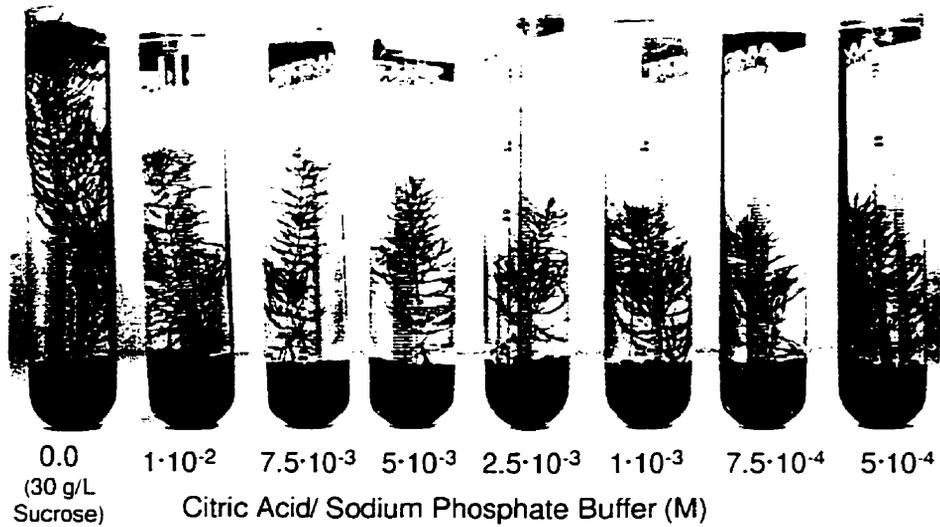


Figure 27: The citric acid/sodium hypophosphate buffer at concentrations ranging between  $1 \cdot 10^{-2}$  and  $5 \cdot 10^{-4}$  M severely inhibited the growth of *Myriophyllum sibiricum*. The plants were axenically cultured in modified Andrews' medium for 14 days.

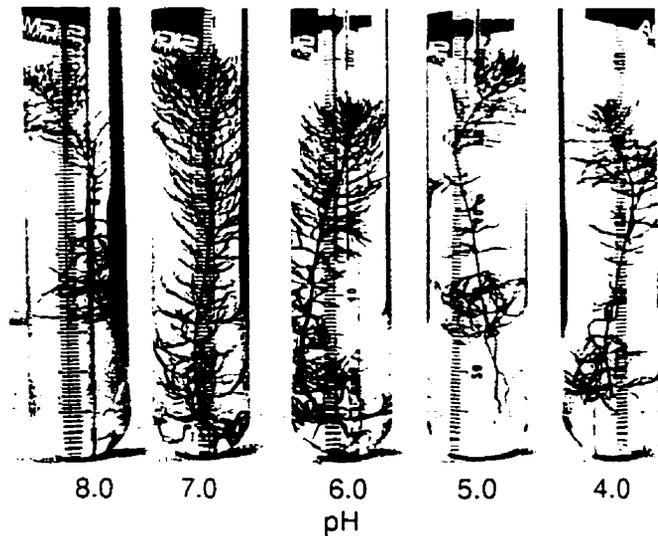


Figure 28: The pH of modified Andrews' medium was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0. The pH of the medium did not statistically affect the growth of *Myriophyllum sibiricum* in axenic culture.

### 4.3.7 Media pH

#### 4.3.7.1 Introduction

The pH of liquid growth media may affect aquatic plant growth in laboratory culture. In a closed system, as the pH increases the amount of dissolved CO<sub>2</sub> decreases and the HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>-</sup> ion concentrations increase (Miller, 1985). In freshwater aquatic systems, only a few submersed macrophytes can survive at pH values below 4.0 and some tolerate periods at pH 10 (Bowes, 1985). The most commonly used pH for aquatic macrophyte laboratory culture is 5.8 (Greenberg *et al.*, 1992; Powell *et al.*, 1996), although a small range about this value is common.

Flemming *et al.* (1991) used a pH of 5.7 for culturing and herbicide testing with *Potamogeton pectinatus*. Selim *et al.* (1989) cultured *Myriophyllum spicatum* with an initial pH between 6.0 and 6.5. In tissue culture, pH values between 5.5 and 5.9 are commonly used (Dixon, 1985). Liquid growth medium at a pH of 5.8 was used for inducing callus formation of *Lemna perpusilla* (Chang and Hsing, 1978) and for tissue culturing of *Neptunia oleracea* Lour., an aquatic legume (Kakkar and Ram, 1986). *Myriophyllum aquaticum* was cultured in liquid growth medium at a pH of 5.7, as adjusted with potassium hydroxide (KOH) (Kane *et al.*, 1988a; 1991). Seven *Myriophyllum* species and two *Proserpinaca* species were cultured *in vitro* at a pH of 5.7 (Kane and Albert, 1989b). A pH of 5.7 was also used for propagating *M. aquaticum*, *M. heterophyllum*, *M. pinnatum* (Walt.) BSP, *M. spicatum* (Kane and Gilman, 1991), *Proserpinaca palustris* L. (mermaidweed) (Kane and Albert, 1987) and *Nelumbo lutea* (Willd.) Pers. (American lotus) (Kane *et al.*, 1988b). Fluctuations in pH during a toxicity test can influence the magnitude of the toxicity (Cooney, 1995).

#### 4.3.7.2 Methods

The influence of liquid growth medium pH on the growth and development of *Myriophyllum sibiricum* in axenic culture was examined in M & S, Hoagland's, Gaudet's and modified Andrews' media. Full and half strength M & S medium was tested at pH's 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Full, three-quarter and half strength Hoagland's medium was tested at pH's equal to 4.0, 5.0, 6.0, 7.0 and 8.0. Gaudet's and modified Andrews' media were tested in separate experiments at full and half strengths with pH values of 4.0, 5.0, 6.0, 7.0 and 8.0. For the micronutrient and macronutrient constituents of these media, refer to Table 17. Each medium was made with nanopure water and 3%

sucrose. The pH was adjusted using hydrochloric acid (HCl) or KOH prior to autoclaving. pH was determined using either a Beckmann Portable Checkmate® Field pH meter or an Accumet® pH meter 925 (Fisherbrand®) prior to and after autoclaving. Forty-five mL of the appropriate medium were added to five replicate test tubes per treatment. No rooting substrate was used in any of these experiments. Three centimetre apical segments, which had been cultured in half strength M & S medium, were transferred into the new medium. A 15 cm measuring rod was added to each test tube and then capped with a translucent test tube closure and sealed with laboratory sealant. Initial plant height was determined, the plants were randomised in test tube racks and incubated. During the 14 day incubation period, plant height was measured an additional 7 times. At the end of the incubation period, plant height, root number, root length, fresh weight, membrane integrity, plant area and chlorophyll *a*, chlorophyll *b* and carotenoid content were determined. The data were tested for normality and analysed with an one-way analysis of variance or the nonparametric Kruskal-Wallis test. If differences were detected at  $\alpha = 0.05$ , multiple comparisons were conducted to determine which treatments differed.

In October 1991, the pH was measured at the Puslinch Lake collection site with a Beckmann Portable Checkmate® Field pH meter.

#### 4.3.7.3 Results and Discussion

The pH trials with different strengths and types of media had similar results. Only the results from the pH comparison of full strength modified Andrews' medium are presented in this section. Representative plants from this experiment may be observed in Figure 28. One or two replicate plants were lost from each treatment due to fungal contamination. Without a rooting substrate, the roots pushed the plants away from the bottom of the test tube. Visually, it appears that pH's of 4.0, 5.0 and 8.0 did not support growth as well as pH's of 6.0 and 7.0. From the growth curves in Figure 29, it appears that a pH of 6.0 supported the greatest increase in plant height. These visual observations were not supported statistically (Table 23). Statistically ( $\alpha = 0.05$ ), there was no significant difference between pH treatments for area under the growth curve, plant length, root number, root length, fresh weight, membrane permeability or plant area. Based on apical fresh weight, chlorophyll *a* and carotenoid content were reduced

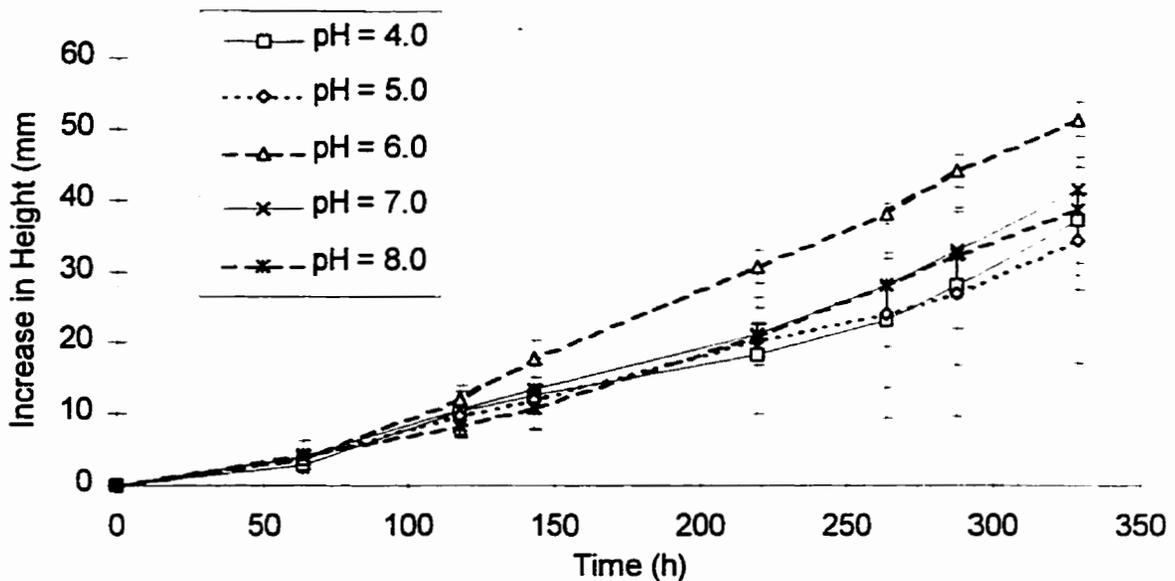


Figure 29: pH of modified Andrews' medium did not statistically affect the shoot growth of axenic *Myriophyllum sibiricum* plants over 14 days of incubation.

at pH values of 4.0 and 5.0 and chlorophyll *b* content was reduced at a pH of 5.0 (Table 23).

Medium pH influenced the effect of heavy metals upon frond multiplication of *L. paucicostata*. If the medium pH was higher than 6.1, a white precipitate formed (Nasu and Kugimoto, 1981). No precipitate was observed at any of the pH values tested in the *Myriophyllum sibiricum* toxicity test.

In the autumn, the pH of Puslinch Lake was 8.4. This pH value was higher than the pH value chosen for the laboratory experiments. A pH of 9.0 was tested with M & S medium but this pH level severely reduced growth so this pH was not tested with the other media. Because there were only minor effects caused by changes in pH, it was decided to use a pH of 5.8 for testing with *M. sibiricum*. Use of an intermediate pH allows for minor pH fluctuations without undue stress to the plants. A pH of 5.8 is commonly used in aquatic plant culturing (Greenberg *et al.*, 1992; Powell *et al.*, 1996) and this allows comparability with other toxicity tests.

Table 23: The effect of pH on the growth and development of *Myriophyllum sibiricum* plants cultured in full strength modified Andrews' medium. The experimental plants were grown in axenic culture for two weeks. Half strength M & S medium was used to culture the stock plants used in this experiment.

pH	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)
4.0	4 772.9 ± 1 693.6 <sup>a</sup>	39.8 ± 8.7 <sup>a</sup>	3.5 ± 0.6 <sup>a</sup>	44.3 ± 21.4 <sup>a</sup>	142.3 ± 38.1 <sup>a</sup>	17.9 ± 9.3 <sup>a</sup>	3.6 ± 1.3 <sup>a</sup>	0.43 ± 0.015 <sup>a</sup>	0.15 ± 0.011 <sup>a,b</sup>	0.15 ± 0.003 <sup>a</sup>
5.0	4 856.7 ± 722.0 <sup>a</sup>	38.0 ± 7.2 <sup>a</sup>	3.3 ± 0.6 <sup>a</sup>	74.6 ± 8.5 <sup>a</sup>	131.3 ± 6.4 <sup>a</sup>	9.7 ± 5.8 <sup>a</sup>	2.8 ± 0.2 <sup>a</sup>	0.41 ± 0.023 <sup>a</sup>	0.15 ± 0.009 <sup>a</sup>	0.15 ± 0.001 <sup>a</sup>
6.0	7 254.8 ± 438.2 <sup>a</sup>	53.3 ± 3.4 <sup>a</sup>	4.0 ± 0.8 <sup>a</sup>	137.0 ± 37.1 <sup>a</sup>	241.6 ± 64.5 <sup>a</sup>	7.9 ± 2.7 <sup>a</sup>	4.8 ± 1.6 <sup>a</sup>	0.54 ± 0.076 <sup>b</sup>	0.19 ± 0.033 <sup>a,b,c</sup>	0.19 ± 0.021 <sup>a,b</sup>
7.0	5 505.2 ± 3 060.2 <sup>a</sup>	42.2 ± 24.6 <sup>a</sup>	3.5 ± 0.6 <sup>a</sup>	159.1 ± 96.7 <sup>a</sup>	239.9 ± 125.8 <sup>a</sup>	9.5 ± 3.9 <sup>a</sup>	4.9 ± 2.4 <sup>a</sup>	0.55 ± 0.035 <sup>b</sup>	0.20 ± 0.015 <sup>b,c</sup>	0.22 ± 0.061 <sup>b</sup>
8.0	5 211.8 ± 934.8 <sup>a</sup>	42.8 ± 6.7 <sup>a</sup>	2.7 ± 0.6 <sup>a</sup>	122.1 ± 25.3 <sup>a</sup>	125.7 ± 26.8 <sup>a</sup>	17.6 ± 6.1 <sup>a</sup>	2.7 ± 0.6 <sup>a</sup>	0.61 ± 0.055 <sup>b</sup>	0.24 ± 0.026 <sup>c</sup>	0.21 ± 0.012 <sup>b</sup>

a,b,c Any two untransformed means ( $\pm$  s.d.) in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to a one-way ANOVA followed by post hoc comparison. Root number was not normal and could not be transformed to meet the requirements for normality. A Kruskal-Wallis test followed by multiple comparisons was used to statistically compare this data. Due to fungal contamination, there were 4 replicates for pH = 4.0, 6.0 and 7.0, while there were 3 replicates for pH = 5.0 and 8.0.

### 4.3.8 Iron Addition and Chelator Use

#### 4.3.8.1 Introduction

The use of metals in nutrient media often requires a natural (e.g., amino acids, humic acids) or synthetic (e.g., ethylenediamine tetraacetic acid (EDTA), nitrilotriacetic acid (NTA)) chelator to keep the metals dissolved and available for plant absorption (Borgmann, 1983). However, these chelators in solution can reduce the toxicity of some compounds, especially metals (Borgmann, 1983; Brown and Rattigan, 1979; Thellen *et al.*, 1989). For duckweed testing, Huebert and Shay (1992a; 1993a) recommended that EDTA be included in the liquid medium because without a chelator, precipitation occurs. EDTA is also important for optimal *Lemna* growth and consistency between replicates (Huebert and Shay, 1992a; 1993a). The presence of chelators becomes questionable when testing the effects of metals, since toxicity is related to free ion activity and not total metal concentration (Anderson and Morel, 1978; Huebert and Shay, 1991; 1992a; 1992b; 1993a; 1993b; Nor and Cheng, 1986). In the presence of EDTA metal toxicity can either increase, as is the case with zinc, or decrease, as it does with mercury, silver and thallium (Canterford and Canterford, 1980). If this toxicity test were to be used to determine the effects of metals upon aquatic macrophytes, then more attention should be given to the concentration of metals and chelators in solution and any interactions that might occur. Because the current toxicity test deals mainly with pesticide effects, the presence of the iron and the chelators are less likely to interfere with the toxicity results.

#### 4.3.8.2 Methods

Selim *et al.* (1989) recommended that 10 mg/L of FeEDTA stock solution be added to the modified Andrews' medium. In 1 L of nanopure water, the stock solution contained 372 mg Na<sub>2</sub>EDTA (disodium ethylenediamine tetraacetate) and 278 mg FeSO<sub>4</sub>·7H<sub>2</sub>O (ferrous sulphate) dissolved by heating to 80 °C (Selim *et al.*, 1989). To prevent degradation, this stock solution was stored in the dark at 4 °C. In this experiment, FeEDTA concentrations of 0.0, 0.865, 1.73, 2.595 and 3.46 mg/L were used. This produced the iron:EDTA ratios of 0.0:0.0, 0.14:0.725, 0.28:1.45, 0.42:2.175 and 0.56:2.9 mg/L. Prior to autoclaving, the appropriate amount of FeEDTA was added to the modified Andrews' medium in 500 mL Erlenmeyer flasks. Forty mL of the appropriate sterile medium were pipetted into five test tubes (2.5 x 15 cm) per

treatment. Each test tube contained 3 g of Turface® that had been autoclaved once on each of three consecutive days. Three centimetre long apical segments, which had been cultured in M & S medium, were transferred into modified Andrews' medium containing the various concentrations of FeEDTA. Once the plant segment had been added to the test tube, a Westergren measuring rod was placed into the centre of the tube, covered with a translucent test tube closure and sealed with parafilm. The plants were incubated (16 hours of light at 25 °C and 8 hours of darkness at 20 °C) for 14 days during which time the plant height was measured every second day. On the fourteenth day, the plants were harvested. The endpoints measured were the amount of dissolved oxygen in the test tube, shoot height, root number and length, total fresh weight, pigment content of a 50 mg apical segment and the membrane permeability of a 100 mg segment. Plant area was not determined because the photocopied plants were too light for accurate detection by the plant area meter. The data were tested for normality. A one-way ANOVA was conducted on the normal parameters. If differences were detected, multiple comparisons were performed (Sokal and Rohlf, 1981). The Kruskal-Wallis test was conducted on non-normal data and if differences were detected, this was followed by multiple comparisons (Conover, 1980). All statistical analyses were conducted using the computer package SYSTAT® (SYSTAT®, 1990).

#### 4.3.8.3 Results and Discussion

Different concentrations of FeEDTA had very little effect on *Myriophyllum sibiricum* growth (Figure 30 and Table 24). Statistically significant differences between the Fe:EDTA concentrations were only detected for area under the growth curve and plant length (Figure 30 and Table 24). For area under the growth curve, plants grown with 0.42 mg/L of Fe and 2.175 mg/L of EDTA were significantly taller than plants cultured with 0.14 mg/L Fe and 0.725 mg/L of EDTA. For plant length, plants grown with 0.42 : 2.175 mg/L of Fe:EDTA were significantly longer than plants cultured with 0.0 : 0.0 and 0.14 : 0.725 mg/L Fe:EDTA. Iron and EDTA should not be eliminated from the medium. In aquatic environments, natural chelators exist so it would not be realistic to grow the *M. sibiricum* plants in medium without a chelator. At iron to EDTA concentrations of 0.28 : 1.45, 0.42 : 2.175 and 0.56 : 2.9 mg/L, there were no significant differences in the endpoint parameters. Therefore, any of these iron to EDTA concentrations could be utilised in the *Myriophyllum sibiricum* toxicity test. For

consistency with the published work conducted by Selim *et al.* (1989) on *M. spicatum* and other studies conducted with *M. sibiricum*, the level of Fe:EDTA was maintained at 0.56 : 2.9 mg/L for the remainder of the experiments. Therefore, 10 mL of concentrated FeEDTA stock solution would be added to each litre of modified Andrews' medium.

Other researchers have utilised different amounts of iron and chelators in their research with macrophytes. For macrophyte testing, Nelson and Fairchild (1994) used Hoagland's medium with EDTA. In the *Lemna* testing protocols (ASTM, 1991b; U.S. EPA, 1985b), it was recommended that the experimental nutrient medium contain no EDTA. Forney and Davis (1981) used one-quarter strength Hoagland's solution with twice the prescribed amount of iron in their research with submersed aquatic plants.

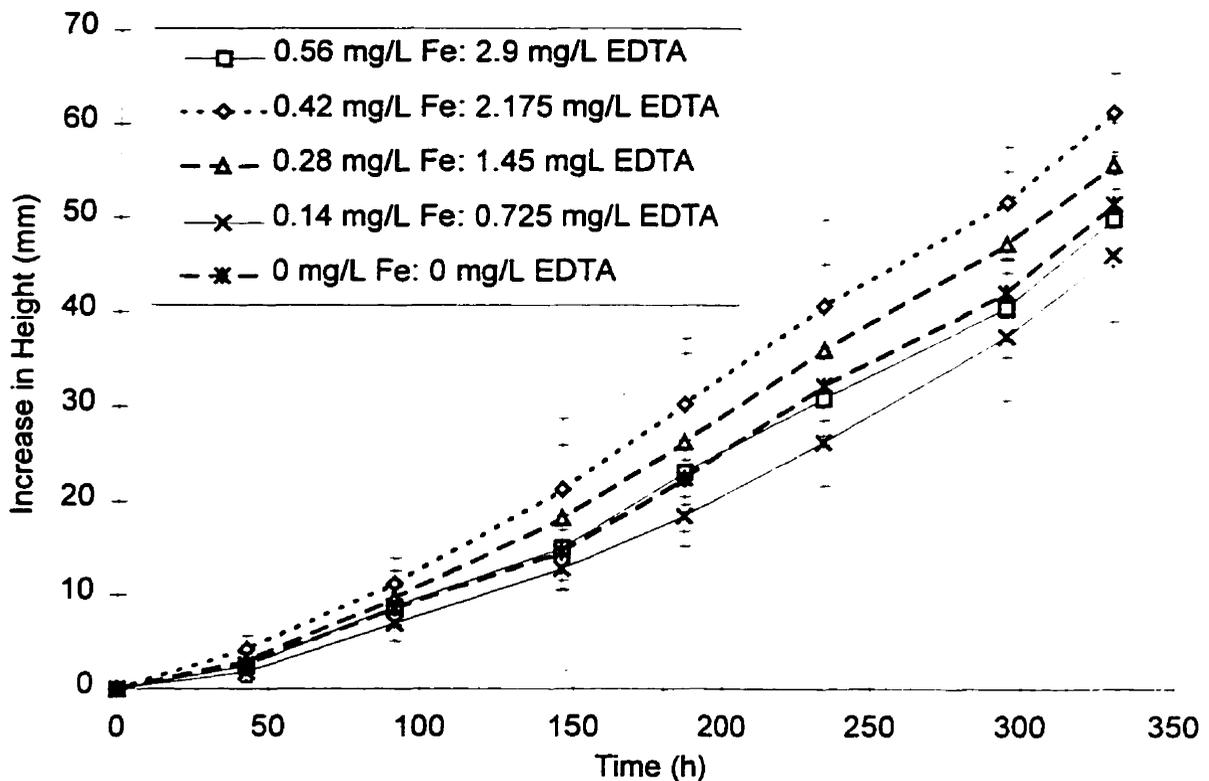


Figure 30: Two week growth curves for *Myriophyllum sibiricum* plants cultured in axenic modified Andrews' medium containing different concentrations of iron and EDTA. For area under the growth curve, only plants cultured with 0.42 mg/L Fe: 2.175 mg/L EDTA differed from those grown with 0.14 mg/L Fe: 0.725 mg/L EDTA. For shoot length measured at the end of the fourteen day growth period, plants cultured in 0.42 mg/L Fe: 2.175 mg/L EDTA differed from those grown with 0.14 mg/L Fe: 0.725 mg/L EDTA and 0.0 mg/L Fe: 0.0 mg/L EDTA.

Table 24: The effect of several Fe and EDTA concentrations in modified Andrews' medium upon the growth and development of *Myriophyllum sibiricum* plants grown in axenic culture for two weeks. Differences were detected between the treatments ( $\alpha = 0.05$ ) for area under the growth curve and plant length. None of the other growth parameters were affected by the FeEDTA concentration.

Fe:EDTA Ratio (mg/L)	Area under the Growth Curve	Plant Length (mm)	Root Number	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g)	Chlorophyll b Content (mg/g)	Carotenoid Content (mg/g)	Membrane Permeability (%)
0.56 : 2.9	6 727.7 ± 1 010.2 <sup>a,b</sup>	54.7 ± 5.0 <sup>a,b</sup>	4.8 ± 1.3 <sup>a</sup>	220.7 ± 46.1 <sup>a</sup>	40.0 ± 13.9 <sup>a</sup>	363.5 ± 113.1 <sup>a</sup>	0.48 ± 0.05 <sup>a</sup>	0.16 ± 0.02 <sup>a</sup>	0.16 ± 0.02 <sup>a</sup>	9.8 ± 3.6 <sup>a</sup>
0.42 : 2.175	8 802.6 ± 1 683.4 <sup>a</sup>	64.3 ± 4.9 <sup>a</sup>	5.6 ± 1.1 <sup>a</sup>	280.5 ± 43.3 <sup>a</sup>	49.4 ± 4.3 <sup>a</sup>	449.4 ± 72.3 <sup>a</sup>	0.44 ± 0.02 <sup>a</sup>	0.16 ± 0.01 <sup>a</sup>	0.15 ± 0.01 <sup>a</sup>	7.8 ± 0.8 <sup>a</sup>
0.28 : 1.45	7 792.6 ± 1 900.6 <sup>a,b</sup>	56.8 ± 5.7 <sup>a,b</sup>	5.6 ± 1.8 <sup>a</sup>	276.3 ± 94.9 <sup>a</sup>	49.0 ± 5.1 <sup>a</sup>	412.3 ± 110.6 <sup>a</sup>	0.46 ± 0.09 <sup>a</sup>	0.15 ± 0.03 <sup>a</sup>	0.16 ± 0.03 <sup>a</sup>	8.2 ± 2.8 <sup>a</sup>
0.14 : 0.725	5 843.8 ± 990.4 <sup>b</sup>	49.6 ± 7.5 <sup>b</sup>	4.8 ± 0.4 <sup>a</sup>	260.2 ± 124.8 <sup>a</sup>	53.6 ± 4.4 <sup>a</sup>	304.5 ± 74.4 <sup>a</sup>	0.41 ± 0.03 <sup>a</sup>	0.13 ± 0.01 <sup>a</sup>	0.14 ± 0.01 <sup>a</sup>	8.1 ± 4.1 <sup>a</sup>
0.0 : 0.0	6 860.0 ± 779.0 <sup>a,b</sup>	52.1 ± 7.6 <sup>b</sup>	3.8 ± 0.8 <sup>a</sup>	193.4 ± 56.9 <sup>a</sup>	51.6 ± 7.6 <sup>a</sup>	343.9 ± 89.0 <sup>a</sup>	0.40 ± 0.04 <sup>a</sup>	0.13 ± 0.02 <sup>a</sup>	0.14 ± 0.02 <sup>a</sup>	7.6 ± 1.9 <sup>a</sup>

a,b Untransformed means ( $\pm$  s.d.) within the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  according to either a one-way ANOVA followed by the Tukey post hoc comparison or a Kruskal-Wallis analysis followed by a multiple comparison. There were five replicates for all treatments except for membrane permeability at 10 mg/L FeEDTA where there were only four replicates.

The toxicity of metals to aquatic plants can be modified by the presence of chelators, such as EDTA, in the liquid growth medium. In studies with *Lemna trisulca*, EDTA reduced metal absorption and antagonised the toxicity of copper (Huebert and Shay, 1993b), cadmium and zinc (Huebert and Shay, 1992a). In algal studies, zinc toxicity decreased as the EDTA concentration increased (Anderson and Morel, 1978; Canterford and Canterford, 1980). When EDTA was present in *Selenastrum capricornutum* growth medium, reduced toxicity was observed for copper, zinc, nickel, chromium, cobalt (Chiaudani and Vighi, 1978) and cadmium (Chiaudani and Vighi, 1978; Thellen *et al.*, 1989). In aquarium culture, copper absorption by *Eichornia crassipes* (Mart.) Solms. (water hyacinth) was inhibited by EDTA and humic acid. The natural ligand fulvic acid did not interfere with the accumulation of copper by *E. crassipes* (Nor and Cheng, 1986). If this axenic macrophyte toxicity test was used to examine the effects of metals upon aquatic plants, the amount of iron and EDTA in solution may be lowered to 0.28 mg/L of iron and 1.45 mg/L of EDTA but should not be lowered further. Lower concentrations of FeEDTA might compromise the *M. sibiricum* control growth. In this experiment with *M. sibiricum*, the FeEDTA was added to the medium prior to autoclaving. Huebert and Shay (1992a; 1993a) recommended that the FeEDTA be filter sterilised and added to the medium after autoclaving. No precipitate or other adverse effects were observed as a response to autoclaving the chelator with the medium.

#### 4.4 TOXICITY TEST DURATION

##### 4.4.1 Introduction

The toxic effect of a compound can vary with the length of time the test organism is exposed to the toxicant. The toxicity can either become greater with longer exposure times (Cowgill *et al.*, 1989b; Powell *et al.*, 1996; Rand *et al.*, 1995; Walsh *et al.*, 1991a) or reduced or more variable with longer exposure times (Cowgill *et al.*, 1989b).

Long testing periods allow the organisms to change the composition of the test medium by releasing organic compounds or changing the pH of the medium. These changes in medium composition can inhibit the growth of the test organism (Patrick, 1973), change the chemical speciation of the toxicants, and thus their toxicity (Peterson, 1991; Versteeg, 1990). The longer growth period can allow the toxicant effects to be

fully expressed (Versteeg, 1990) and any toxicant metabolism by the test organism to be observed.

The influence of the duration of toxic exposure can be species specific. Toxicant exposure times for algae have ranged from one hour to three weeks (Lewis, 1995) but the most commonly used exposure time is 96 hours (ASTM, 1990; St. Laurent *et al.*, 1992). Duckweed is usually exposed to toxicants for seven days (ASTM, 1991b; Cowgill *et al.*, 1989b; Day and Hodge, 1996) but longer exposures, such as eight (Huang *et al.*, 1993) or fourteen days (Cowgill *et al.*, 1989b; Huebert and Shay, 1992a; 1992c; 1993b), have been used. For rooted aquatic macrophytes, the test duration has ranged from one to six weeks (Lewis, 1995). This experiment was conducted to determine whether seven or fourteen days was the appropriate time period for the axenic *Myriophyllum sibiricum* toxicity test.

#### 4.4.2 Methods

*Myriophyllum sibiricum* plants, which had been cultured in modified Andrews' medium, were transferred into test tubes containing 3 g of Turface® and 40 mL of modified Andrews' medium with 3% sucrose and various concentrations of zinc chloride. Zinc chloride was chosen for this experiment because it is a standard reference toxicant and it was tested three times in the *Myriophyllum sibiricum* axenic toxicity test with a 14 day exposure period (Section 5.8.2). ZnCl<sub>2</sub> concentrations tested were 0.0, 4.26, 8.52, 17.04, 34.07, 68.15, 136.3 and 272.59 mg/L. Five replicates of each concentration were incubated for either seven or fourteen days. A longer time period (e.g. twenty-one days) was not tested because larger culture tubes would be required to supply the plants with adequate nutrients. Larger test tubes would add an additional expense to the toxicity test. It would also be more time consuming and expensive to conduct longer tests. During the growth period, height of every plant was measured daily. At the end of the appropriate growth period, the plants were harvested and plant height, root number, root length, dissolved oxygen, fresh weight, plant area, pigment content and membrane integrity were determined. All other procedures followed those outlined in Section 4.6.

#### 4.4.3 Results and Discussion

Representative plants cultured with ZnCl<sub>2</sub> for seven or fourteen days are displayed in Figure 31 and Figure 32, respectively. The plants cultured for seven days did not have as much time to differentiate in response to the toxicant as those exposed

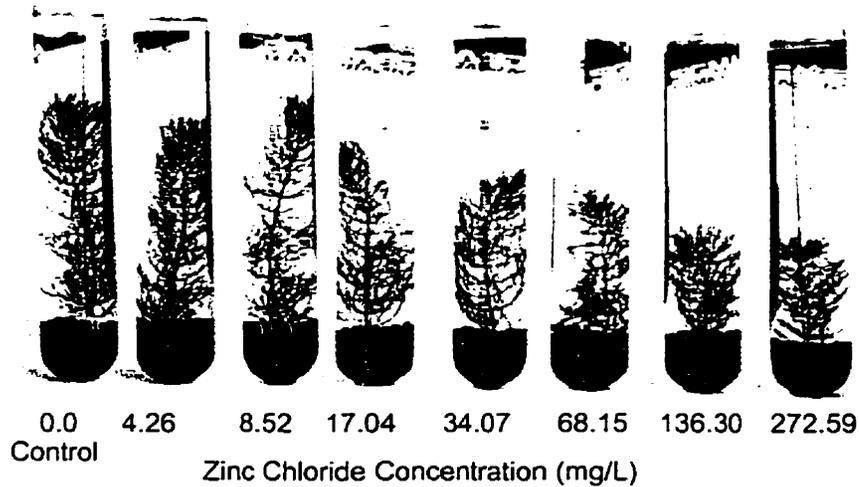


Figure 31: This photograph shows *Myriophyllum sibiricum* plants exposed to 0.0, 4.26, 8.52, 17.04, 34.07, 68.15, 136.30 and 272.59 mg/L of zinc chloride for 7 days. The plants were axenically cultured in modified Andrews' medium containing 3% sucrose. Turface<sup>®</sup> was used as the rooting substrate. The highest concentration of ZnCl<sub>2</sub> completely inhibited the growth of this aquatic plant.

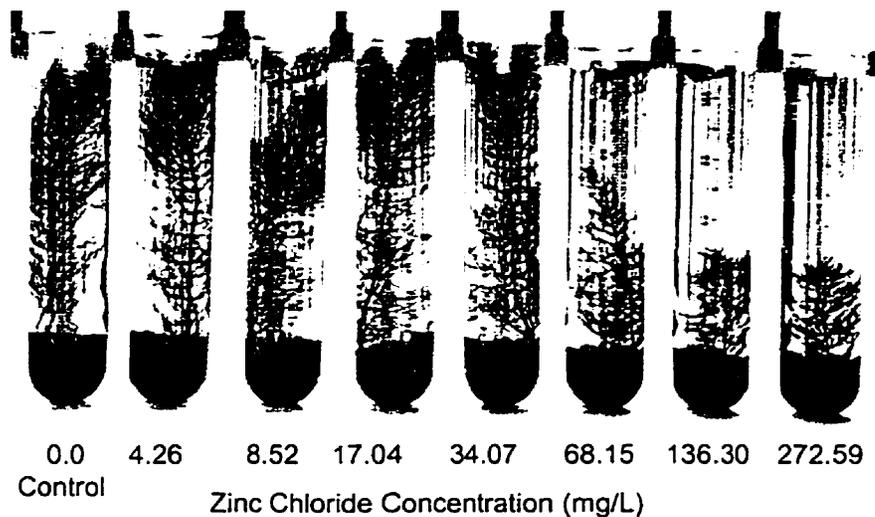


Figure 32: The effect of ZnCl<sub>2</sub> (0.0, 4.26, 8.52, 17.04, 34.07, 68.15, 136.30 and 272.59 mg/L) upon the growth of *Myriophyllum sibiricum* after 14 days of exposure. Axenic plants were cultured in test tubes containing Turface<sup>®</sup> and modified Andrews' medium supplemented with 3% sucrose. The highest concentration of ZnCl<sub>2</sub> completely inhibited plant growth.

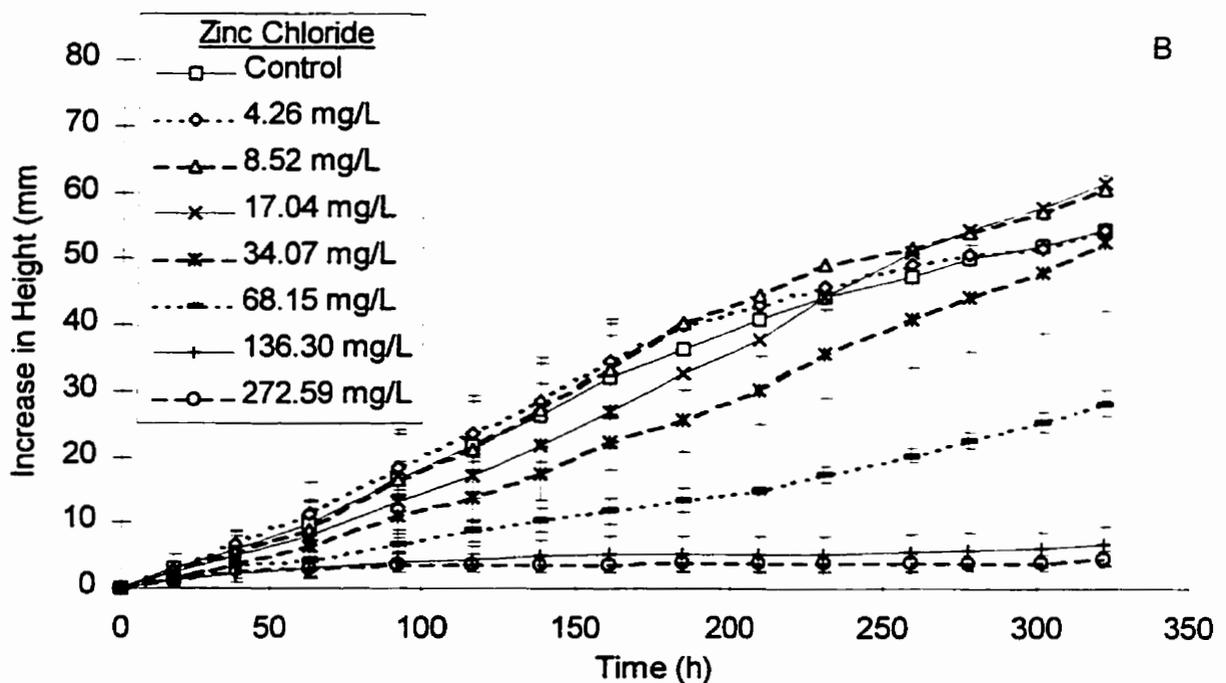
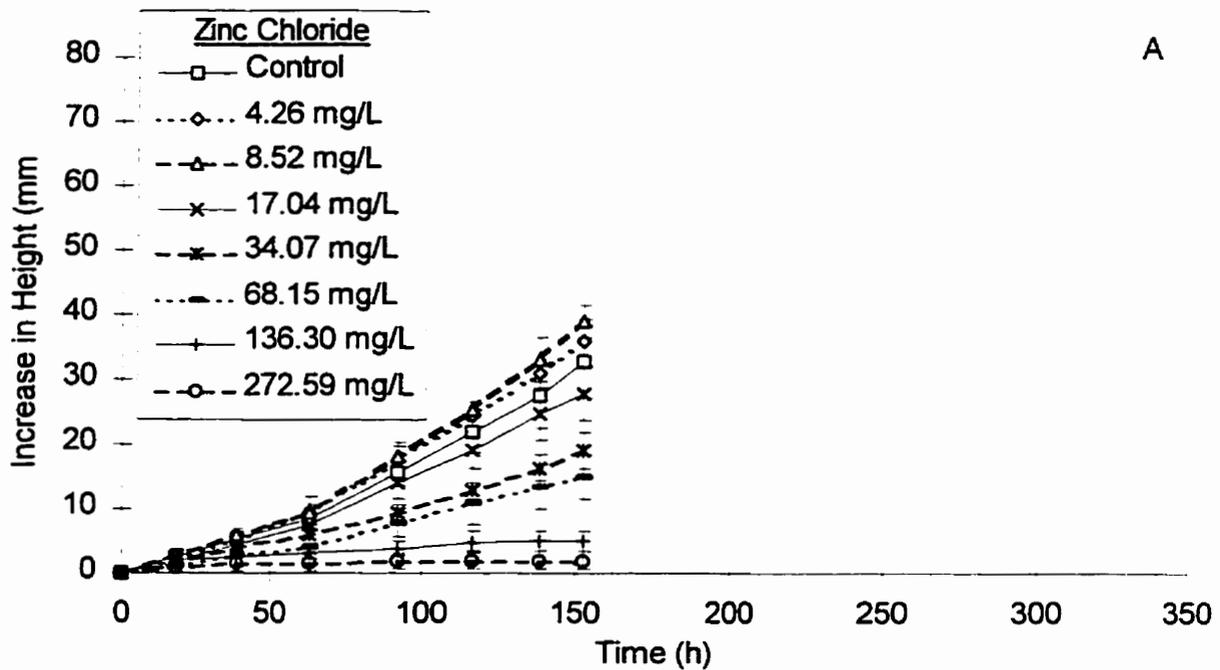


Figure 33: Increase in *Myriophyllum sibiricum* shoot height after seven (A) and fourteen (B) days of exposure to various concentrations of  $ZnCl_2$ . The plants were exposed to the toxicant added to sterile modified Andrews' medium. Fourteen days of toxicant exposure allowed the plants to differentiate in their response to the toxicant. Within a single concentration, growth rate did not decrease, which suggested that nutrients were not limited.

Table 25: In sterile modified Andrews' medium, *Myriophyllum sibiricum* plants were exposed to seven ZnCl<sub>2</sub> concentrations (4.26, 8.52, 17.04, 34.07, 68.15, 136.3 and 272.59 mg/L) plus a control treatment for seven or fourteen days. The phytotoxic effects of this compound upon *M. sibiricum* growth and development are presented as NOEC, LOEC and the IC50 with 95% confidence intervals.

GROWTH PARAMETER	7 Day			14 Day		
	NOEC (mg/L)	LOEC (mg/L)	IC50 (mg/L)	NOEC (mg/L)	LOEC (mg/L)	IC50 (mg/L)
Area Under the Growth Curve	34.07	68.15	52.8 (35.9, 77.7) <sup>a</sup>	17.04	34.07	58.2 (49.9, 67.8) <sup>a</sup>
Increase in Shoot Length	4.26	34.07	62.3 (40.0, 97.0) <sup>a</sup>	4.26	8.52	76.6 (58.8, 99.9) <sup>a</sup>
Root Number	34.07	68.15	39.2 (28.1, 54.7) <sup>a</sup>	17.04	34.07	37.3 (31.3, 44.4) <sup>a</sup>
Root Length	17.04	34.07	20.0 (16.9, 23.6) <sup>a</sup>	4.26	8.52	23.1 (15.4, 34.7) <sup>a</sup>
D.O.	4.26	8.52	46.7 (35.7, 61.1) <sup>b</sup>	34.07	68.15	180.1 (118.9, 273.0) <sup>a</sup>
Fresh Weight	17.04	34.07	71.3 (36.1, 140.6) <sup>b</sup>	17.04	34.07	46.8 (34.2, 63.9) <sup>b</sup>
Membrane Integrity	68.15	136.3	> 272.59 <sup>a,b</sup>	8.52	17.04	> 272.59 <sup>a,b</sup>
Plant Area	17.04	34.07	130.7 (63.6, 268.8) <sup>b</sup>	17.04	34.07	66.2 (37.8, 115.7) <sup>b</sup>
Chlorophyll a (fresh wt)	17.04	136.3	> 272.59 <sup>a,b</sup>	4.26	8.52	163.8 (127.7, 210.1) <sup>a</sup>
Chlorophyll b (fresh wt)	17.04	136.3	> 272.59 <sup>a,b</sup>	4.26	136.3	201.3 (142.8, 283.6) <sup>a</sup>
Carotenoid (fresh wt)	17.04	272.59	> 272.59 <sup>a,b</sup>	4.26	136.3	> 272.59 <sup>a,b</sup>
Chlorophyll a (dry wt)	< 4.26	34.07	69.3 (36.4, 132.1) <sup>a</sup>	8.52	17.04	87.4 (40.7, 187.5) <sup>a</sup>
Chlorophyll b (dry wt)	< 4.26	34.07	101.0 (49.9, 204.1) <sup>a</sup>	17.04	34.07	108.0 (58.2, 200.4) <sup>a</sup>
Carotenoid (dry wt)	17.04	34.07	107.6 (36.2, 319.8) <sup>a</sup>	17.04	34.07	170.7 (47.3, 615.7) <sup>a</sup>

a The cumulative normal statistical model was utilised to determine the IC50 values and 95% confidence intervals.

b The IC50 and 95% confidence intervals were determined using the weibull model.

for fourteen days. The growth curves are shown in Figure 33 A and B for seven and fourteen days of exposure, respectively. The plant growth rate did not decrease during the fourteen day exposure period, indicating that nutrient limitation was not a problem. Table 25 contains the NOEC, LOEC and IC50 values for these two exposure times. For most of the endpoint parameters, there was not much difference between the IC50 values for the two exposure periods because their 95% confidence intervals overlapped. ZnCl<sub>2</sub> was more toxic after fourteen days of exposure when the IC50 values for chlorophyll *a* and chlorophyll *b* (fresh weight) were compared. When comparing the NOECs, the NOEC was lower for the fourteen day exposure period than for the seven day exposure period for more endpoint parameters (area under the growth curve, root number, root length, membrane integrity and pigment content on a fresh weight basis). With the seven day exposure duration, the variability within treatments was greater so that differences between treatments and the control were not as consistent. This was also evident from the LOEC values in Table 25. For the seven day exposure period, the LOEC was not the next highest concentration to the NOEC for six of the endpoints [shoot length, chlorophyll *a* (fresh weight), chlorophyll *b* (fresh weight), carotenoid (fresh weight), chlorophyll *a* (dry weight) and chlorophyll *b* (dry weight)]. Only chlorophyll *a* and *b*, expressed in terms of fresh weight, had variability between the NOEC and the LOEC after fourteen days of exposure to ZnCl<sub>2</sub>. The fourteen day NOEC and IC50 values obtained in this experiment are similar to those values obtained in the three reference toxicant experiments discussed in Section 5.8.2. The NOECs from the other fourteen day experiments with ZnCl<sub>2</sub> are given in Table 89. The NOEC values were identical to the ones for the fourteen day experiment in Table 25 for about half of the endpoints measured in the second dilution series and for three parameters from the first dilution experiment (Table 89). For the fourteen day IC50 values found in Table 25 and the second dilution series experiment in Table 90, there was overlap of the 95% confidence intervals for thirteen of the fourteen endpoint parameters measured. The results from the fourteen day ZnCl<sub>2</sub> experiment discussed in this section (Table 25), the range-finding test (Table 90) and the first dilution series (Table 90) were not as closely correlated. This was possibly due to the different media composition used in culturing the plants before experimentation. The first two experiments were started using stock plants that had been cultured in half strength M & S medium while the stock plants utilised in the second reference toxicant experiment and the ones discussed in this

chapter were cultured in full strength modified Andrews' medium. This re-emphasised the possibility that growth media may influence the toxicity of certain compounds.

The results determined in this experiment with *M. sibiricum* correlate with some of the results from other aquatic plant studies but there were some differences. In the emergent macrophyte toxicity test (Powell *et al.*, 1996), *Oryza sativa* was tested with boron over a one, two, and four week exposure period. In that study it was determined that exposure time did influence the level of toxicity. The general trend was that as exposure time increased, the calculated toxicity values (IC50) for dry weight and chlorophyll *a* content decreased. The between treatment variation also increased as exposure duration increased. Differences based upon chlorophyll *a* concentration could be detected after only a short exposure period but differences based upon dry weight values required a longer exposure duration to be detectable. Native emergent species studied by Powell *et al.* (1996) were exposed to the toxicant for two or three weeks. After two weeks of exposure there was no significant difference in dry weight for *Spartina pectinata* and *Polygonum muhlenbergh*. With *S. pectinata*, dry weight differences were noted after three weeks of exposure at the highest concentration. Boron accumulation in leaf tissue was the only data presented for native emergent plant species exposed over a four week period (Powell *et al.*, 1996).

In tests with *Echinochloa crusgalli*, the LOEC became greater with longer exposure time of up to fourteen days (Walsh *et al.*, 1991a). The effects of exposure time can be species specific. Walsh *et al.* (1991b) grew *E. crusgalli* for two weeks, while *Spartina alterniflora* was cultured for four weeks.

Cowgill *et al.* (1988; 1989b) compared a seven to fourteen day exposure period for *Lemna gibba* and several clones of *L. minor*. The seven day exposure period produced consistent results for the species and endpoints examined while the results from the fourteen day exposure period were more variable. There was a significant decrease in chlorophyll *a* production in the plants after fourteen days of chemical exposure. For biomass, several of the *L. minor* clones exhibited greater sensitivity after seven days of exposure than after fourteen days while the reverse was observed for *L. gibba*. It was hypothesised that the additional variability over the fourteen day exposure period resulted from a lack of nutrients in the medium (Cowgill *et al.*, 1988; 1989b).

Several toxicants were tested upon *Selenastrum capricornutum* to compare the sensitivities of the standard 4 day population growth test with 30 minute carbon dioxide

fixation and 30 minute oxygen generation tests. Based upon EC50 values, it was determined that the population growth test was more sensitive for detecting toxicant effects than were the shorter tests (Versteeg, 1990). The toxicity of hexazinone and atrazine to *Anabaena flos-aquae* and *Selenastrum capricornutum*, as measured by chlorophyll *a* content, decreased with increasing exposure time (Abou-Waly *et al.*, 1991b).

Growth rate should not decrease over time as this might indicate nutrient stress or inadequate volume (Huebert and Shay, 1993b). In the submersed macrophyte toxicity test presented in this thesis, the test tube size did not permit the experiment to be conducted for longer than two weeks. If larger test tubes were used, it might be possible to conduct the experiment for a longer test period. Conducting tests for a longer duration would be more expensive from an equipment standpoint (larger test tubes, longer measuring rods, more growth cabinet use) and from a personnel standpoint (longer work period). From the data presented in this section, it was apparent that better comparisons of NOEL, LOEL and IC50 values were possible with the two week exposure duration as compared to the one week duration. The *Myriophyllum sibiricum* toxicity test should be conducted for 2 weeks to reduce variability within treatments and increase variability between treatments. However, this toxicity test could be conducted for 7 days if direct comparison with duckweed toxicity testing was desired.

## 4.5 TROUBLE SHOOTING

There are several problems that can develop during the toxicity testing procedure. The problem with sterility has been discussed previously (Section 3.4.1). Unclean glassware and volatile compounds are the other two areas where problems can develop.

### 4.5.1 Cleaning Procedure

During the course of this thesis it became very apparent that cleanliness of the dishes was very important to the success of the toxicity test. On several occasions, control or stock plants would display symptoms similar to a pesticide effect. For example, Figure 34 shows two control plants with the plant on the left showing normal,

healthy development while the plant on the right shows symptoms consistent with one of the bleaching herbicides. These control plants were started during an experiment that did not use either bleaching herbicides as the treatment. It was obvious that the cross contamination was transferred from improperly washed glassware. Either the glass measuring rod, the glass test tube or the Tygon® tube cap contained herbicide residue. Two solutions to this cross-contamination problem exist. If sufficient funds allow, disposable test tubes and pipettes can be used and new test tube closures can be purchased for every experiment. The other, more economical procedure is to efficiently



Figure 34: Two control plants with the plant in the right test-tube showing symptoms similar to the bleaching herbicide effect.

wash the glassware and remove all trace contaminants. Regardless of which method is used for the test tubes, pipettes and closures, proper cleaning procedures must be followed for the remaining glassware. Several slightly different glassware washing techniques for use with biological testing systems are recommended by the various testing agencies (ASTM, 1990; ASTM, 1993; Environment Canada, 1992; Miller *et al.*, 1978). All reusable glassware used during this thesis was cleaned using a stiff-bristle brush, warm water and detergent. After triple rinsing with tap water and triple rinsing with deionized water, all glassware was rinsed with 10% HCl (v/v) to remove trace metals. This was followed by three more deionized water rinses, a rinse with acetone to remove any organic contaminants and another triple rinse with deionized water. Most glassware was

allowed to air dry upside down. Occasionally when clean glassware was required quickly, it was placed in an oven at 105 °C until dry. Section 3.7.8 contains a more detailed description of the recommended glassware cleaning procedure. It is important that all personnel be properly trained in glassware washing procedures. It is particularly difficult to ensure that all washing solutions enter the hollow inside of the glass measuring rods and the inside of the Tygon® tube caps.

#### 4.5.2 Media Storage

The other factor that apparently affected the growth of the stock plants was contamination by volatile compounds. Occasionally, the stock plants would demonstrate the epinasty and stem rupture associated with the auxin mimicking herbicides (2,4-D and triclopyr) (see Section 4.3.1; Figure 2). There were two possible reasons for this contamination of the media.

At the beginning of this thesis, half strength Murashige and Skoog medium was used to culture the *M. sibiricum* plants, as recommended by Kane and Albert (1989b) and Kane *et al.* (1988a; 1991) for other *Myriophyllum* species. The M & S medium was obtained from Sigma Chemicals. During the period of these investigations, Sigma changed the packaging of the M & S medium three times. Originally it was purchased in amber vials with rubber stoppers fastened in place with metal pull-tabs. The vials were later closed by screw tops. Finally, the M & S medium was purchased in aluminium pouches. One possible cause of the malformed plants could be a by-product of the packaging material since the problem began shortly after the aluminium pouches were introduced.

At about the same time, one of our refrigerators required repair so the media were temporarily stored in a refrigerator that also contained pesticides and pesticides stored in solvent. These pesticides included triclopyr and 2,4-D. It was possible that volatile compounds entered the M & S storage pouches and contaminated the medium. Even with the purchase of new pouches of M & S medium, the epinastic disfiguration continued. At this point, it was decided to use the modified Andrews' medium for both stock and experiment plants. It was also decided that media stock solutions should be stored in cold areas where pesticides were not allowed. These experiences emphasised the importance of separate facilities for storage of test compounds and nutrient solutions as recommended under good laboratory practices (GLP) (DiGiulio and Malloy, 1995; U.S. EPA, 1983a; 1983b).

#### 4.6 SUMMARY OF THE *M. SIBIRICUM* AXENIC TOXICITY TEST TECHNIQUE

This toxicity test is in the process of being published with the American Society for Testing and Material (ASTM). A draft of the ASTM protocol can be found in Appendix 8.6. Without permission from ASTM headquarters, citation of the ASTM

document is not allowed until it is published. This section contains a summary of the methods recommended for conducting the axenic *Myriophyllum sibiricum* toxicity test.

The axenic toxicity testing technique involves exposing the test organism to selected concentrations of the test chemical in individual tubes. Each test tube contains 3 g of Turface® and 40 mL of nutrient medium previously spiked with the test chemical. A 3 cm apical segment of *M. sibiricum* is added the tube. The tubes are incubated for 14 days, during which time the increase in plant height may be measured and growth curves established. Other possible toxicity test endpoints that may be measured include shoot length, root number and total root length, total fresh weight, chlorophyll *a*, chlorophyll *b* and carotenoid content, membrane integrity, plant area and oxygen production. Appendix 8.5 contains a complete list of the equipment (Table 100 and Table 101) and reagents (Table 102) that are required to conduct this toxicity test.

#### 4.6.1 Culturing *Myriophyllum sibiricum*

*M. sibiricum* is to be cultured and maintained in a laboratory facility where temperature and lighting can be controlled. The culture should be isolated from the toxicity testing chamber to minimise the risk of culture contamination by volatile chemicals released from sample test solutions. The test species must be cultured for at least 8 weeks in the facility before testing can be initiated.

#### 4.6.2 Starting from Field Collected, Non-axenic Plants

- 1) In the autumn, collect *Myriophyllum sibiricum* turions.
- 2) Place the turions into a 20 L aquarium containing 5 cm of soil and 18 L of distilled water. Aerate the aquarium and maintain the culture at a temperature of 15 °C and a fluence rate of 200 - 300  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 16 hours per day (Figure 35a).
- 3) Plants are removed from the aquarium and rinsed under flowing deionized water for about ½ hour. Using aseptic technique in a Laminar Airflow Cabinet, the plants are disinfected in a 3% (w/v) sodium hypochlorite solution containing 0.01% Tween-20 for 20 min (Figure 35b).
- 4) Transfer segments with several nodes into 45 mL of full strength modified Andrews' medium (Selim *et al.*, 1989), as described below (Chapters 4.6.3 and Table 26).

### 4.6.3 Starting with an Axenic Culture

- 1) In a Fernbach flask, make 2 L of full strength modified Andrews' medium with 60 g of sucrose. Remove the modified Andrews' stock solutions (Table 26) from the cold room. Fresh stock solutions should be prepared every 6 months. Allow the stock solutions to warm up to room temperature. Place 60 g of sucrose in a 2 L volumetric flask and dissolve in ultrapure water. Add the correct volumes of the eleven nutrient stock solutions (Table 26) and fill with ultrapure water. Stir until mixed. Adjust the pH to  $5.8 \pm 0.1$  with 1N KOH and HCl. Cover with aluminium foil. Each 2 L batch of medium will start approximately 40 plants. Ten to twelve days before each experiment, transfer double the number of plants necessary for the experiment. This permits the selection of healthy plants for the experiment while leaving the additional plants for new stock plant creation. Apical shoots are ready for experimental use when they are at least 3 cm in length.
- 2) Autoclave the liquid medium at 121 °C for 20 min with a slow exhaust portion at the end of the autoclave cycle. A dry load containing forceps, scalpel handles, glass Petri plates (all wrapped in aluminium foil), pipettes, and round bottomed test tubes (2.5 x 15 cm) is autoclaved at 121 °C for 20 min with a final fast exhaust segment.
- 3) Under the Laminar Airflow Cabinet, pipette 45 mL of the autoclaved medium into each test tube and cover with a plain test tube cap that has been labelled on the side. Transfer several mL of the remaining medium into a disposable Petri plate containing Trypticase Soy Agar (TSA). Use laboratory sealant film to secure the TSA plate.
- 4) After the medium has cooled to room temperature, aseptically transfer segments from 10 to 12 day old stock plants (Figure 35c). Use large forceps (25 cm) to remove the stock plant from the test tube and to place the new segments in the fresh medium (Figure 35d). Between the cover and the bottom of a large glass Petri plate, manipulate the plants with a small forceps and a scalpel (Figure 35c). Each segment should contain several nodes and visible buds. If the new stock plants are to be utilised for an experiment in 10 to 12 days, they should contain only one visible bud no longer than one cm. Between stock plant transfers, rinse the utensils in 95% reagent alcohol and flame dry. Be careful not to singe the plant or get any alcohol in the fresh medium. After completing several plant transfers, change scalpel blades, forceps and Petri plates.

Record when the utensils were changed. From randomly selected stock plant test tubes, transfer several mL of medium onto TSA plates to check for microorganisms.

- 5) Secure each new test tube shut with laboratory sealant film before removing them from the Laminar airflow Cabinet.
- 6) Record the source of each segment and count the number of nodes and buds.
- 7) The tubes (20 tubes per rack) should be alternated in test tube racks (12 x 30 cm with 40 spaces). Place the racks in a growth cabinet set at 16/8 hour photoperiod and a 25/20 °C temperature regime. The fluence rate at the base of the test tube rack should range between 100 - 150  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Figure 35e).
- 8) After 10 - 12 days, the segments should have produced shoots at least 3 cm long. At this point the shoots are ready to be transferred for experimentation. Do not use plants that were growing in medium contaminated with bacteria or fungi. Avoid using plants transferred in the same Petri plate as plants that became contaminated.

#### 4.6.4 Methods for *Myriophyllum sibiricum* Experimental Plant Transfer

- 1) In a Fernbach flask, make 2 L of modified Andrews' medium with 60 g sucrose (Chapter 4.6.3). Adjust the pH to  $5.8 \pm 0.1$ . Cover with aluminium foil.
- 2) Autoclave the medium at 121 °C for 20 min with a slow exhaust portion. Also autoclave a dry load containing the utensils, glass Petri plates, measuring rods (wrapped in aluminium foil), pipettes, 500 mL Erlenmyer flasks, graduated cylinders and round bottomed test tubes (2.5 x 15 cm) covered with clear plastic test tube closures (I.D. = 25 mm, 38 mm in height) containing 3.5 cm sections of Tygon<sup>®</sup> tube (I.D. = 7 mm, O.D. = 10 mm). Each test tube should contain 3 g Turface<sup>®</sup> that has been rinsed in deionized water, dried at 105 °C, and sterilised three times at 24 hours intervals. Label the side of each cap.
- 3) Randomly select 10 - 12 day old shoots that are at least 3 cm long. Use plants of the same age for each experiment.
- 4) Under the Laminar Airflow Cabinet, dispense 250 mL of autoclaved medium into the autoclaved 500 mL Erlenmyer flasks.
- 5) For water soluble chemicals, make up the correct concentrations of toxicant in either sterile nanopure water or sterile modified Andrews' medium. Add 1 mL of each concentration to 250 mL of medium. Use 1 mL of sterile nanopure water for the control.

If the chemical is not soluble in water, dissolve the chemical in methanol. Add 1 mL of each toxicant/methanol concentration to the 250 mL of medium. Use 1 mL of methanol as a solvent control and 1 mL of sterile nanopure water as a water control (Figure 35f). For pesticides, the concentration that is expected to be found in the environment may be tested and compared to the controls. If this concentration is phytotoxic, then a test containing a series of dilutions may be conducted. In the dilution series, it is recommended that five to seven test concentrations plus the control are tested and replicated five times. All chemical manipulation must be conducted using aseptic technique. Ensure that the toxicant/medium solution is thoroughly combined.

6) Under the Laminar Airflow Cabinet, aseptically pipette 40 mL of the toxicant/medium solution into each test tube, five replicates of each concentration (Figure 35g). Cut 3 cm apical lengths from the *M. sibiricum* stock plants. Remove all buds, branches and roots. Using long forceps, transfer the apex into a randomly selected test tube, ensuring that the cut end of the apex is touching the Surface<sup>®</sup> (Figure 35h). Carefully add a 15 cm measuring rod (shortened Westergren Blood Sedimentation Tube) (Figure 35h). The top end of the measuring rod is inserted into the 3.5 cm length of Tygon<sup>®</sup> tubing. Between apical transfers, rinse the utensils in 95% reagent alcohol and flame dry. Be careful not to singe the plant. Do not get any alcohol or test chemical into the next tube. Continue transferring until all experimental tubes have been filled. Record the order in which the plants were transferred. From randomly selected experimental stock plant tubes, transfer several mL of medium onto TSA plates to check for contamination.

7) Use laboratory sealant film to secure each lid to the tube before removing from the Laminar Airflow Cabinet.

8) Record the source of each apex. Count the number of nodes and measure the length of each plant using the measuring rod. Length is measured from the cut end of the plant to the top of the apex. Do not include any leaves that extend above the apex. A sample worksheet is included as Table 103 in Appendix 8.5.

9) The tubes should be randomised in alternating holes in test tube racks (12 x 30 cm with 40 spaces). Place the racks in a growth cabinet set at 16/8 hour photoperiod and a 25/20 °C temperature regime. The fluence rate at the base of the test tube rack should be between 100 - 150  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

10) Measure the pH and conductivity of the unused toxicant/medium solutions. If residue analysis is to be conducted on the solutions, store the solutions in screw cap glass tubes and place in a -20 °C freezer (or other appropriate storage method for the test chemical).

11) Every second day, measure the plant length to the tip of the apex, excluding any leaves that extend above the apical meristem (Table 103). The plant length data (mm) are used to establish a growth curve and area under the growth curve is calculated by:

$$\text{Area under Curve} = \sum_{i=2}^n \frac{IH_{i-1} + IH_i}{2} \cdot (T_i - T_{i-1}) \quad , \quad (7)$$

where IH is the increase in height from the start of the experiment and T is the time at each subsequent measurement point, in hours from time zero (Boutin *et al.*, 1993). The number of roots and branches produced plus the number of nodes may be measured every second day or less frequently. Terminate the experiment on Day 14. Discard any plants that become contaminated with a bacterial or fungal infestation. There must be at least three surviving replicates of the controls and treatments for the experiment to be valid.

#### 4.6.5 Endpoint Determination for the *Myriophyllum sibiricum* Toxicity Tests

The steps listed below are the maximum number of endpoints that have been developed to date (Figure 35i). A sample worksheet has been included as Table 104 in Appendix 8.5. If the toxicant's mode of action does not affect one of the systems examined, that endpoint can be eliminated.

- 1) If a visual record of the experiment is required, take photographs.
- 2) Visually assess the length and number of nodes.
- 3) One at a time, remove the laboratory sealant film and measuring rod from each tube. Measure the D.O. immediately.
- 4) If the determination of chemical concentration is required, remove about half of the medium at this point. Place the medium in glass screw top tubes and place in a -20 °C freezer (or other appropriate storage method) until analysis can be conducted.
- 5) Using the long forceps, remove the plant from the test tube. Measure the calliper length of the shoot and each root. Return the plant to the appropriate test tube to prevent desiccation.

- 6) To determine the plant area, remove the plants, pat dry on paper towels and photocopy each plant onto photocopy acetates. For each photocopier, the darkness setting needs to be calibrated to get a realistic copy. On each acetate sheet, also photocopy a black circle or square with a known area for calibration purposes. Label the acetate with the plant's number. Return the plants to the test tubes.
- 7) Individually, pat the plant dry on paper towels. Place the plant into a weigh boat and determine the fresh weight.
- 8) Working quickly, cut off the apex to  $50 \pm 2$  mg. Place the apex into a glass scintillation vial containing 10 mL of 80% ethanol. Store the scintillation vials in a dark cold room (4 °C). These apices will be used to determine chlorophyll/carotenoid content.
- 9) To determine membrane permeability, the next  $100 \pm 5$  mg of the shoot is rinsed three times in nanopure water, placed into a flat bottomed tube containing 20 mL of nanopure water, loosely covered and left at room temperature overnight. In order to avoid excess cellular leakage, this 100 mg sample should consist of only one plant section.
- 10) The extra portion of the plant is weighed, placed into an envelope and dried at 80 °C for a minimum of 24 h. Weigh the dried plants.
- 11) Record the time at which all the plants are weighed and segmented. The 24 hour measurements are made 24 hours from this point.
- 12) Measure the conductivity and pH of the medium in each test tube.
- 13) If residue analysis is to be conducted, save the remainder of the medium in screw cap tubes in a -20 °C freezer (or alternative storage technique). If no analysis is to be conducted, discard the medium and contaminated Turface® in a manner appropriate to the toxicant being tested.

#### 4.6.6 Twenty-four hour measurements for the *Myriophyllum sibiricum* toxicity test

##### 4.6.6.1 Measurement of Membrane Integrity

- 1) Measure the conductivity of the water/plant solution in the flat bottomed tubes.

- 2) Place the flat bottomed tubes in an enamel container filled with a small amount of water. Boil for 20 min.
- 3) Remove the tubes from the water. Allow to cool down to room temperature. Measure the conductivity of the solution.
- 4) Membrane injury is determined as percentage of total electrolyte leakage:

$$\% \text{ Membrane Leakage} = \frac{\text{Conductivity before boiling}}{\text{Conductivity after boiling}} \cdot 100. \quad (8)$$

#### 4.6.6.2 Measurement of Chlorophyll/Carotenoid Content of the Apices

- 1) After the apices have been soaking in the 80% ethanol for 24 hours, measure the pigment content spectrophotometrically at 447, 647 and 663 nm. Calculate values for chlorophyll *a*, chlorophyll *b* and carotenoid content based on either the fresh or dry weight of the apices.

#### 4.6.6.3 Plant Area Measurement

- 1) Cut each individual plant out of the acetate.
- 2) At the plant area meter, remove the plant number and run the photocopied plant through the area meter. Determine the area of the photocopied circle or square. If the area of the photocopied circle/square varies from the area of the actual circle/square, adjust the values of the photocopied plants accordingly.

#### 4.6.7 Calculations

Depending on the data to be analyzed and the purpose of the test, a variety of procedures can be used to calculate the results from a test.

- 1) If the test contains both a nutrient medium control and a nutrient solution/solvent control, the growth and development of the plants in the two controls should be compared using a t-test or a nonparametric test, such as the Mann-Whitney U-test. Another method of comparing the controls would be to conduct an LSD test. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.
- 2) If a statistically significant difference in growth or development is detected between the two controls, only the solvent control can be used in the calculation of results. If no

statistically significant difference is detected, the data from both controls can be pooled and used in the calculations.

3) If the test consisted of only one test concentration and the control(s), a %I for this concentration may be determined. A t-test, for normal data, or Mann Whitney U-test, for nonparametric data, may be used on the transformed or raw data to determine if the treatment is statistically significantly different from the control(s).

4) For data from a dilution series, the data may be examined for the presence of outliers and tested for heterogeneity before a randomized complete block analysis of variance (ANOVA) is conducted. If the data are not normal and can not be transformed, a nonparametric equivalent, such as the Kruskal-Wallis test, should be conducted.

5) After the ANOVA or the Kruskal-Wallis test, the treatments can be compared to the control using an appropriate mean comparison procedure. The highest concentration not significantly different from the control is designated the no-observable-effect-concentration (NOEC). The percent inhibition actually observed at the NOEC should be calculated.

6) If an IC50 is to be determined, first calculate the percent inhibition (%I) for each test chamber in each treatment other than the control(s). Percent inhibition is usually calculated by:

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean}} \cdot 100. \quad (9)$$

On occasion, it might be necessary to use a modified formula to calculate percent inhibition. This is useful for endpoint parameters, such as membrane integrity, where treatment values increase as toxicity increases.

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean} - \text{most toxic value}} \cdot 100. \quad (10)$$

In situations where 100% inhibition is not equivalent to zero, the following percent inhibition formula can be substituted:

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean} - \text{minimum value}} \cdot 100. \quad (11)$$

7) The IC<sub>50</sub> is calculated using a linear or a nonlinear regression model, such as that developed by Anderson *et al.* (in press). The different methods are compared in Section 5.6.1. The type of model and estimation method should be described along with the 95% confidence intervals about the estimates.

#### 4.6.8 Washing of Glassware

To remove all trace metals and organics, all reusable glassware (test tubes, Erlenmeyer, Fernbach and volumetric flasks, pipettes, graduated cylinders, etc.) must be cleaned and treated according to the following method:

- 1) place in warm water with a dish detergent and allow to soak overnight;
- 2) using a stiff-bristle brush, loosen any material stuck to the inside or outside of the glassware;
- 3) triple rinse with tap water;
- 4) triple rinse with deionized water;
- 5) rinse with reagent grade 10% HCl (v/v); for large containers partially fill and swirl the HCl solution so that the entire inner wall is bathed (wear acid proof gloves during this procedure);
- 6) triple rinse with deionized water;
- 7) rinse with acetone;
- 8) triple rinse with deionized water;
- 9) let air dry upside down or place in an oven at 105 °C until dry; and,
- 10) store in boxes or covered with appropriate cap or aluminium foil.

#### 4.6.9 Axenic Culturing Technique

All the plant manipulations must be conducted using sterile techniques. This requires that all liquids be placed in loosely covered containers and autoclaved for 121 °C for 20 min with a slow exhaust portion at the end of the autoclave cycle. The container lids should be tightened before the container is removed from the autoclave. If the liquids cannot be autoclaved because the chemical properties of the solution may be altered, then filter sterilisation to remove all particulates larger than 0.2 µm may be utilised. All dry equipment should be wrapped in aluminium foil and autoclaved at 121 °C for 20 min with a fast exhaust portion to the autoclave cycle.

a) In aquaria, turions of *Myriophyllum sibiricum* are grown into mature plants.



b) The plant segments are disinfected in a 3% NaOCl and 0.01% Tween®-20 solution for 20 minutes.



d) The segments are placed into modified Andrews' media (Selim *et al.*, 1989) supplemented with 3% sucrose.

e) Plant cultures are incubated at  $25 \pm 2$  °C (16 hr photoperiod) for 10 to 12 days until new growth is formed. Plants are used for experimentation (Steps f - i) or divided to maintain sterile stock cultures (Steps c - e).



f) The toxicant or nanopure water is added to the treatment or control media.

g) Forty mL of treatment or control media is pipetted into test tubes containing 3 g Turface®. Each concentration is replicated 5 times.



h) Aseptically, 3 cm apical plant shoots and a measuring rod are transferred into the tubes.

i) After 2 weeks of incubation, the plants are examined for:

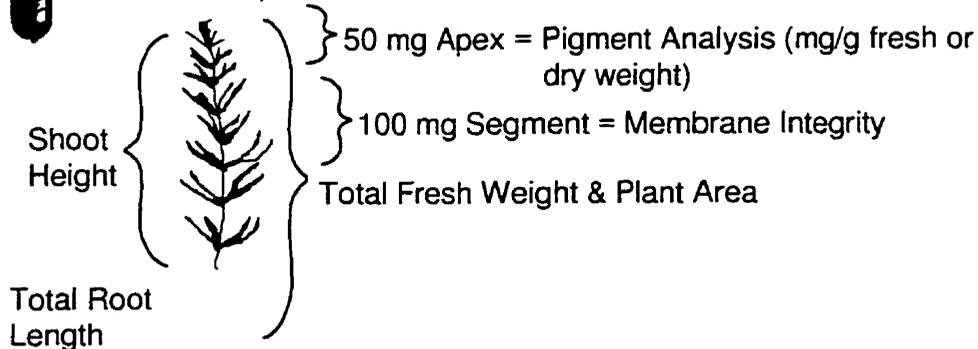


Figure 35: Flow chart displaying the *Myriophyllum sibiricum* axenic bioassay culturing and experimentation procedures.

Solution Number	Salt	Weight of salt per 1 L stock solution	mL stock solution per 2 L final volume
1	KNO <sub>3</sub>	16.16 g	10.0
2	Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	37.76 g	10.0
3	MgSO <sub>4</sub> •7H <sub>2</sub> O	19.72 g	10.0
4	KH <sub>2</sub> PO <sub>4</sub>	5.44 g	10.0
5	KCl	746 mg	2.0
6	H <sub>3</sub> BO <sub>3</sub>	155 mg	2.0
7	MnSO <sub>4</sub> •H <sub>2</sub> O	169 mg	2.0
8	ZnSO <sub>4</sub> •7H <sub>2</sub> O	115 mg	2.0
9	CuSO <sub>4</sub> •5H <sub>2</sub> O	12.5 mg	2.0
10	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> •4H <sub>2</sub> O	3.7 mg	2.0
11	FeEDTA	372 mg Na <sub>2</sub> EDTA 278 mg FeSO <sub>4</sub> •7H <sub>2</sub> O heat to 80 °C	20.0

Note: Solution 11 (FeEDTA) is made by dissolving 372 mg Na<sub>2</sub>EDTA in 1000 mL nanopure water. Once this is dissolved, add 278 mg FeSO<sub>4</sub>•7H<sub>2</sub>O and heat to approximately 80 °C. Ten mL of this solution are added per litre of final culture solution. These stock solutions may be stored at 4 °C for a maximum of 6 months. The cold storage room must be free from volatile compounds.

After sterilisation of the liquids and dry equipment, all further manipulations must occur in the sterile environment of a Laminar Airflow Cabinet or an ultraviolet sterilization hood. The work surface of the cabinet must be surface sterilised with 75% reagent ethyl alcohol. The exterior surfaces of all equipment entering the cabinet must also be surface sterilised with 75% reagent ethyl alcohol. The workers hands must be surface sterilised by either direct contact with 75% ethyl alcohol or by utilising gloves. The utensils (forceps, scalpels) should be dipped into 95% reagent ethyl alcohol and flamed dry between each plant manipulation.

Plating randomly selected medium onto TSA plates (or similar) allows for contamination to be monitored. Close the tubes and TSA plates with laboratory sealant film before removing them from the Laminar Airflow Cabinet. The TSA plates should be monitored and within several days of inoculation, bacterial and fungal contamination will be visible.

## **5. LABORATORY VALIDATION OF THE *MYRIOPHYLLUM SIBIRICUM* TOXICITY TEST**

### **5.1 INTRODUCTION**

Once the toxicity test parameters, such as type of media, growing conditions, and endpoint measurements, had been developed, the next step was to determine the sensitivity of this toxicity test to numerous toxicants. Eight herbicides (see Section 5.7) and two reference toxicants (see Section 5.8) were selected for evaluation.

In order to determine the effect of pesticides on this aquatic plant species, the first step was to determine the toxicity of the pesticide at the expected environmental concentration (EEC). The EEC was calculated as the concentration resulting from a pesticide application at the recommended maximum label rate (MLR) for each pesticide into a 15 cm deep column of water (Boutin *et al.*, 1993). When the EEC was determined to be phytotoxic to the *Myriophyllum* plants, the magnitude of the toxic response was quantified by assessing the toxicity of the pesticide over a range of concentrations, hereafter called a dilution series. NOEC, IC50 and SC20 levels were then determined from the results of the dilution series.

### **5.2 CHEMICAL SELECTION**

The pesticides evaluated were chosen from different pesticide families that exhibit a range of modes of phytotoxic action (Table 27). This was done to determine whether this toxicity testing system was particularly sensitive or insensitive to some mechanisms of phytotoxicity and to facilitate comparison of this toxicity test with other non-target aquatic plant toxicity tests.

### **5.3 COMPARISON OF TECHNICAL AND FORMULATED PESTICIDES**

#### **5.3.1 Introduction**

The proposed Environment Canada guidelines for pesticide registration recommend using technical grade herbicides during Tiers I and II and formulated product testing during the Tier III stage (Boutin *et al.*, 1993). The effect of the active ingredient upon the non-target organism can be determined using the technical pesticide while any additional effect of the surfactant or adjuvant or any interaction between active and inactive ingredients can be determined in tests with the formulated product. With fish, amphibians and aquatic invertebrates, it has been shown that some pesticide

formulations can vary in toxicity from the technical compound (Buhl and Faerber, 1989; Folmar *et al.*, 1979; Perkins, 1997). Aquatic plant research has been conducted using either technical or formulated pesticides or both. For example, Richardson *et al.* (1979), Rubin *et al.* (1984) and St. Laurent *et al.* (1992) used technical herbicides while Day (1993), Goldsborough and Brown (1988), Hartman and Martin (1984; 1985), Hernando *et al.* (1989), Lockhart *et al.* (1989) and Sullivan *et al.* (1981) used the formulated products. Cooley and Foy (1986) and Walsh (1972) compared technical and formulated herbicides. Peterson *et al.* (1994) tested mainly technical compounds except for a few cases where the formulated product was substituted and one instance where both the formulated and technical products were compared. In laboratory and pond experiments, deNoyelles and Kettle (1985) compared the effects of reagent grade atrazine with the commercial product on phytoplankton photosynthesis and determined that both compounds had the same effect. In the current research, technical grade herbicides were used in most cases. A small comparison was initiated to compare the MLR of technical glyphosate with the isopropylamine salt formulations Rodeo<sup>®</sup> and Round-up<sup>®</sup>, manufactured by Monsanto Company. Round-up<sup>®</sup> contains 356 g ae/L and a surfactant (polyoxyethyleneamine) while Rodeo<sup>®</sup> contains 480 g ae/L and no surfactant. Technical triclopyr acid was compared with the formulated products Garlon<sup>®</sup> 3A (0.36 kg triclopyr/L as triethylamine (TEA) salt) and Garlon<sup>®</sup> 4 (0.48 kg triclopyr/L as the butoxyethyl ester), which are products of Dow Elanco Inc.

### 5.3.2 Methods

The technical glyphosate and triclopyr and the formulated products were obtained from the manufacturers. Pesticide stock solutions were prepared on the day of the experimental transfer. For the glyphosate treatments, 1 mL of sterile nanopure water or 1 mL of concentrated pesticide stock solution was added to 250 mL of autoclaved medium to obtain the control or the EEC of 2.99 mg/L (Table 27). Because of lower water solubility of the technical triclopyr, the triclopyr treatments were delivered as 5 mL of concentrated pesticide stock solution into 250 mL of sterile medium to produce the EEC of 6.67 mg/L (Table 27). The control medium for the triclopyr experiment contained 5 mL of sterile water added to 250 mL of medium. Three centimetre long apical segments were transferred from half strength M & S medium into full strength modified Andrews' medium. The experiment was established as outlined in

Table 27: Properties and maximum label rates of the pesticides tested in the *Myriophyllum sibiricum* toxicity test.

Pesticide	Mode of Action	Water Solubility (mg/L) <sup>3,5</sup>	Half-life (days) <sup>4</sup>	Maximum Label Rate (kg/ha) <sup>4</sup>	Molecular Weight (g/mol) <sup>5</sup>	Concentration in a 15 cm column of water (mg/L) (EEC)	Concentration in a 15 cm column of water (M) (EEC)
2,4-D	Auxin Mimic <sup>1</sup> DNA, RNA and Protein Disrupter <sup>2</sup>	890 <sup>5</sup> 900 <sup>3</sup>	10	2.20	221.0	1.47	6.64·10 <sup>-6</sup>
Atrazine	Ps II Inhibitor <sup>4</sup>	33 <sup>3,5</sup>	60	4.48	215.7	2.99	1.38·10 <sup>-5</sup>
Diquat (dibromide salt)	Contact Membrane Toxin <sup>1</sup>	718 000 <sup>3,5</sup>	1 000	4.48	344.06	2.99	8.25·10 <sup>-6</sup>
Fluridone	Carotenoid Synthesis Inhibitor (Aquatic) <sup>3</sup>	10 <sup>5</sup> 12 <sup>3</sup>	21	4.50	329.3	3.00	9.11·10 <sup>-6</sup>
Glyphosate	Aromatic Amino Acid Inhibitor <sup>4</sup>	900 000 <sup>5</sup>	47	4.48	169.1	2.99	1.77·10 <sup>-5</sup>
Hexazinone	Ps II Inhibitor (Forestry) <sup>1</sup>	33 000 <sup>5</sup>	90	12.00	252.3	8.00	3.17·10 <sup>-5</sup>
Metolachlor	Growth Inhibitor/ Anti-gibberellic Acid <sup>3,4</sup>	530 <sup>5</sup> 488 <sup>3</sup>	90	4.48	283.8	2.99	1.05·10 <sup>-5</sup>
Triclopyr	Hormone (Auxin Mimic) <sup>3</sup> DNA, RNA and Protein Disrupter <sup>3</sup>	430 <sup>3</sup>	46	10.00	256.5	6.67	2.60·10 <sup>-5</sup>

1 Information obtained from Ashton and Crafts (1981)

2 Information obtained from Chen *et al.* (1972; 1973)

3 Information obtained from WSSA (1994)

4 Information obtained from WSSA (1989)

5 Information obtained from Wauchope *et al.* (1992)

Section 4.6. After 14 days of incubation, the plants were examined for numerous endpoint parameters. The data were transformed when necessary and compared using a one-way ANOVA. The Tukey-Kramer multiple comparison test was conducted when significant differences between treatments were detected. For comparison with literature values and with other sections in this document, percent inhibition was calculated on the same data.

### 5.3.3 Results and Discussion

Representative *Myriophyllum sibiricum* plants treated with the maximum label rate of technical and formulated glyphosate and triclopyr are pictorially displayed in Figure 36 and Figure 37, respectively. The growth curves for the MLR of technical and formulated glyphosate and triclopyr are displayed in Figure 38 and Figure 39, respectively. For both pesticides, there was a statistically significant difference between the control plants and the treated plants for most of the endpoint parameters examined. The means and s.d. for the endpoints are tabulated in Table 28 and Table 29 for glyphosate and triclopyr, respectively. Percent inhibition values for glyphosate are shown in Table 30, and for triclopyr the percent inhibition values are found in Table 31.

For glyphosate, the control plants were statistically different from all treatments for all endpoint parameters except for oxygen content and membrane permeability (Table 28). For the other parameters, there was no difference between the effects of the technical and formulated products. Percent inhibition was greater than 40% for the endpoints other than oxygen content, pigment content and membrane permeability (Table 30). Round-up® has been reported to inhibit the growth of *Chlorella pyrenoidosa* cells at concentrations ranging from 0.1 to 0.75 mM (Hernando *et al.*, 1989). Increases in *Selenastrum capricornutum* biomass were inhibited by 50% with 3.83 µg/L (95% c.i. = 3.82 - 3.84 µg/L) of Round-up® as determined in a 21 day flask test (Turbak *et al.*, 1986). Round-up® and technical glyphosate did not significantly differ in their affect upon the dry weight of *Lemna gibba* (Cooley and Foy, 1986). The toxicity of Rodeo® (1 L/ha) to *Lemna gibba* growth was examined in a field microcosm study conducted at two wetland sites by Gardner and Grue (1996). When the frond number was examined 24 hours postspray, there was no significant difference between the

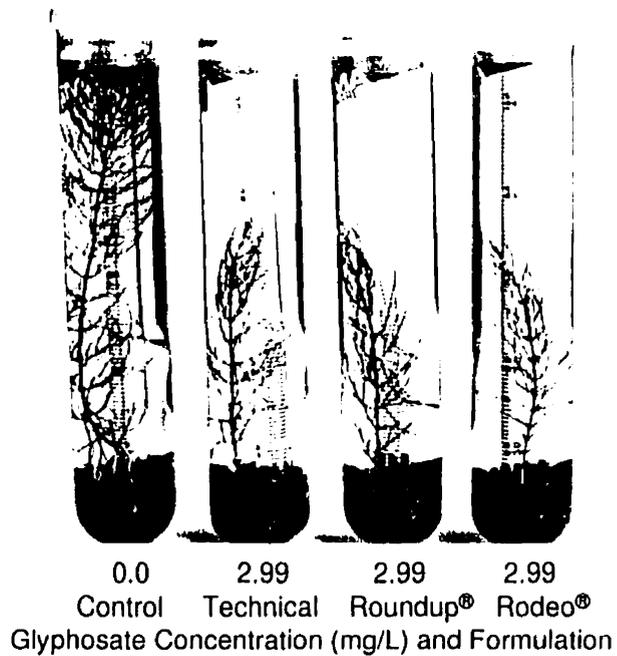


Figure 36: The effect of different formulations of glyphosate at 2.99 mg a.i./L on *Myriophyllum sibiricum* growth over 14 days in axenic culture.

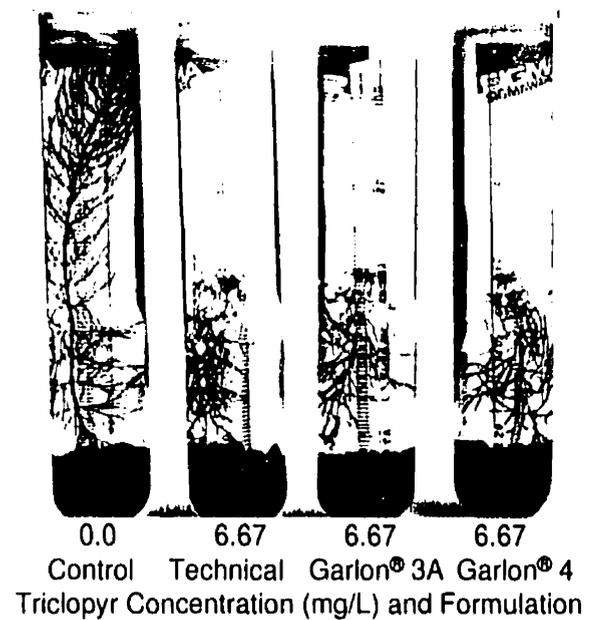


Figure 38: The effect of triclopyr formulations (6.67 mg a.i./L) on the growth of *Myriophyllum sibiricum*. After 14 days of axenic culture, the treated plants were twisted and the leaves were epinastic.

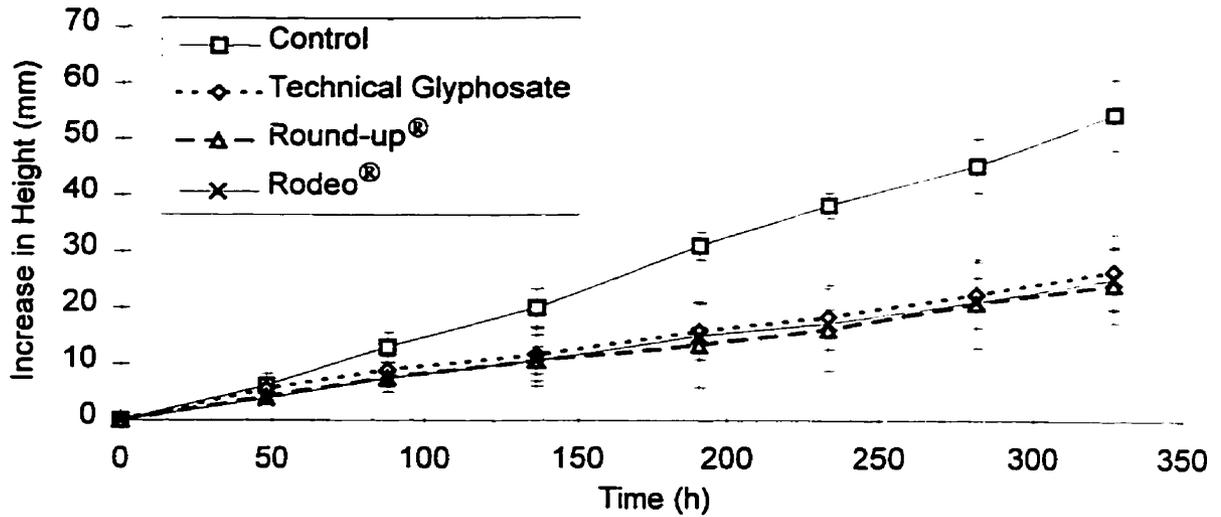


Figure 38: Effects of technical and formulated glyphosate (2.99 mg/L) on the growth of *Myriophyllum sibiricum* apices over 14 days as tested in an axenic test tube culture with modified Andrews' medium. After 14 days, the three chemical treatments were significantly different from the control but were not significantly different from each other ( $\alpha = 0.05$ ).

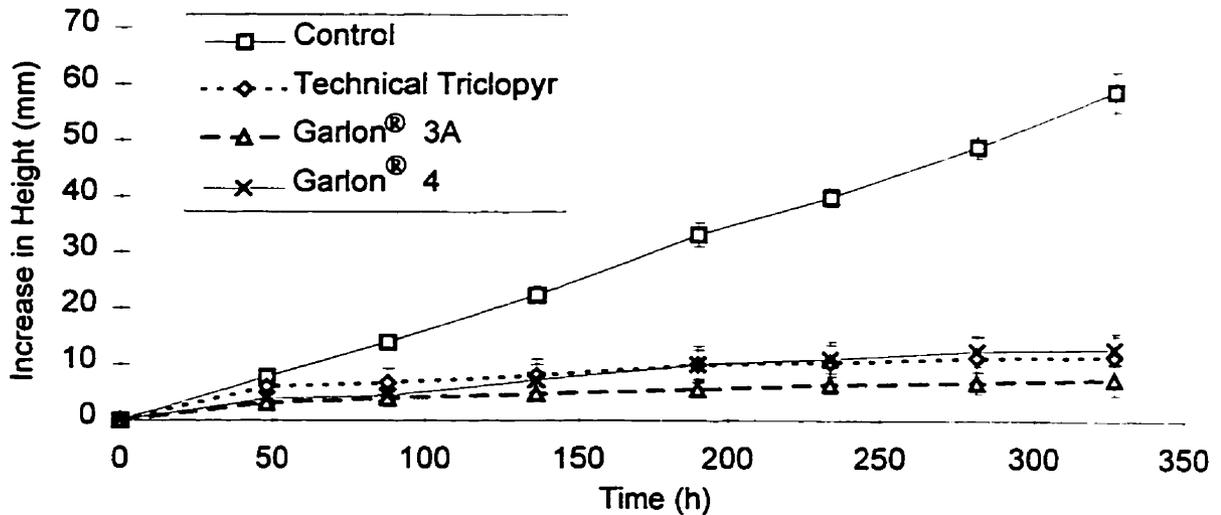


Figure 39: Growth of *Myriophyllum sibiricum* apices over 14 days when exposed to technical and two formulations of triclopyr (6.67 mg/L). Plants were cultured axenically in modified Andrews' medium. After 14 days of incubation, all the treatments were statistically different from the controls but not from each other ( $\alpha = 0.05$ ).

Table 28: Effects of technical and two formulations of glyphosate, applied at the EEC of 2.99 mg/L, on the growth and development of axenic *Myriophyllum sibiricum* apices after 14 days of incubation in modified Andrews' medium.

Treatment	Area under the Growth Curve	Increase in Plant Length (mm)	Root #	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
Control	8 485.1 ± 721.9 <sup>a</sup>	58.5 ± 7.1 <sup>a</sup>	5.8 ± 0.8 <sup>a</sup>	298.3 ± 31.0 <sup>a</sup>	74.0 ± 3.9 <sup>a</sup>	452.4 ± 58.8 <sup>a</sup>	0.51 ± 0.02 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>	8.4 ± 1.3 <sup>a</sup>	4.3 ± 0.9 <sup>a</sup>
Technical Glyphosate	4 497.8 ± 1 552.1 <sup>b</sup>	34.6 ± 3.7 <sup>b</sup>	0.8 ± 0.8 <sup>b</sup>	1.8 ± 3.0 <sup>b</sup>	81.0 ± 5.4 <sup>a</sup>	117.9 ± 18.9 <sup>b</sup>	0.68 ± 0.06 <sup>b</sup>	0.26 ± 0.03 <sup>b</sup>	0.21 ± 0.01 <sup>b</sup>	11.2 ± 2.2 <sup>a,b</sup>	1.2 ± 0.8 <sup>b</sup>
Round-up <sup>®</sup>	3 977.1 ± 760.6 <sup>b</sup>	30.2 ± 2.1 <sup>b</sup>	0.6 ± 0.9 <sup>b</sup>	0.7 ± 1.1 <sup>b</sup>	84.2 ± 9.0 <sup>a</sup>	129.2 ± 22.0 <sup>b</sup>	0.69 ± 0.08 <sup>b</sup>	0.27 ± 0.03 <sup>b</sup>	0.22 ± 0.01 <sup>b</sup>	11.7 ± 1.6 <sup>a,b</sup>	1.3 ± 0.6 <sup>b</sup>
Rodeo <sup>®</sup>	4 105.9 ± 636.6 <sup>b</sup>	30.0 ± 2.8 <sup>b</sup>	0.8 ± 1.3 <sup>b</sup>	2.5 ± 4.9 <sup>b</sup>	80.2 ± 6.9 <sup>a</sup>	112.0 ± 41.0 <sup>b</sup>	0.71 ± 0.05 <sup>b</sup>	0.28 ± 0.03 <sup>b</sup>	0.22 ± 0.02 <sup>b</sup>	13.7 ± 3.2 <sup>b</sup>	0.8 ± 0.6 <sup>b</sup>

a, b Any two means in the same column with the same superscript are not significantly different at  $\alpha = 0.05$ . Data presented are the untransformed mean  $\pm$  s.d. Five replicates per treatment, except for the membrane integrity of the control plants where  $n = 4$ .

**Table 29: Effects of technical triclopyr and two formulations of triclopyr at the EEC of 6.67 mg/L upon the growth and development of *Myriophyllum sibiricum* apices after 14 days of exposure in axenic test tube culture.**

Treatment	Area under the Growth Curve	Plant Length (mm)	Root #	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
Control	9 157.5 ± 336.6 <sup>a</sup>	63.5 ± 1.6 <sup>a</sup>	5.4 ± 1.7 <sup>a</sup>	269.9 ± 72.9 <sup>a</sup>	53.4 ± 5.0 <sup>a</sup>	473.4 ± 15.5 <sup>a</sup>	0.54 ± 0.04 <sup>a</sup>	0.20 ± 0.01 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>	6.8 ± 0.5 <sup>a</sup>	4.5 ± 1.9 <sup>a</sup>
Technical Triclopyr	2 716.6 ± 946.8 <sup>b</sup>	14.5 ± 4.3 <sup>b,c</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	28.2 ± 13.3 <sup>b</sup>	120.3 ± 55.2 <sup>b</sup>	0.10 ± 0.05 <sup>b,c</sup>	0.05 ± 0.03 <sup>b,c</sup>	0.05 ± 0.02 <sup>b,c</sup>	19.0 ± 8.5 <sup>b</sup>	1.4 ± 1.1 <sup>b</sup>
Garlon® 3A	1 613.3 ± 370.4 <sup>c</sup>	12.5 ± 1.1 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	38.2 ± 7.2 <sup>a,b</sup>	90.4 ± 15.8 <sup>b</sup>	0.15 ± 0.04 <sup>b</sup>	0.07 ± 0.02 <sup>b</sup>	0.08 ± 0.02 <sup>b</sup>	22.2 ± 4.6 <sup>b</sup>	0.9 ± 0.5 <sup>b</sup>
Garlon® 4	2 588.8 ± 659.6 <sup>b,c</sup>	16.9 ± 1.4 <sup>c</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	29.0 ± 5.5 <sup>b</sup>	129.6 ± 31.0 <sup>b</sup>	0.09 ± 0.02 <sup>c</sup>	0.03 ± 0.01 <sup>c</sup>	0.04 ± 0.01 <sup>c</sup>	21.2 ± 4.0 <sup>b</sup>	1.0 ± 0.7 <sup>b</sup>

a, b, c Any two means in the same column are not statically significantly different at  $\alpha = 0.05$  as determined using a one-way ANOVA. Data presented are the untransformed mean  $\pm$  s. d. Each treatment contained 5 replicates.

Table 30: Percent inhibition of the endpoint parameters for *Myriophyllum sibiricum* plants treated with the EEC (2.99 mg/L) of technical glyphosate and two formulations of glyphosate. A negative value indicates stimulation over the control values.

Treatment	Area under Growth Curve	Increase in Plant Length	Root #	Total Root Length	D.O. (%)	Total Fresh Weight	Chlorophyll a Content	Chlorophyll b Content	Carotenoid Content	Membrane Permeability	Plant Area (cm <sup>2</sup> )
Technical Glyphosate	47.0 ± 18.3	40.9 ± 6.3	86.2 ± 14.4	99.4 ± 1.0	-9.5 ± 7.3	73.9 ± 4.2	-33.2 ± 11.8	-46.0 ± 15.2	-24.7 ± 8.5	5.7 ± 4.4	72.5 ± 17.7
Round-up®	53.1 ± 9.0	48.4 ± 3.6	89.7 ± 15.4	99.8 ± 0.4	-13.8 ± 12.2	71.4 ± 4.9	-34.4 ± 15.2	-46.8 ± 18.5	-27.4 ± 7.6	6.7 ± 3.3	69.3 ± 12.9
Rodeo®	51.6 ± 7.5	48.7 ± 4.7	86.2 ± 22.5	99.2 ± 1.6	-8.4 ± 9.3	75.2 ± 9.1	-38.4 ± 8.9	-54.6 ± 15.6	-30.2 ± 9.5	10.7 ± 6.5	82.1 ± 13.6

Table 31: Percent inhibition for *Myriophyllum sibiricum* plants treated with the EEC of technical triclopyr and two of its formulations.

Treatment	Area under Growth Curve	Plant Length	Root Number	Total Root Length	D.O.	Total Fresh Weight	Chlorophyll a Content	Chlorophyll b Content	Carotenoid Content	Membrane Permeability	Plant Area (cm <sup>2</sup> )
Technical Triclopyr	70.3 ± 10.3	77.1 ± 6.7	100.0 ± 0.0	100.0 ± 0.0	47.2 ± 25.0	74.6 ± 11.7	80.7 ± 9.3	73.2 ± 15.2	70.6 ± 13.6	19.9 ± 13.0	68.6 ± 24.7
Garlon® 3A	82.4 ± 4.0	80.4 ± 1.8	100.0 ± 0.0	100.0 ± 0.0	28.5 ± 13.5	80.9 ± 3.3	71.4 ± 6.8	65.5 ± 9.9	57.0 ± 9.5	29.9 ± 9.0	79.5 ± 11.0
Garlon® 4	71.7 ± 9.6	73.4 ± 2.9	100.0 ± 0.0	100.0 ± 0.0	45.7 ± 15.4	129.6 ± 31.0 <sup>p</sup>	83.9 ± 8.5	82.8 ± 12.1	75.8 ± 12.9	27.9 ± 9.1	78.5 ± 10.8

treatments and the controls but 48 hours postspray there was a statistically significant decrease in number of living fronds when the treatments were compared to the controls at both wetland study sites. It was hypothesised that the toxicity of Rodeo® could be from the direct contact with the herbicide spray rather than from water dissolved herbicide (Gardner and Grue, 1996). The technical glyphosate used by Richardson *et al.* (1979) inhibited *Euglena gracilis* growth at concentrations equal to or greater than  $3 \cdot 10^{-3}$  M (507.3 mg/L). There was no effect on *E. gracilis* cell number at glyphosate concentrations of  $3 \cdot 10^{-4}$  M (50.73 mg/L) or less. The results reported by Richardson *et al.* (1979) differ from the results reported in the current study.

The EEC of glyphosate (2.99 mg/L) did not significantly inhibit oxygen production in *Myriophyllum sibiricum* (Table 28 and Table 30). This differs from results reported in the literature. Richardson *et al.* (1979) found that technical glyphosate at concentrations of  $6 \cdot 10^{-6}$  to  $1.2 \cdot 10^{-4}$  M (1.01 to 20.29 mg/L) inhibited oxygen release from *Euglena gracilis* cells. Hernando *et al.* (1989) determined that Round-up® at concentrations of 0.1 to 0.75 mM (16.91 to 126.8 mg/L) inhibited oxygen evolution in *Chlorella pyrenoidosa* cultures. The concentration range used by Richardson *et al.* (1979) overlaps the EEC of 2.99 mg/L used in the current research while the concentrations used by Hernando *et al.* (1989) were greater. Oxygen evolution in *Selenastrum capricornutum* was inhibited by 50% after 24 hours of exposure to 10.0 µg/L (c.i. = 9.09 - 11.0 µg/L) of Round-up® (Turbak *et al.*, 1986).

Membrane permeability of the control plants did not differ significantly ( $\alpha = 0.05$ ) from the membrane integrity of plants treated with technical glyphosate or Round-up®. Rodeo® damaged the plant membranes to a greater extent than the control treatment but the damage inflicted by Rodeo® did not differ significantly from the other glyphosate treatments (Table 28).

The other interesting observation was that pigment concentration (mg/g fresh weight of the apex) in glyphosate treated plants was significantly stimulated when compared to the control (Table 28 and Table 30). For chlorophyll *b*, stimulation of pigment content in the apices was approximately 50% (Table 30). This is contradictory to the research conducted by Hernando *et al.* (1989), Kitchen *et al.* (1981a) and Richardson *et al.* (1979). Hernando *et al.* (1989) determined that Round-up® (0.1 to 0.75mM) inhibited chlorophyll synthesis and decreased the carotenoid content in

*Chlorella pyrenoidosa*. Kitchen *et al.* (1981a) found that Rodeo® at concentrations ranging from 1.0 to 0.0001 mM, decreased chlorophyll content in barley (*Hordeum vulgare* L.) seedlings. The effect of Rodeo® upon chlorophyll content of corn (*Zea mays* L.) seedlings was tested at concentrations of 10 to 0.01 mM (1691 to 1.69 mg/L). All concentrations but 0.01 mM reduced pigment content (Kitchen *et al.*, 1981a). In the study by Richardson *et al.* (1979), chlorophyll content of *E. gracilis* was reduced by technical glyphosate concentrations greater than  $1.2 \cdot 10^{-3}$  M. Concentrations less than  $3 \cdot 10^{-3}$  M had no effect on pigment content (Richardson *et al.*, 1979). The range used by Hernando *et al.* (1989) and Richardson *et al.* (1979) were more concentrated while the range used by Kitchen *et al.* (1981a) overlapped the EEC used in the current study. The stimulation of pigment content seen in the current study might be an artefact of calculating the pigment concentration based on fresh weight rather than dry weight. In future experiments, it was decided to dry the apices after measuring the pigment content so that pigment content could be calculated on both a fresh and dry weight basis. The other possible explanation for the discrepancy between the current results and published values could be that the current pigment concentrations were presented for the subsample of plant tissue (i.e., 50 mg of apex). To explore this possibility, an approximation of total pigment content for the total plant weight was calculated (Table 32). In this format, the pigment contents were inhibited between 57.6 and 68.5% when compared to the control (Table 33). They correlate with the pigment inhibition reported in the literature (see above). This method of estimating pigment content for the whole plant is not completely valid in this study because the total fresh weight of the plants included the weight of the roots, which do not contain photosynthetic pigments.

The control plants were statistically different from all the triclopyr treatments (Table 29). Visually, all the triclopyr treatments showed signs of epinasty. The endpoints measured for technical and formulated triclopyr showed more variation than the endpoints measured for glyphosate formulations. There was no difference between the technical and formulated products for root number, root length, membrane permeability or plant area. For area under the growth curve, the technical triclopyr acid differed from Garlon® 3A but Garlon® 4 was not statistically different from either the technical triclopyr or Garlon® 4. For the other endpoints, Garlon® 4 was more toxic than Garlon® 3A with neither formulation being significantly different from the technical

triclopyr acid (Table 29). Percent inhibition was extreme for root number and root length since these parameters were inhibited by 100%. Membrane integrity was inhibited by 46.3% or greater and dissolved oxygen was inhibited by 28.5% or greater when compared to the control. The other endpoints showed inhibitions greater than 57% (Table 31).

Table 32: Pigment content of *Myriophyllum sibiricum* plants treated with technical or formulated glyphosate after fourteen days of incubation in sterile modified Andrews' medium.

Treatment	Chlorophyll a Content (mg/g total fresh weight)	Chlorophyll b Content (mg/g total fresh weight)	Carotenoid Content (mg/g total fresh weight)
Control	0.23 ± 0.03 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>
Technical Glyphosate	0.08 ± 0.01 <sup>b</sup>	0.03 ± 0.004 <sup>b</sup>	0.03 ± 0.003
Round-up <sup>®</sup>	0.09 ± 0.02 <sup>b</sup>	0.03 ± 0.009 <sup>b</sup>	0.03 ± 0.006 <sup>b</sup>
Rodeo <sup>®</sup>	0.08 ± 0.02 <sup>b</sup>	0.03 ± 0.007 <sup>b</sup>	0.02 ± 0.007 <sup>b</sup>

a, b Data are the untransformed means of 5 replicates, any two means in the same column with the same superscript are not significantly different at  $\alpha = 0.05$ .

Table 33: Percent inhibition data for pigment content based on total plant fresh weight for axenic *Myriophyllum sibiricum* plants treated with technical glyphosate, Round-up<sup>®</sup> or Rodeo<sup>®</sup> after fourteen days of incubation in modified Andrews' medium.

Treatment	Chlorophyll a Content	Chlorophyll b Content	Carotenoid Content
Technical Glyphosate	65.4 ± 5.0	62.3 ± 4.7	67.7 ± 3.9
Round-up <sup>®</sup>	61.2 ± 9.9	57.6 ± 11.3	63.5 ± 7.6
Rodeo <sup>®</sup>	66.4 ± 9.5	62.9 ± 8.7	68.5 ± 8.5

Information comparing the effects of both technical and formulated triclopyr on aquatic macrophytes is limited. In a field microcosm study conducted by Gardner and Grue (1996), the toxicity of Garlon<sup>®</sup> 3A (5 L/ha) to *Lemna gibba* was examined at 24 and 48 hours postspray. In both wetland sites studied, the average number of duckweed fronds did not differ significantly from the controls at either time period. It was hypothesised that a longer observation period might have shown a more toxic effect of the Garlon<sup>®</sup> 3A because the herbicide is systemic (Gardner and Grue, 1996). In a study by Sprecher and Stewart (1995), *Myriophyllum spicatum* treated with Garlon<sup>®</sup> 3A

showed curvature and epidermal rupture within 3 days after treatment with 1.0 and 2.5 mg a.i./L. By day 14, no viable stems or leaves remained. Five weeks after application, there was no *M. spicatum* biomass remaining in either treatment. *Elodea canadensis* and *Vallisneria americana* were not affected by either triclopyr treatment but *Potamogeton pectinatus* showed a reduction in biomass at the 2.5 mg/L treatment level (Sprecher and Stewart, 1995). In an aquarium study, *M. spicatum* demonstrated apical leaf curvature within 12 hours of treatment and shoot bending and twisting and epidermal rupture 36 hours after treatment with Garlon® 3A at concentrations ranging between 0.25 to 2.5 mg ae/L (Netherland and Getsinger, 1992). Triclopyr amine, in a single field application of 12 kg/ha, destroyed root systems and mature *Lythrum salicaria* L. (purple loosestrife) plants. Grasses were not affected but sedges (*Carex* spp.) declined in density after treatment with this herbicide (Gabor *et al.*, 1995). The toxicity of triclopyr triethylamine salt to *Lemna gibba* over a 14 day period was examined by Cowgill and Milazzo (1987). The triclopyr TEA salt had a pronounced effect above 36 mg/L. The EC50 for plant number for 100% triclopyr TEA salt was determined to be 9 mg/L with a range of 2.1 to 38.4 mg/L, while for the formulated product, the EC50 was 19.9 mg/L with a 95% c.i. of 4.6 to 85.2 mg/L. The EC50 for frond number was determined to be 9.1 mg/L (95% c.i. = 2.9 - 28.4 mg/L) for the active ingredient and 20.1 (95% c.i. = 6.4 - 63.0 mg/L) for the formulation. The formulation was slightly less toxic to *L. gibba* than the technical TEA salt (Cowgill and Milazzo, 1987). This study was expanded by Cowgill *et al.* (1988) to examine the effects of Garlon® 3A on two duckweed species (*L. gibba* and *L. minor*). After 7 days of exposure, it was determined that this compound was only slightly toxic to these species with EC50s between 35 and 93 mg/L depending upon the endpoint examined (Cowgill *et al.*, 1988). These two studies on duckweed (Cowgill and Milazzo, 1987; Cowgill *et al.*, 1988) suggest that duckweed was not as sensitive to triclopyr as was the *M. sibiricum* used in the current study. Prasad (1984) concluded that technical glyphosate was more toxic than the commercial formulations to *Lemna minor*. However, the formulations tested were not identified (Prasad, 1984).

Using *Myriophyllum sibiricum*, EEC experiments with technical glyphosate and triclopyr were repeated and dilution series were conducted to determine the phytotoxicity of these herbicides. The glyphosate results are in Section 5.7.5 and the triclopyr results are in Section 5.7.8. The comparison of technical and formulated

glyphosate and triclopyr and their effect upon non-target organisms was expanded by Perkins (1997). In that research, dilution series of technical and formulated glyphosate and triclopyr were compared for their toxicity to *Lemna gibba* and *Myriophyllum sibiricum*. It was determined that there was no effect from surfactants on these two species of aquatic plants. *M. sibiricum* was more sensitive to these compounds than was *L. gibba* (Perkins, 1997).

The results generated in the above comparison confirm that both technical grade and formulated pesticides can be used in the *Myriophyllum* toxicity test. The literature surveyed showed that different species react differently to formulated and technical pesticides and thus examination of both can be important. The choice of technical or formulated pesticide will depend upon the problem being examined. If the concern is about the immediate effect of a direct overspray of a pesticide on water, it would be best to test the formulated product. On the other hand, if the concern is for longer term effects or for the effects of pesticide runoff into water, results from technical grade pesticides would be just as relevant. Except for the above study with glyphosate and triclopyr where formulated and technical grade products were compared, all of the pesticides in this project were examined as the technical grade chemicals.

#### 5.4 PESTICIDE AND CHELATOR INTERACTION

##### 5.4.1 Introduction

There is evidence that a few pesticides may interact with natural and synthetic chelators in liquid nutrient medium and natural waters. Glyphosate (Carlisle and Trevors, 1988; Duke, 1988; Glass, 1984; Hensley *et al.*, 1978; Moshier and Penner, 1978; Subramaniam and Hoggard, 1988; Trotter *et al.*, 1990), amitrole (Sund, 1956) and fluridone (Spencer and Ksander, 1989) have been shown to complex with metal ions and become inactivated. The high concentrations of iron, calcium and magnesium found in groundwater and soils can also combine with glyphosate to form insoluble complexes (Subramaniam and Hoggard, 1988). Likewise, EDTA has been shown to alter the phytotoxicity of glyphosate by adsorbing the metal ions in solution (Shea and Tupy, 1984).

#### 5.4.2 Methods

Glyphosate was selected to determine if there was an effect or interaction between chelator/metal concentration and pesticide in this toxicity test. Either no pesticide or glyphosate at the EEC concentration of 2.99 mg/L were used as the pesticide treatment. For the EDTA treatments, the appropriate amount of FeEDTA (Stock Solution # 11 (Table 26)) was added to the medium to obtain a final FeEDTA concentration of 0.0, 0.865, 1.73, 2.595 and 3.46 mg/L. Each amount of FeEDTA was added to two 500 mL flasks containing 250 mL of modified Andrews' medium prior to autoclaving. After autoclaving, glyphosate or sterile water was added to the flasks. After complete mixing, 40 mL of medium from each treatment was pipetted into five test tubes containing 3 g of sterile Turface®. Ten day old apical segments that had been cultured in M & S medium were transferred into the test tubes. The plants were incubated for fourteen days according to the conditions outlined in Section 4.6. On the fourteenth day, the plants were harvested and shoot height, root number and length, total fresh weight, pigment content of the apical segment, membrane permeability and plant area were measured. The data were tested for normality. Two-way ANOVAs were conducted using the computer package SYSTAT® (SYSTAT®, 1990). If no interaction was found, the treatments could be pooled and examined independently (Sokal and Rohlf, 1981).

#### 5.4.3 Results and Discussion

For all endpoint parameters, plants treated with glyphosate were adversely affected compared with plants in the growth medium without glyphosate (Figure 40, Figure 41, Table 34, Table 35, Table 36, Table 37, Table 38 and Table 39). The two figures clearly divide the treatments into two populations based on glyphosate content. For most endpoints (plant length, root number, root length, fresh weight, membrane integrity, plant area, chlorophyll *a* and carotenoid content), the two-way ANOVA indicated no interaction between FeEDTA and glyphosate. For the same endpoints, there was also no FeEDTA effect. A characteristic sample of two-way ANOVA results is presented in Table 34. From the ANOVA results, it was concluded that the data for all of these parameters could be pooled based on plants treated with and without glyphosate. For all of these endpoint parameters, the glyphosate treatment was significantly different from the treatment without glyphosate at  $\alpha = 0.05$  (Table 35).

presence or absence of glyphosate was detected (Table 38 and Table 39). As in Section 4.3.8, area under the growth curve was affected by FeEDTA. Thus, for most endpoint parameters and concentrations of FeEDTA, there will not be an interaction or effect upon glyphosate phytotoxicity to *M. sibiricum* in axenic culture. Although this research indicated that FeEDTA and glyphosate interactions were minor, it may be of interest to confirm this with other pesticides that may interact with the FeEDTA. FeEDTA was utilised in the remainder of the experiments at the levels recommended by Selim *et al.* (1989). If this bioassay is utilised for other pesticides, it should be remembered that there may be an interaction between certain herbicides and FeEDTA.

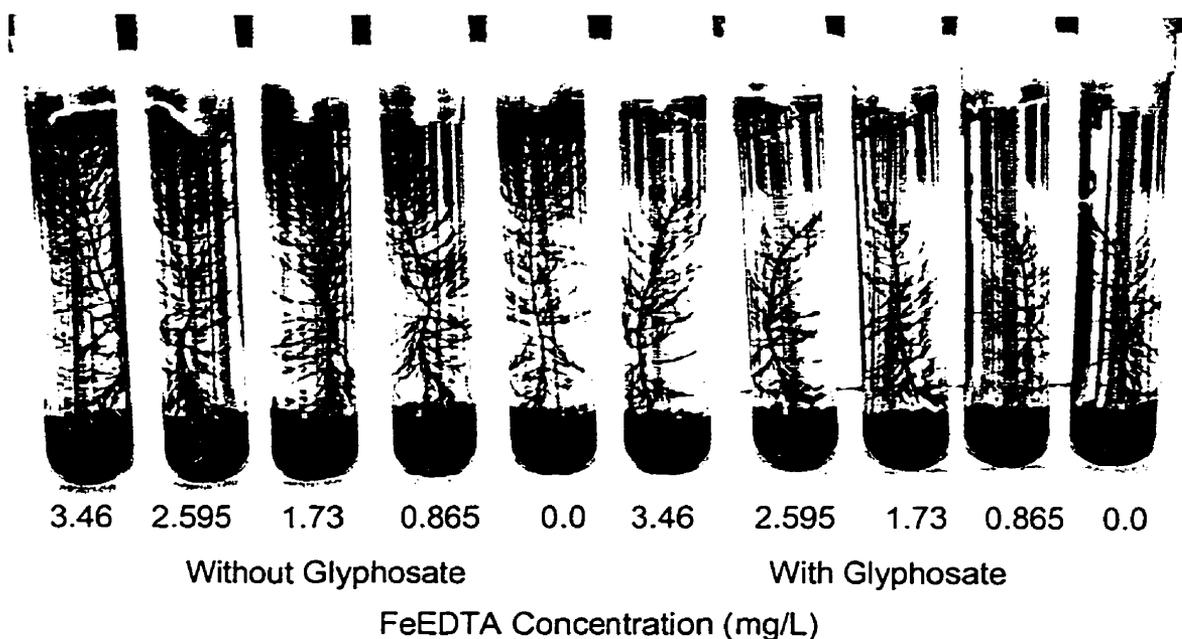


Figure 40: The five plants on the right were cultured without glyphosate added to the modified Andrews' medium. The five plants on the left were cultured in modified Andrews' medium containing 2.99 mg/L of glyphosate. Within each set of five plants, the EDTA concentrations decreased from 3.46 mg/L in the medium in the tube on the left to 0.0 mg/L in the test tube on the right.

More information regarding the toxicity and environmental properties of glyphosate may be found in Sections 5.3 and 5.7.5. Section 4.3.8 gives more information on the effect of metals and chelators on aquatic plant growth. Glyphosate would combine with soil in terrestrial environments and with suspended and deposited sediment in aquatic systems (Bowmer *et al.*, 1986; Sprankle *et al.*, 1975a; 1975b). In the *M. sibiricum* bioassay, the highest FeEDTA concentration reduced glyphosate effects on growth rate (see area under the growth curve (Figure 41, Table 38 and Table

Chlorophyll *b* content of the apices was the only endpoint parameter that demonstrated differences caused by the various FeEDTA concentrations. For this parameter, there was no interaction between the FeEDTA and glyphosate concentrations (Table 36). At an FeEDTA concentration of 2.595 mg/L, the chlorophyll *b* content was greater than in plants treated with 0.0 mg/L (Table 37). For area under the growth curve, an interaction between FeEDTA concentration and the presence or absence of glyphosate was detected (Table 38 and Table 39). As in 4.3.8, area under the growth curve was affected by FeEDTA. Thus, for most endpoint parameters and concentrations of FeEDTA, there will not be an interaction or effect upon glyphosate phytotoxicity to *M. sibiricum* in axenic culture. Although this research indicated that FeEDTA and glyphosate interactions were minor, it may be of interest to confirm this with other pesticides that may interact with FeEDTA. FeEDTA was utilised in the remainder of the experiments at the levels recommended by Selim *et al.* (1989). If this toxicity test is utilised for other pesticides, it should be considered that an interaction may exist between certain herbicides and FeEDTA.

More information regarding the toxicity and environmental properties of glyphosate may be found in Sections 5.3 and 5.7.5. Section 4.3.8 gives more information on the effect of metals and chelators on aquatic plant growth. Glyphosate combines with soil in terrestrial environments and with suspended and deposited sediment in aquatic systems (Bowmer *et al.*, 1986; Sprankle *et al.*, 1975a; 1975b). In the *M. sibiricum* toxicity test, the highest FeEDTA concentration reduced glyphosate effects on growth rate (see area under the growth curve (Figure 41, Table 38 and Table 39)). It can be speculated that higher FeEDTA concentrations interacted with the glyphosate and further reduced phytotoxicity. In natural environments, the phytotoxicity might be reduced even further than found in this toxicity test.

Walsh and Alexander (1980) determined that EDTA in an algal growth medium had no effect upon the toxicity of carbaryl to *Skeletonema costatum*, *Nitzschia angularum*, *Chlorococcum* sp. or *Chlorella* sp. after twelve days of exposure (Walsh and Alexander, 1980). The toxicity of fluridone to *Hydrilla verticillata* was not affected by iron concentration (0 to 5 mg/L as FeEDTA) in the medium during toxicity testing but was reduced by the iron concentration in

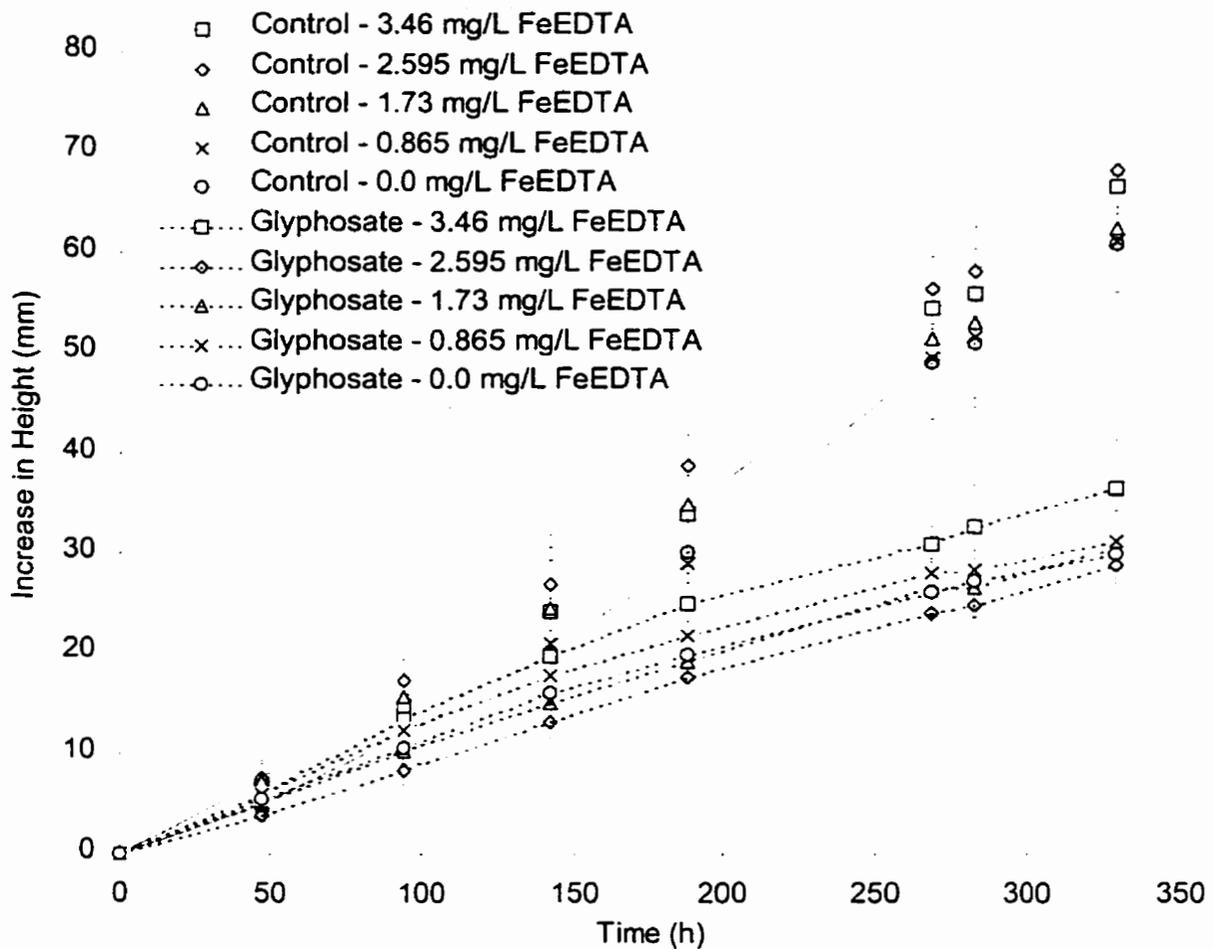


Figure 41: The interaction between iron chelate (FeEDTA) and glyphosate on the growth of *Myriophyllum sibiricum* during two weeks of exposure. Glyphosate was either absent from the medium or tested at a concentration of 2.99 mg/L. FeEDTA was added to the modified Andrews' medium prior to autoclaving at FeEDTA concentrations of 0.0, 0.865, 1.73, 2.595 and 3.46 mg/L.

*Hydrilla* tissue prior to the addition of fluridone (Spencer and Ksander, 1989). EDTA increased glyphosate phytotoxicity to wheat seedlings (Shea and Tupy, 1984). In the current *M. sibiricum* study, effects from iron could not be separated from EDTA effects but there were no major changes in glyphosate toxicity caused by the addition of FeEDTA.

The complexing of metal ions with glyphosate usually inactivates its herbicidal properties (Carlisle and Trevors, 1988; Duke, 1988; Hensley *et al.*, 1978; Moshier and Penner, 1978; Trotter *et al.*, 1990). The activity of glyphosate upon the growth of wheat seedlings was reduced by calcium (Shea and Tupy, 1984). Glyphosate phytotoxicity, as measured in a *Sorghum vulgare* Pers. root bioassay, was decreased in two mineral soils and by several metals (FeCl<sub>2</sub>, FeCl<sub>3</sub> and AlCl<sub>3</sub>). The glyphosate concentrations tested were 0.66, 1.3, 2.7, 5.3, 11.0 and 21.0·10<sup>-4</sup> M (11.2, 22.0, 45.7, 89.6, 186.0 and 355.1 mg/L) and the iron concentrations tested ranged from 6.3 to 101.4 mg/L (Hensley *et al.*, 1978). These concentrations were much higher than those used in the *M. sibiricum* assay. Inactivation of glyphosate might occur at a faster rate with lower initial glyphosate and higher metal concentrations. The one exception found in the literature was that of Bowmer *et al.* (1986) who did not observe a reduced phytotoxicity with complexation of glyphosate. At the levels of FeEDTA utilised in the current study with axenic *M. sibiricum*, there did not appear to be reduced phytotoxicity caused by complexation with the metal or chelator.

Table 34: An example of the ANOVA results from the experiment examining the interaction of glyphosate with FeEDTA. This example was for chlorophyll *a* and demonstrated that the presence or absence of glyphosate was the only effect detected. There was no interaction or effect of FeEDTA on the production of chlorophyll *a* or most of the other endpoint parameters.

Source	Sum of Squares	d.f.	Mean-Square	F-Ratio	p
Glyphosate	0.018	1	0.018	11.133	0.002**
FeEDTA	0.011	4	0.003	1.714	0.168 <sup>n.s.</sup>
Glyphosate*FeEDTA	0.005	4	0.001	0.775	0.549 <sup>n.s.</sup>
Error	0.057	36	0.002		

\*\* Significantly different at  $\alpha = 0.01$ .

n.s. Not significantly different at  $\alpha = 0.05$ .

Table 35: Influence of glyphosate at 2.99 mg/L combined with various FeEDTA concentrations on the growth of *Myriophyllum sibiricum* in axenic culture with modified Andrews' medium. Glyphosate phytotoxicity was not significantly altered by varying the concentrations of FeEDTA (0.0, 0.865, 1.73, 2.595 and 3.46 mg/L). Glyphosate at 2.99 mg/L reduced plant growth as determined by these eight parameters ( $\alpha = 0.05$ ).

Treatment	Shoot Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Chlorophyll <i>a</i> Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant area (cm <sup>2</sup> )
Control	66.7 ± 5.4 <sup>a</sup>	7.8 ± 1.2 <sup>a</sup>	423.8 ± 81.2 <sup>a</sup>	448.9 ± 69.0 <sup>a</sup>	0.53 ± 0.04 <sup>a</sup>	0.178 ± 0.01 <sup>a</sup>	6.7 ± 0.8 <sup>a</sup>	11.3 ± 2.3 <sup>a</sup>
Glyphosate	35.7 ± 4.2 <sup>b</sup>	5.0 ± 1.4 <sup>b</sup>	31.4 ± 13.0 <sup>b</sup>	137.0 ± 20.8 <sup>b</sup>	0.57 ± 0.04 <sup>b</sup>	0.192 ± 0.01 <sup>b</sup>	12.1 ± 1.7 <sup>b</sup>	3.4 ± 0.9 <sup>b</sup>

a,b Any two means (untransformed mean ± s.d.) in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  as determined with either a t-test or Mann-Whitney U-test.  $n = 22$  for the control and  $n = 24$  for the glyphosate treatment.

**Table 36: ANOVA results for chlorophyll *b* from the experiment examining the interaction of glyphosate with FeEDTA. There was no interaction between FeEDTA concentration and the presence of glyphosate. FeEDTA concentration and the presence or absence of glyphosate affected this parameter independently.**

Source	Sum of Squares	d.f.	Mean-Square	F-Ratio	p
Glyphosate	0.013	1	0.013	38.796	0.000**
FeEDTA	0.004	4	0.001	2.692	0.046*
Glyphosate*FeEDTA	0.001	4	0.000	0.581	0.678 <sup>n.s.</sup>
Error	0.012	36	0.000		

\*\* Significantly different at  $\alpha = 0.01$ .

\* Significantly different at  $\alpha = 0.05$ .

n.s. Not significantly different at  $\alpha = 0.05$ .

**Table 37: The effect of FeEDTA and glyphosate on chlorophyll *b* content of *Myriophyllum sibiricum* plants grown in axenic culture.**

FeEDTA (mg/L)	Chlorophyll <i>b</i> (mg/g fresh weight)	
	Glyphosate - 0.00 mg/L	Glyphosate - 2.99 mg/L
3.46	0.18 ± 0.015 <sup>a,b</sup>	0.22 ± 0.010 <sup>c,d</sup>
2.595	0.20 ± 0.031 <sup>a</sup>	0.23 ± 0.013 <sup>c</sup>
1.73	0.19 ± 0.029 <sup>a,b</sup>	0.22 ± 0.017 <sup>c,d</sup>
0.865	0.18 ± 0.011 <sup>a,b</sup>	0.22 ± 0.015 <sup>c,c</sup>
0.0	0.17 ± 0.015 <sup>b</sup>	0.20 ± 0.021 <sup>d</sup>

a,b,c,d In the above table, any two means followed by the same superscript were not significantly different ( $\alpha = 0.05$ ).

**Table 38: ANOVA results for area under the growth curve from the experiment examining the interaction of glyphosate with FeEDTA. There was an interaction between FeEDTA concentration and the presence of glyphosate. FeEDTA concentration did not affect this parameter but area under the growth curve was significantly affected by the presence or absence of glyphosate.**

Source	Sum of Squares	d.f.	Mean-Square	F-Ratio	p
Glyphosate	0.623	1	0.623	205.258	0.000**
FeEDTA	0.025	4	0.006	2.028	0.111 <sup>n.s.</sup>
Glyphosate*FeEDTA	0.048	4	0.012	3.932	0.009**
Error	0.109	36	0.003		

\*\* Significantly different at  $\alpha = 0.01$ .

n.s. Not significantly different at  $\alpha = 0.05$ .

Table 39: The effect of glyphosate and FeEDTA on area under the growth curve for *Myriophyllum sibiricum* plants grown in axenic culture for two weeks.

FeEDTA (mg/L)	Area Under the Growth Curve	
	Glyphosate - 0.00 mg/L	Glyphosate - 2.99 mg/L
3.46	10 091.2 ± 868.0 <sup>a</sup>	6 714.5 ± 892.5 <sup>b,c</sup>
2.595	10 870.2 ± 1091.8 <sup>a</sup>	4 848.0 ± 270.9 <sup>d</sup>
1.73	9 907.3 ± 1715.7 <sup>a</sup>	5 402.3 ± 618.0 <sup>c,d</sup>
0.865	8 919.7 ± 1337.7 <sup>a,b</sup>	5 973.8 ± 967.4 <sup>c,d</sup>
0.0	8 887.7 ± 1063.3 <sup>a,b</sup>	5 501.3 ± 603.8 <sup>c,d</sup>

a, b,c,d In this table, any two means followed by the same superscript were not significantly different at  $\alpha = 0.05$ . An interaction between glyphosate and FeEDTA was detected.

## 5.5 COMPARISON OF SOLVENT CARRIERS

### 5.5.1 Introduction

The reported solubility for the test chemicals in water is not necessarily identical to the solubility in the nutrient medium (Conder *et al.*, 1995). Dissolving the compound in a solvent carrier is occasionally required before addition to a toxicity test medium. The U.S. EPA (1985b) recommends that the concentration of solvent should not exceed 0.1 mL/L, while other authors suggest a maximum level of 0.5 mL of solvent per litre of dilution water (Parrish, 1985; Petrocelli, 1985). After conducting numerous studies on solvent-pesticide interactions with several different microorganisms, Stratton and Smith (1988) concluded that solvent concentrations up to 1.0% should be acceptable. Bowman *et al.* (1981) tested solvents on four invertebrate species and found tolerance to 2% by volume. Various solvents have been used including dimethyl sulfoxide (DMSO) (Greenberg *et al.*, 1992; Huang *et al.*, 1991; Petrocelli, 1985; Selim *et al.*, 1989; Stratton, 1987a; Turbak, 1986; Versteeg, 1990; WSSA, 1989), acetone (Baarschers *et al.*, 1988; Cunningham *et al.*, 1984; Fleming *et al.*, 1991; Hutber *et al.*, 1979; Walsh and Alexander, 1980; Walsh *et al.*, 1982), ethanol (Blanck *et al.*, 1984; Maule and Wright, 1984), methanol (Abou-Waly *et al.*, 1991a; Bowman *et al.*, 1981; Delistraty and Hershner, 1984; Fedtke, 1982; Hutber *et al.*, 1979; Jones and Winchell, 1984; Kemp *et al.*, 1985; O'Brien and Prendeville, 1979; Parrish, 1985; Shehata, 1993; Stratton, 1987a; Versteeg, 1990; WSSA, 1989; Zweig *et al.*, 1968), triethylene glycol (Petrocelli, 1985), dimethyl formamide (Hughes and Vilkas, 1983; Petrocelli, 1985; Stratton, 1987a),

benzene, hexane, ethyl ether, (WSSA, 1989) and acetonitrile (Bowman *et al.*, 1981; WSSA, 1989).

A number of solvent carriers have been shown to impose an additional stress upon the test organism (Bowman *et al.*, 1981). For use in this axenic *Myriophyllum* toxicity test, it was therefore important to determine which solvent should be used to dissolve chemicals with low water solubilities.

### 5.5.2 Methods

When it became apparent that some of the pesticides chosen for use in this toxicity test had low water solubilities, two experiments were conducted to determine the effect of some possible solvents on the growth and development of *Myriophyllum sibiricum* in axenic culture. The first experiment examined the effects of ethanol on *M. sibiricum* plants, while the second experiment determined the effects of acetone, acetonitrile, benzene, dimethyl sulfoxide, ethyl ether, hexane and methanol on *M. sibiricum* plants. All solvents used were pesticide or optima grade and were tested at a concentration of 1 mL per 250 mL of modified Andrews' medium (0.4% v/v). The methods followed those outlined in Section 4.6 except that the stock plants used in this experiment were cultured in Murashige and Skoog medium (Sigma Chemical Company). The controls from the two experiments were compared using the nonparametric Mann-Whitney U-test. The data for all treatments were transformed as required, analysed using a one-way ANOVA. If significant differences were detected, the means were compared using the Tukey-Kramer multiple comparison test.

### 5.5.3 Results and Discussion

The comparison of the two sets of control plants showed that they were not statistically different except for the amount of chlorophyll *a* and *b*. Therefore, the control data from both experiments were combined for the other parameters. The growth curves are in Figure 42 and the data for the other endpoint parameters are summarised in Table 40. A generalised ranking of the eight solvents examined based on their toxicity (most toxic to least toxic) to *Myriophyllum sibiricum* in axenic culture would be: benzene > ethanol > acetonitrile > ethyl ether > acetone > DMSO > hexane = methanol.

The most toxic solvent to the *Myriophyllum* plants was benzene. Benzene severely inhibited plant growth (Figure 42), root formation and pigment production and it also disrupted membrane integrity (Table 40). This was surprising since benzene was

not very toxic to the green alga *Scenedesmus quadricauda*. Based on cell counts, the benzene concentration where inhibitory action began was greater than 1 400 mg/L (Bringmann and Kühn, 1980). After 96 hours of incubation, the growth of *Selenastrum capricornutum* was not severely affected by benzene, as indicated by a NOEC of 600 mg/L (Slooff *et al.*, 1983). However, on the basis of the results reported here, benzene should not be employed as a solvent in the axenic *Myriophyllum* toxicity test.

Ethanol was the second most toxic solvent (Figure 42 and Table 40) to the *Myriophyllum* plants at the 0.4% v/v concentration tested. As reported by other investigators, the effects of ethanol on aquatic plants have been variable. Ethanol was used at final concentrations of 2% (v/v) and 0.5% (v/v) to solubilize metolachlor in two separate experiments conducted by Mellis *et al.* (1982). When using *Lemna minor*, ethanol concentrations should be kept below 0.5% (Chollet, 1992a; 1992b). Ethanol

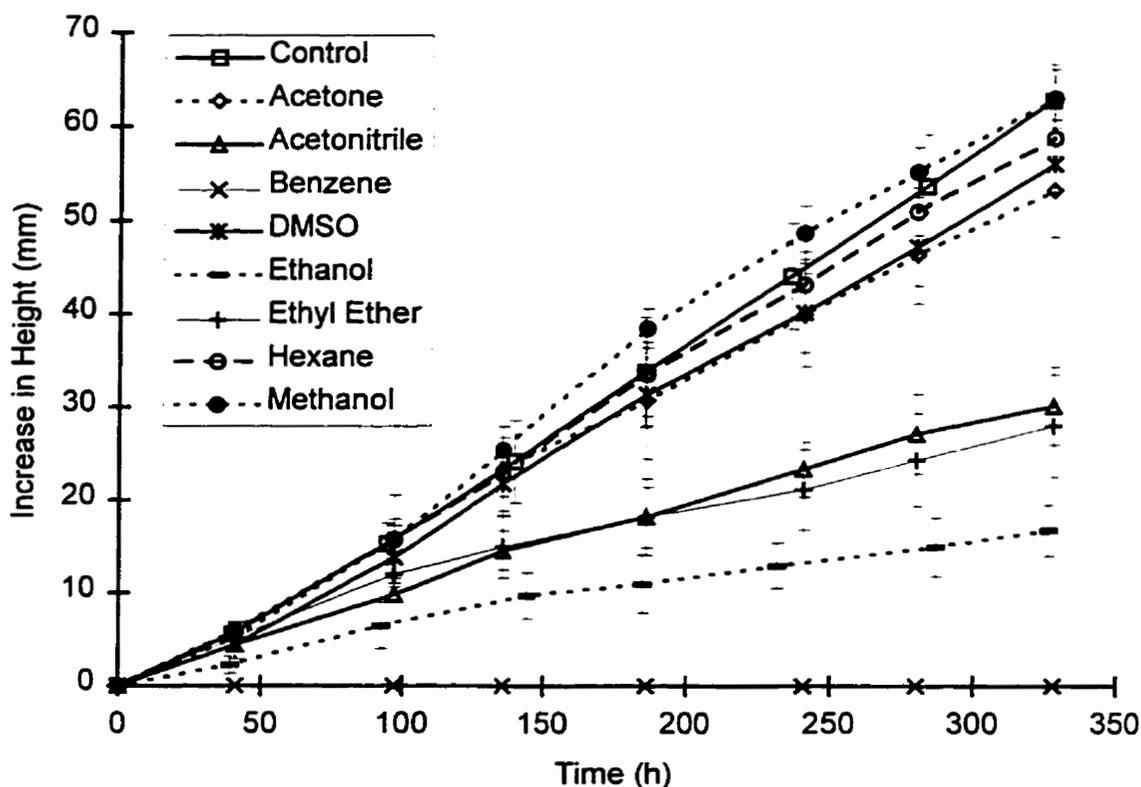


Figure 42: The effect of eight different solvents (0.4% v/v) on *Myriophyllum sibiricum* plant height in modified Andrews' medium. The plants were incubated for 14 days. Area under the growth curve and shoot length of plants treated with acetone, DMSO, hexane and methanol did not differ statistically from the controls.

Table 40: The effect of eight solvents (mean  $\pm$  s.d) upon *Myriophyllum sibiricum* growth and development.

Treatment Concentration Solvent/Medium (% v/v)	Area under the Growth Curve	Plant Length (mm)	Root #	Total Root Length (mm)	Total Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight) <sup>g</sup>	Chlorophyll b Content (mg/g fresh weight) <sup>g</sup>	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
Control (0.0)	9 711.9 $\pm$ 1 173.7 <sup>a</sup>	65.2 $\pm$ 4.4 <sup>a</sup>	6.4 $\pm$ 1.3 <sup>a</sup>	330.3 $\pm$ 59.2 <sup>a</sup>	511.6 $\pm$ 87.3 <sup>a</sup>	0.46 $\pm$ 0.04 <sup>a</sup> ; 0.54 $\pm$ 0.08 <sup>f</sup>	0.16 $\pm$ 0.02 <sup>a</sup> ; 0.20 $\pm$ 0.03 <sup>e</sup>	0.17 $\pm$ 0.02 <sup>a</sup>	9.1 $\pm$ 2.0 <sup>a,d</sup>	6.6 $\pm$ 1.3 <sup>a</sup>
Acetone (0.4)	8 825.9 $\pm$ 1 309.8 <sup>a</sup>	56.1 $\pm$ 4.8 <sup>a</sup>	6.8 $\pm$ 1.3 <sup>a</sup>	12.1 $\pm$ 2.1 <sup>b</sup>	328.3 $\pm$ 48.0 <sup>b</sup>	0.54 $\pm$ 0.04 <sup>a,b,c</sup>	0.20 $\pm$ 0.03 <sup>b</sup>	0.19 $\pm$ 0.02 <sup>a</sup>	11.5 $\pm$ 5.8 <sup>a,b,d</sup>	6.3 $\pm$ 0.9 <sup>a</sup>
Acetonitrile (0.4)	5 286.0 $\pm$ 697.7 <sup>b</sup>	34.8 $\pm$ 4.6 <sup>b</sup>	0.4 $\pm$ 0.5 <sup>b</sup>	0.3 $\pm$ 0.4 <sup>c</sup>	152.7 $\pm$ 32.3 <sup>c,d</sup>	0.78 $\pm$ 0.05 <sup>d</sup>	0.31 $\pm$ 0.02 <sup>c</sup>	0.27 $\pm$ 0.01 <sup>b</sup>	12.3 $\pm$ 2.0 <sup>b,c</sup>	2.5 $\pm$ 0.6 <sup>b</sup>
Benzene (0.4)	0.0 $\pm$ 0.0 <sup>c</sup>	0.4 $\pm$ 0.5 <sup>c</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>c</sup>	79.4 $\pm$ 23.2 <sup>e</sup>	0.007 $\pm$ 0.005 <sup>e</sup>	0.001 $\pm$ 0.001 <sup>d</sup>	0.001 $\pm$ 0.001 <sup>c</sup>	60.5 $\pm$ 9.5 <sup>e</sup>	0.9 $\pm$ 0.5 <sup>c</sup>
DMSO (0.4)	8 763.6 $\pm$ 821.6 <sup>a</sup>	58.8 $\pm$ 3.3 <sup>a</sup>	6.2 $\pm$ 1.1 <sup>a</sup>	331.6 $\pm$ 84.3 <sup>a</sup>	525.1 $\pm$ 83.7 <sup>a</sup>	0.57 $\pm$ 0.06 <sup>c</sup>	0.19 $\pm$ 0.02 <sup>a,b</sup>	0.20 $\pm$ 0.02 <sup>a</sup>	10.8 $\pm$ 3.5 <sup>a,b,d</sup>	7.8 $\pm$ 1.4 <sup>a</sup>
Ethanol (0.4)	3 074.3 $\pm$ 700.7 <sup>d</sup>	19.5 $\pm$ 3.6 <sup>d</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>c</sup>	145.3 $\pm$ 52.7 <sup>d</sup>	0.28 $\pm$ 0.01 <sup>e</sup>	0.14 $\pm$ 0.01 <sup>f</sup>	0.14 $\pm$ 0.01 <sup>d</sup>	15.1 $\pm$ 3.9 <sup>b,c</sup>	2.1 $\pm$ 0.8 <sup>b</sup>
Ethyl Ether (0.4)	5 169.2 $\pm$ 1 100.6 <sup>b</sup>	30.0 $\pm$ 5.1 <sup>b</sup>	3.4 $\pm$ 0.9 <sup>c</sup>	11.8 $\pm$ 5.3 <sup>b</sup>	227.5 $\pm$ 55.9 <sup>b,c</sup>	0.57 $\pm$ 0.06 <sup>c</sup>	0.21 $\pm$ 0.02 <sup>b</sup>	0.18 $\pm$ 0.01 <sup>a</sup>	10.0 $\pm$ 0.6 <sup>a,b,d</sup>	3.3 $\pm$ 0.7 <sup>b</sup>
Hexane (0.4)	9 468.2 $\pm$ 7 78.21 <sup>a</sup>	62.2 $\pm$ 3.6 <sup>a</sup>	5.4 $\pm$ 1.1 <sup>a,c</sup>	326.9 $\pm$ 35.7 <sup>a</sup>	491.3 $\pm$ 40.4 <sup>a</sup>	0.50 $\pm$ 0.02 <sup>a,c</sup>	0.17 $\pm$ 0.01 <sup>a,b</sup>	0.18 $\pm$ 0.01 <sup>a</sup>	7.5 $\pm$ 0.7 <sup>d</sup>	6.6 $\pm$ 0.3 <sup>a</sup>
Methanol (0.4)	10 390.9 $\pm$ 481.5 <sup>a</sup>	66.9 $\pm$ 5.3 <sup>a</sup>	7.3 $\pm$ 1.0 <sup>a</sup>	345.3 $\pm$ 37.2 <sup>a</sup>	571.0 $\pm$ 34.6 <sup>a</sup>	0.46 $\pm$ 0.02 <sup>a,b</sup>	0.15 $\pm$ 0.01 <sup>a</sup>	0.16 $\pm$ 0.01 <sup>a,d</sup>	9.6 $\pm$ 0.9 <sup>a,b,d</sup>	6.9 $\pm$ 1.3 <sup>a</sup>

a, b, c, d, e, f identical superscripts in the same column indicate that the means are not significantly different ( $\alpha = 0.05$ ).

g Top value for control chlorophyll is compared to chlorophyll data for all solvents except ethanol. The bottom control chlorophyll value is compared to the ethanol chlorophyll data. n = 10 for the control; n = 5 for acetone, acetonitrile, DMSO, ethyl ether, and hexane; and, n = 4 for benzene, ethanol, and methanol. Data presented are the untransformed means  $\pm$  s.d.

had no effect upon the membrane permeability of *Lemna paucicostata* at a concentration of 0.3% (Duke and Kenyon, 1992). As a solvent carrier, concentrations of ethanol <1 mM had a negligible effect upon the growth of *Lemna gibba* G3 (Barber *et al.*, 1995). *L. gibba* and four clones of *L. minor* were not severely affected by ethanol; the seven day EC50 values ranged from a low of 3 250 mg/L (c.i. = 42 -251 291 mg/L) for the frond number of one *L. minor* clone to a high of 43 505 mg/L (c.i. = 0 - 87 520 mg/L) for the plant number of another *L. minor* clone. The lowest NOEL for the effect of ethanol upon duckweed was 280 mg/L for frond and plant number of *L. gibba*. Duckweed dry weight was not a sensitive endpoint to the effects of ethanol (Cowgill *et al.*, 1991) but weight of *Myriophyllum sibiricum* was a sensitive endpoint in the current study (Table 40). An inhibitory effect of ethanol was noted on the cell multiplication of *Scenedesmus quadricauda* at 5 000 mg/L (Bringmann and Kühn, 1980). An EC50 value of 11 619 mg/L (c.i. = 7 923 - 15 314 mg/L) and NOEL of 5 400 mg/L were obtained for *Skeletonema costatum* cell count after five days of exposure to ethanol (Cowgill *et al.*, 1989a). Ethanol was one of the most toxic solvents tested on the growth of *Chlorella pyrenoidosa* with an EC50 of 1.18% v/v (Stratton and Smith, 1988). Ethanol was highly toxic to five blue-green algal species with EC50 values between 0.8% and 2.87%. Total inhibition was observed at concentrations between 1.5% and 3.0% with several species of *Anabaena* (Stratton, 1987a). In studies with fungi, ethanol was toxic and resulted in EC50s between 0.72% and 2.24% (Stratton, 1985). Ethanol has also been used to solubilize 2,4-dichlorophenol (Ensley *et al.*, 1994).

Ethyl ether and acetonitrile were intermediate in toxicity to *Myriophyllum sibiricum* with respect to plant height (Figure 42 and Table 40) but the acetonitrile was more toxic to root formation. Neither one of these solvents should be used in the *Myriophyllum* toxicity test. Acetonitrile was the second most toxic solvent tested by Bowman *et al.* (1981). This compound was relatively non-toxic to *Scenedesmus quadricauda*; inhibition of cell multiplication began at acetonitrile concentrations of 7 300 mg/L (Bringmann and Kühn, 1980). When using *Lemna minor*, it has been recommended that acetonitrile be avoided (Chollet, 1992a; 1992b).

Acetone did not severely affect the *Myriophyllum sibiricum* plant height or root number but the total root length was severely inhibited. Furthermore, fresh weight of plants treated with acetone was reduced when compared to the control plants (Table 40). At the 0.4% solvent level tested on *Myriophyllum*, acetone did not significantly

disrupt the membrane integrity. Acetone has been commonly used as a solvent in algal toxicity testing (Adams *et al.*, 1986). Inhibition to *Scenedesmus quadricuada* cell multiplication began at acetone concentrations of 7 500 mg/L (Bringmann and Kühn, 1980). The growth of *Scenedesmus pannonicus* and *Selenastrum capricornutum* was not severely affected by acetone because NOEC of 4 740 mg/L and 7 000 mg/L were obtained, respectively (Slooff *et al.*, 1983). After five days of exposure to acetone, an EC50 of 11 798 mg/L (c.i. = 2 133 - 21 463 mg/L) was obtained for the cell count of *Skeletonema costatum*. Acetone concentrations up to 6 000 mg/L caused no observable effect upon *S. costatum* cell number (Cowgill *et al.*, 1989a). When using *Lemna minor*, acetone may be used at concentrations no higher than one percent (Chollet, 1992a; 1992b). Acetone was not inhibitory to *Lemna gibba* and four clones of *L. minor* as suggested by the seven day EC50 values that ranged from a low of 9 793 mg/L (c.i. = 3 631 -15 955 mg/L) for the frond number of one *L. minor* clone to a high of 19 158 mg/L (c.i. = 9 - 38 948 mg/L) for the plant number of another *L. minor* clone. The lowest NOEL for acetone on duckweed was 3 240 mg/L for dry weight, plant and frond number of one *L. minor* clone (Cowgill *et al.*, 1991). Other researchers have questioned its use. Stratton (1989) found that acetone at concentrations above 0.5% over a 96 hour exposure period caused cellular leakage in *Chlorella pyrenoidosa*. Photosynthesis in *Anabaena cylindrica* was inhibited and cells were lysed by acetone concentrations greater than 0.6% (Stratton, 1981). Acetone has been reported to be unusable in algal toxicity studies (Conder *et al.*, 1995) and microcosm studies. In the microcosm studies, it was shown that the addition of acetone caused a large decrease in pH (Kersting, 1995). The effect of acetone on the number of cells produced by a diatom (*Nitzschia linearis* (Ag.) W. Sm.) resulted in a 50% reduction between 11 493 - 11 727 mg/L (Patrick *et al.*, 1968). Using the *Selenastrum capricornutum* flask assay, St. Laurent *et al.* (1992) determined an acetone NOEC of 0.23% v/v but with the microplate assay, the NOEC for acetone increased to >0.91% v/v (St. Laurent *et al.*, 1992). Acetone, at concentrations between 0.5 and 1.5%, caused a loss of cellular contents from *Chlorella pyrenoidosa* cells. Over longer exposure periods, lower acetone concentrations caused increased cellular leakage (Stratton, 1989). *Anabaena cylindrica* showed intracellular damage and cell lysis when exposed to acetone concentrations greater than 0.6% (Stratton *et al.*, 1980). High concentrations of acetone (3.33%) disintegrated the cell membrane and cytoplasm of *C. pyrenoidosa* cells (Parasher *et al.*,

1978). Concentrations of 3.02% v/v acetone caused a 50% reduction in *C. pyrenoidosa* growth (Stratton and Smith, 1988). *Anabaena* spp. and *Nostoc* sp. were affected (EC50) by acetone concentrations between 0.4 and 4.4% (Stratton, 1987a). Acetone concentrations greater than 1.0% were required to severely inhibit photosynthesis, nitrogen fixation and heterocyst formation in *Anabaena inaequalis* (Kuetz) Bornet & Flahault while significant inhibitions of photosynthesis in *A. cylindrica* were caused by acetone concentrations above 0.6% (Stratton and Corke, 1981a; Stratton *et al.*, 1980). Acetone was of intermediate toxicity to three fungal species since EC50s ranged between 1.99 and 12.07% (Stratton, 1985). Acetone was the most toxic solvent tested by Bowman *et al* (1981).

In the experiment with *M. sibiricum*, the only endpoint parameter where DMSO statistically differed from the control was chlorophyll a content. DMSO was not recommended for use in the *Myriophyllum* toxicity tests because of the moderately high human toxicity (The Merck Index, 1983; MSDS, 1992) even though it did not detrimentally effect the *Myriophyllum* plants when compared to the controls (Figure 42 and Table 40). In other studies, DMSO has been used to solubilize metolachlor (Deal and Hess, 1980; Deal *et al.*, 1980). In toxicological studies with *Lemna gibba*, DMSO concentrations up to 1% (v/v) have been used without deleterious effects (Greenberg *et al.*, 1992; Chollet, 1992a; 1992b). When tested on *Chlorella pyrenoidosa* growth, DMSO produced an EC50 of 2.01% v/v (Stratton and Smith, 1988). DMSO was of intermediate toxicity to *Anabaena* spp. and *Nostoc* sp., producing EC50 values between 0.36% and 4.38%. Total growth inhibition was induced by DMSO at concentrations between 1.5% and 4% for the *Anabaena* spp. but levels of 6.0 to 10.0% were required for total growth inhibition of *Nostoc* sp. (Stratton, 1987a). Three species of fungi were not severely affected by DMSO as seen in EC50 values between 2.49 and 3.33% (Stratton, 1985). DMSO, up to 2% by volume, was tolerated by four invertebrate species (Bowman *et al.*, 1981).

In the current research, no statistically significant difference was observed between the control plants and methanol treated plants for any of the endpoints examined (Figure 42 and Table 40). Methanol has been used to dissolve pesticides in numerous studies with aquatic plants (Jones and Winchell, 1984; Kemp *et al.*, 1985; O'Brien and Prendeville, 1979; Shehata *et al.*, 1993; Stratton, 1987a). St Laurent *et al.* (1992) recommended that methanol at concentrations less than 0.91% (v/v) be used as

a carrier for *Selenastrum capricornutum* toxicity tests because they obtained NOECs of 0.68% v/v with the flask bioassay and >0.91% v/v with the microplate assay. Day and Hodge (1996) used methanol as a solvent carrier for algae and *Lemna gibba* testing. For the algal experiments, they used methanol below the 0.91% concentration determined by St. Laurent *et al.* (1992). They determined that concentrations of methanol up to 0.7% (v/v) did not significantly inhibit *Lemna* growth rate (Day and Hodge, 1996). When using *Lemna minor*, methanol concentrations should be kept below 0.1% (Chollet, 1992a; 1992b). Of the six solvents tested on *Chlorella pyrenoidosa* growth, methanol was the least toxic with an EC50 of 3.60% v/v (Stratton and Smith, 1988). Methanol was not toxic to several *Anabaena* species and *Nostoc* sp. Total growth inhibition was only noted at methanol concentrations above 4.0 to 6.0% with the *Anabaena* cultures and at 10% with for *Nostoc* sp. (Stratton, 1987a). Inhibition of cell multiplication in *Scenedesmus quadricauda* cultures began at methanol concentrations of 8 000 mg/L (Bringmann and Kühn, 1980). This solvent was used at a concentration of 3% (v/v) in studies with *Chlorella pyrenoidosa* (Zweig *et al.*, 1968). Methanol was toxic to three fungal species (EC50s ranging between 1.08 and 2.29%) (Stratton, 1985). At 2%, methanol was tolerated by four invertebrate species (Bowman *et al.*, 1981).

Because hexane did not significantly affect plant growth, it could also be used as a solvent in the *Myriophyllum* toxicity test. Hexane was not very toxic to growth of *C. pyrenoidosa*; an EC50 of 2.66% v/v was determined (Stratton and Smith, 1988). Hexane was not very toxic to blue-green algae because EC50 values up to 8.0% were obtained (Stratton, 1987a). Hexane was chosen as the least toxic solvent to three fungal species. EC50 values between 4.47 and 36.6% were obtained as calculated from changes in growth (Stratton, 1985).

The possible interaction between solvent and pesticide was not examined for any of the pesticides or solvents tested in the *Myriophyllum* toxicity test. Other authors have found that solvents and pesticides in other toxicity tests interact synergistically, antagonistically and additively depending on the type and concentration of solvent, pesticide and the organism utilised (Stratton, 1985; 1989; Stratton and Corke, 1981a; Stratton *et al.*, 1982; Stratton and Smith, 1988).

In conclusion, the eight solvents can be ranked from most toxic to least toxic to *Myriophyllum sibiricum* in axenic culture: benzene > ethanol > acetonitrile > ethyl ether

> acetone > DMSO > hexane = methanol. Based on the similarity between methanol treated plants and the control plants, methanol was chosen as the solvent to be used for pesticides with low water solubilities in the *Myriophyllum sibiricum* toxicity test. According to the solubilities reported in the WSSA Herbicide Handbook (WSSA, 1989), methanol was capable of solublizing the pesticides with low water solubility used in this study. Because only one concentration of solvent was tested (0.4% v/v), this level should not be exceeded without further testing.

## 5.6 STATISTICAL ANALYSIS

### 5.6.1 Dilution Series Data

There is a debate regarding the appropriate method to analyse toxicity dose response data. NOEC's and LOEC's have been used in toxicity testing and ecological risk assessment and these parameters are currently being criticised. The determination of these values is not always biologically relevant because they are by definition always one of the test concentrations. Furthermore, poor experimental design can underestimate the toxicity of a substance, most of the information in the dose response curve (e.g. the slope and the confidence limits) is ignored and the hypothesis testing is conclusionary (toxic or not) rather than descriptive (level causing a specific effect). Descriptive statistics, such as the point estimation of an  $IC_p$ , are being recommended because it is possible to extrapolate to untested concentrations, poor experimental design will be reflected in the confidence limits but will not affect the  $EC_x$  estimation and all the information in the dose response curve is utilised in the analysis (Bruce and Versteeg, 1992; Dhaliwal *et al.*, 1997; Environment Canada, 1993; Hoekstra and Van Ewijk, 1993; Moore and Caux, 1995; 1997).

If NOECs and LOECs are used in statistical analysis, care should be taken to ensure their correct usage. In current usage, NOEC is defined as "the highest concentration of a material in a toxicity test that has no statistically significant adverse effect on the exposed population of test organisms as compared with the controls", while LOEC is defined as "the lowest concentration of a material used in a toxicity test that has a statistically significant adverse effect on the exposed population of test organisms as compared with the controls" (Rand and Petrocelli, 1985a; 1985b). Their explicit definitions are currently under review by the ASTM subcommittee on Terminology (E47-11.02) to be included in the ASTM document on Terminology E 943 (ASTM, under

consideration). Occasionally, the dose response data do not follow an orderly pattern, that is, not all the responses above a certain concentration are significantly different from the control. In these cases, the results at one concentration are significantly different from the control, not different at a higher concentration and then significantly different at another higher concentration. At the November 1996 ASTM E47 meeting in Washington, D.C., it was decided that the highest concentration that did not differ significantly from the control would be the NOEC, the higher concentrations that differed from the control would be termed a zone of ambiguity and the concentration beyond which all the concentrations differed from the control would be the LOEC. For some of the endpoints in several of the experiments described in this document there was a zone of ambiguity. Using the microcomputer statistical package Systat<sup>®</sup>, NOEC and LOEC were determined for each endpoint as affected by the various pesticides using one-way ANOVA for normal data sets and the Kruskal-Wallis test for non-normal data sets. These analyses were followed by multiple comparison tests (Eilersieck and La Point, 1995).

For quantal and lethality data, EC50s and LC50s are calculated using linear (Parrish, 1985), probit (Eilersieck and La Point, 1995; Hubert, 1992) or logit (Hubert, 1992) statistical procedures. These types of analyses are not suitable for quantitative response experiments that generate continuous response data measured in plant research. Non-linear regression analysis is more appropriate for data that have more than two responses. Numerous non-linear techniques exist for determination of IC<sub>p</sub> values (Andersen, submitted; Bruce and Versteeg, 1992; Caux and Moore, 1997; Christensen and Nyholm, 1984; Environment Canada, 1993; Moore and Caux, 1997; Streibig, 1980; Stuekl, 1988) and several were compared in this discussion. The weibull method (Andersen *et al.*, submitted; Christensen and Nyholm, 1984), the cumulative normal analysis (Andersen *et al.*, submitted) and several curve fitting techniques (i.e., linear, logarithmic and polynomial) were used in calculating IC<sub>p</sub> values for the continuous data generated in the axenic *Myriophyllum sibiricum* toxicity test. The weibull and the cumulative normal programs were programmed into Lotus 1-2-3<sup>®</sup> by K.O. Kusk<sup>15</sup>. The

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curve fitting techniques were conducted using Excel® and the IC<sub>p</sub> values were obtained using the “Goal Seek” and “Solver” options.

A data set collected from an experiment that determined the effect of zinc chloride on the shoot growth of *Myriophyllum sibiricum* after fourteen days of exposure in axenic culture was analysed using the above methods. The results from the weibull analysis are presented in Figure 43; Figure 44 shows the results from the cumulative normal method; Figure 45 shows the data overlain with four curves determined using curve fitting techniques. The IC<sub>p</sub> values are summarised in Table 41. Obviously, the linear method did not correctly model the data (Figure 45 and Table 41). There was good correlation between the IC<sub>p</sub> values as determined by the other five methods. One advantage of the weibull and cumulative normal analysis techniques was that 95% confidence intervals could be determined around the data. With the non-linear curve fitting techniques, it was not possible to obtain confidence intervals around the IC<sub>p</sub> values.

Table 41: Summation of the IC<sub>p</sub> values obtained using various linear and non-linear regression techniques. The data were obtained from an experiment that was conducted to determine the effect of zinc chloride on the shoot growth of *Myriophyllum sibiricum* after fourteen days of incubation.

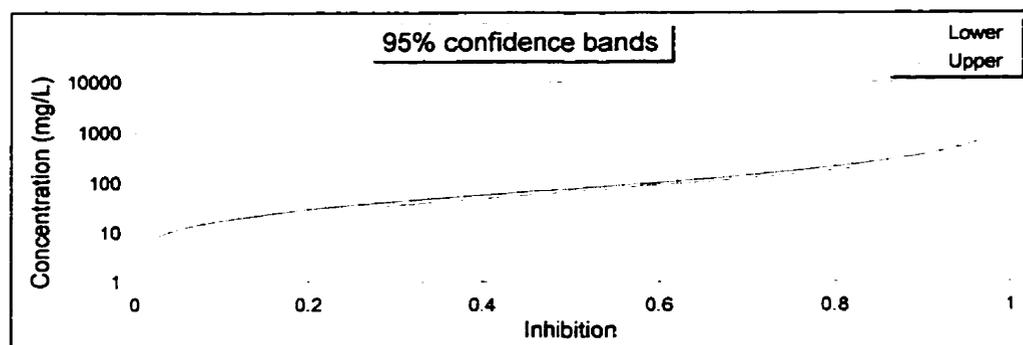
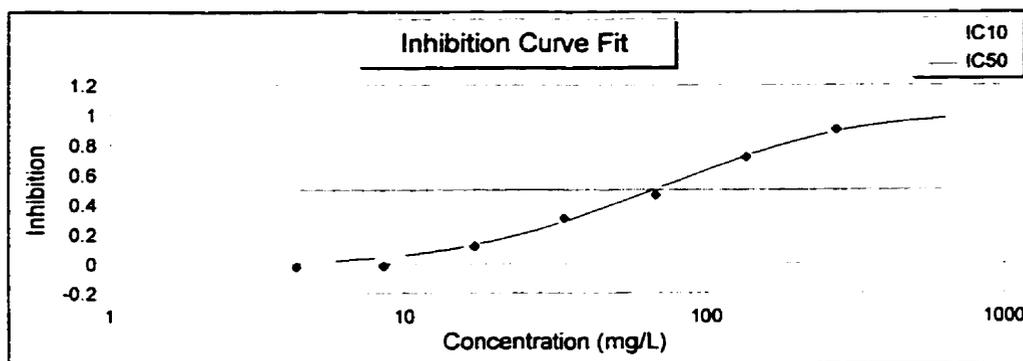
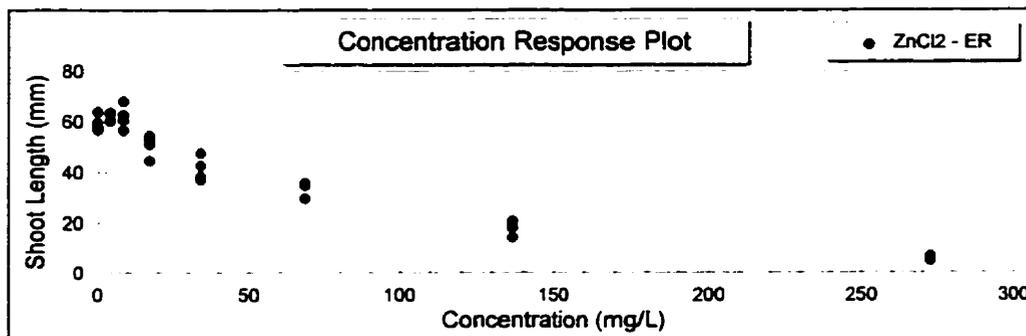
IC <sub>p</sub> Value	Concentration (mg/L)					
	Weibull <sup>a</sup>	Cumulative Normal <sup>a</sup>	Third Order Polynomial <sup>b</sup>	Second Order Polynomial <sup>b</sup>	Logarithmic <sup>b</sup>	Linear <sup>b</sup>
10	13.9	14.3	16.1	16.5	11.6	3.2
20	23.8	24.2	26.3	30.0	17.7	32.3
25	29.3	29.5	32.0	37.1	21.9	46.9
50	66.8	65.8	68.6	77.3	63.2	119.8

a Data were calculated using a program written in Lotus® by K.O. Kusk.

b IC<sub>p</sub> values were obtained using the curve fitting techniques, “goal seek” and “solver” portions of Excel®.

There were several problems encountered with the weibull and cumulative normal programs. The weibull equation forces the data through zero (Andersen *et al.*, submitted) and this technique often encountered problems if there was stimulation in plant growth. Streibig (1980) also recommended that a non-zero asymptote would produce a better fit for stimulation data (Streibig, 1980). The cumulative normal program also encountered difficulties if the endpoint was stimulated above control

***Myriophyllum sibiricum***  
**Compound: Zinc chloride**

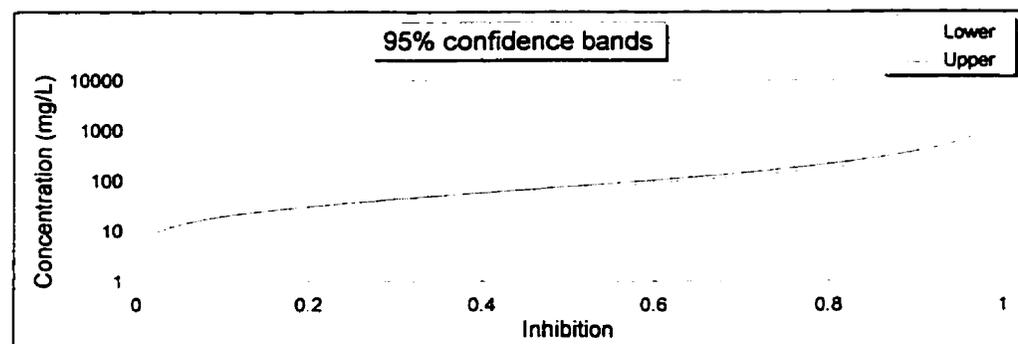
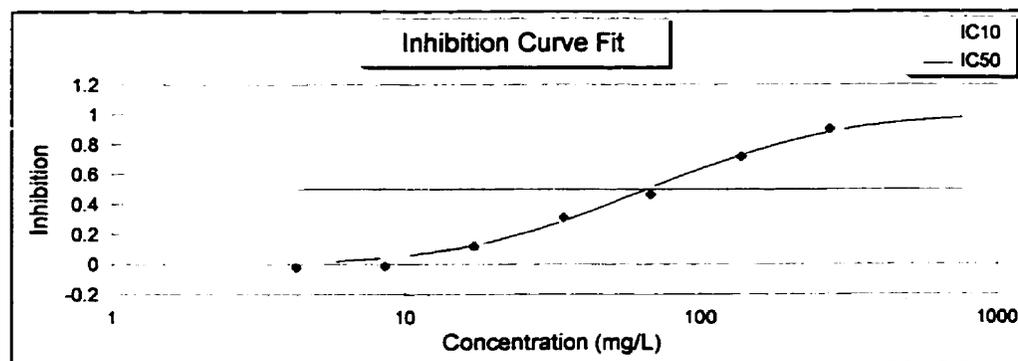
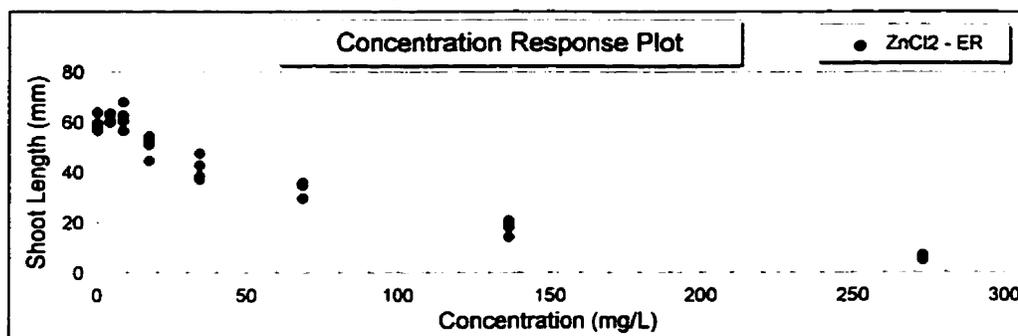


IC	Estimated IC values mg/L		
	95 % Confidence limits		
10	13.88	10.77	17.89
20	23.81	19.17	29.66
25	29.28	23.98	35.75
50	66.77	58.28	76.49

File: ZnCl<sub>2</sub> (ER Series) Page: A Shoot Length Weibull

Figure 43: The results obtained from weibull statistical analysis of shoot length data from a zinc chloride experiment with *Myriophyllum sibiricum*. These three graphs and the table of IC<sub>p</sub> values were generated by the Lotus 1-2-3® program written by K.O. Kusk, Technical University of Denmark.

***Myriophyllum sibiricum***  
**Compound: Zinc chloride**



IC	Estimated IC values mg/L		
	95 % Confidence limits		
10	14.32	10.52	19.49
20	24.18	19.05	30.70
25	29.52	23.84	36.56
50	65.83	57.19	75.77

File: ZnCl<sub>2</sub> (ER Series) Page: A Shoot Length Cumulative Normal

Figure 44: These three graphs and the table of IC<sub>p</sub> values were obtained directly from the cumulative normal program written for Lotus 1-2-3® by K.O. Kusk, Technical University of Denmark. The above shoot length data were analysed from an experiment to determine the effect of zinc chloride on *Myriophyllum sibiricum* apices.

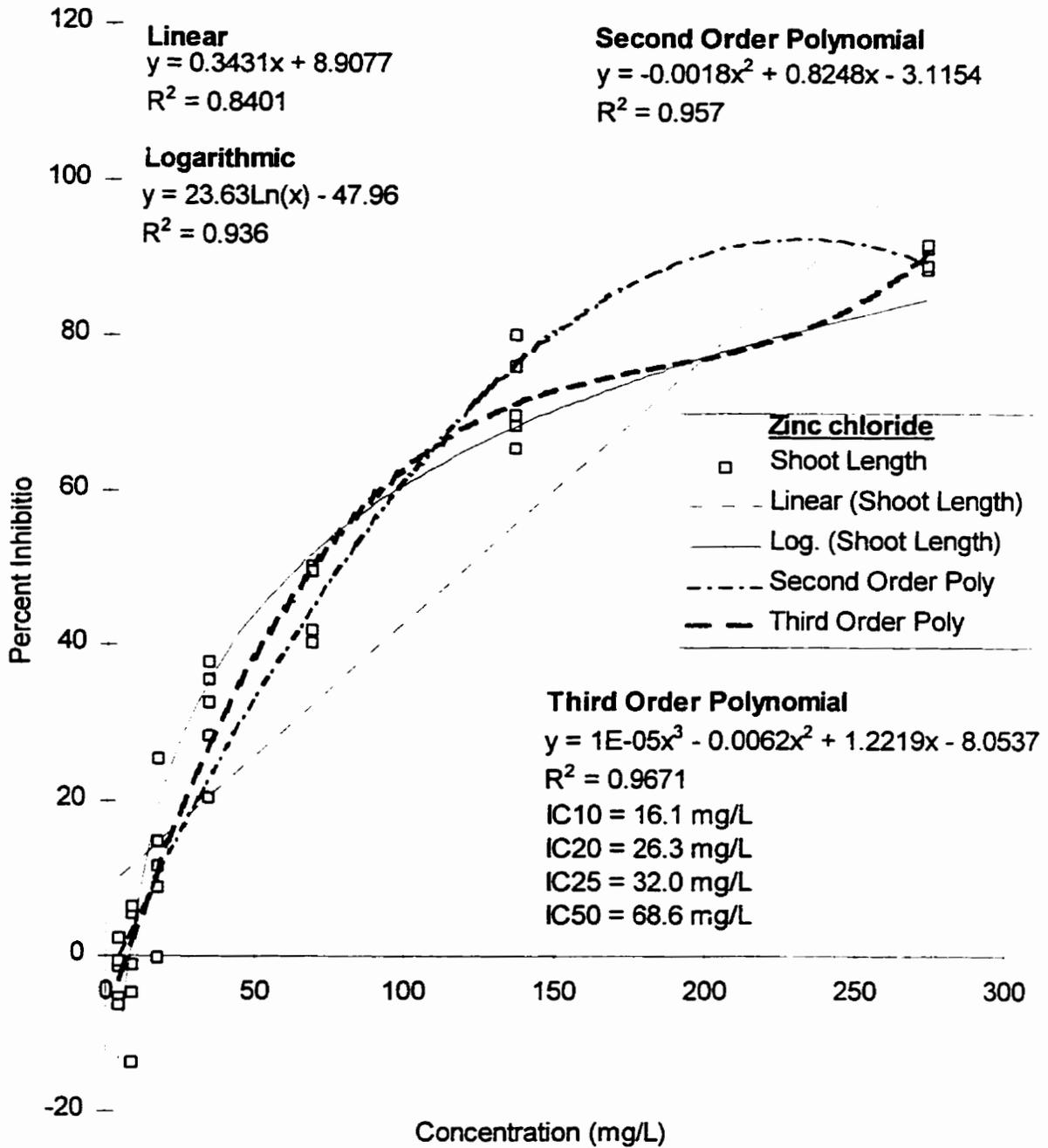


Figure 45: Curve fitting techniques were used to fit four types of curves to the data from an experiment that examined the effect of zinc chloride upon the growth of *Myriophyllum sibiricum*. Inhibition values were calculated for each curve. The third order polynomial is the best fit of this data set.

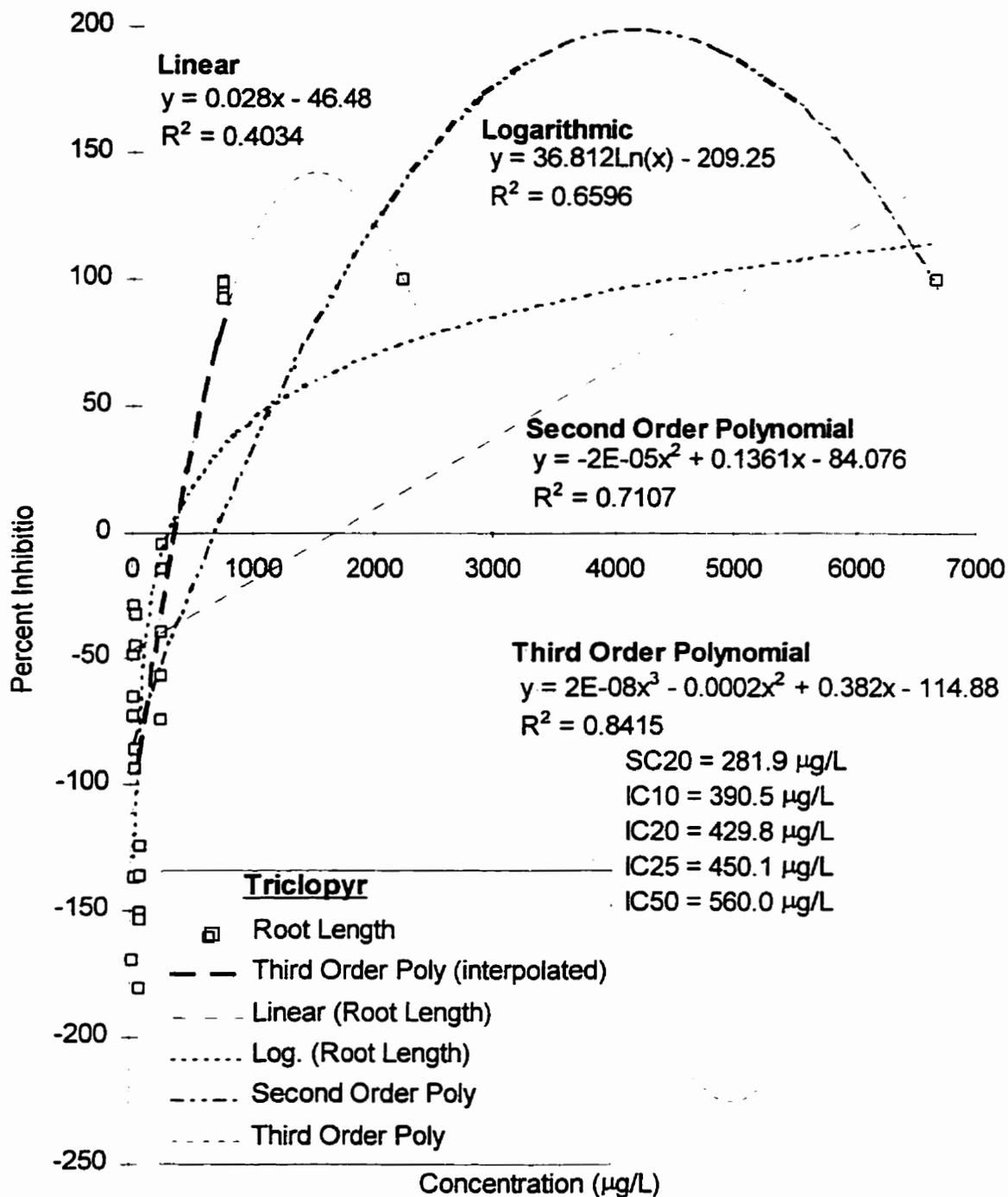


Figure 46: Curve fitting techniques were used to determine  $IC_p$  values for data that displayed stimulation at low concentrations of the toxicant. This example was taken from an experiment that examined the effects of triclopyr on total root length of *Myriophyllum sibiricum* plants axenically cultured for fourteen days in modified Andrews' medium. The linear line did not fit the data. The third order polynomial curve between 0 and 1 000 µg/L was the best fit of the data.

levels. If the concentration range was widely spaced, the weibull program was unable to analyse the data. Both of these programs encountered difficulties if the slope of the curve was steep (i.e., if the data went from stimulation or very low inhibition to high inhibition at the next concentration). Polynomial curves are periodic and visual assessment of the data and the curves must be conducted to eliminate parts of the curves that are not biologically relevant. Interpolation of the appropriate IC values must be obtained from within the range of the tested toxicant concentrations. Extrapolating values outside the collected data is not appropriate with any of the regression techniques discussed in this section.

Growth stimulation at low toxicant concentrations occurs commonly in plant toxicity studies (Streibig, 1980; Walsh and Alexander, 1980; Walsh *et al.*, 1980). Usually this stimulation is just reported as being present but the stimulation data is deleted from the statistical analysis (Streibig, 1980). Walsh *et al.* (1980) determined that stimulation at twenty percent above control values was statistically significant. They defined this concentration as the SC20 or the calculated concentration that would stimulate growth by twenty percent (Lewis, 1995; Walsh *et al.*, 1980). Since the weibull and cumulative normal programs were unable to calculate IC<sub>p</sub> values for data sets that contained stimulation, curve fitting techniques were used to determine IC<sub>p</sub> and SC20 values. Figure 46 gives a representative example of the analysis of a data set with stimulation. This example was from an experiment that examined the effect of triclopyr upon root growth of *Myriophyllum sibiricum* after fourteen days of incubation. Root growth was maximally stimulated up to an average of 149% at 82 µg/L. An SC20 of 281.9 µg/L was obtained using the third order polynomial equation. This equation was only valid at concentrations less than 1 000 µg/L because of the cyclic nature of polynomial equations.

In the following sections describing the effects of pesticide dilution series on the non-target aquatic macrophyte *Myriophyllum sibiricum*, NOEC, LOEC, IC<sub>p</sub> and SC20 values are calculated where appropriate and compared with values cited in the scientific literature. Based on a visual and statistical assessment of each data set, the appropriate IC<sub>p</sub> technique is employed. Any problems encountered with the different statistical techniques are discussed.

## 5.7 PESTICIDE TOXICITY TESTS

Pesticides with various modes of phytotoxic action (Table 27) were selected for testing in the *Myriophyllum sibiricum* toxicity test. As explained in Section 2.3.1, the tiered testing system recommended by Environment Canada (Boutin *et al.*, 1993) was followed. For each of the pesticides studied, the first step was to determine if the EEC (Table 27) was toxic (Tier I). No further testing was conducted if this concentration was not toxic. If the EEC was toxic, then further *Myriophyllum* testing was conducted using geometric dilution series until an IC50 value and NOEC could be calculated (Tier II).

### 5.7.1 Atrazine

#### 5.7.1.1 Introduction

Since its introduction in 1958, atrazine (Figure 47) has been used as a selective triazine herbicide to control dicotyledonous and monocotyledonous weeds in corn and sorghum (*Sorghum vulgare Pers.*). It may be applied preplant, preemergence or postemergence (Jones *et al.*, 1982; McEwen and Stephenson, 1979; WSSA, 1989). It is also occasionally used for weed control in sugar cane (*Saccharum officinarum L.*), pineapple (*Ananas comosus Merr.*), rangeland, turfgrass and conifer reforestation (McEwen and Stephenson, 1979).

The mode of action of atrazine is to inhibit the electron transport chain in photosystem II and thereby inhibit the cleavage of water during the photosynthetic process. It binds to the thylakoid membranes in the chloroplasts and blocks electron transport between photosystems I and II (Ashton and Crafts, 1981).

Atrazine is actively absorbed by the roots and absorbed to a smaller extent through the foliage (OMAF, 1994a). *Hydrilla verticillata*, a submersed aquatic macrophyte, quickly removed atrazine from solution and the tissue concentrations reached equilibrium with the surrounding solution within 1 hour for shoot tissue and within 2 hours for root tissue. When this species was exposed to atrazine treated substrates, atrazine was translocated acropetally (Hinman and Klaine, 1992).

There is an amazing amount of scientific literature dealing with atrazine. It is moderately mobile and moderately persistent, which allows it to move into non-target

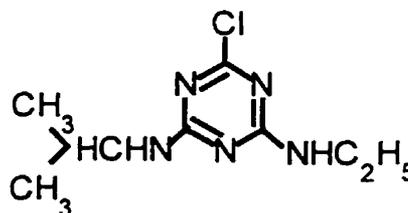


Figure 47: The structural formula for atrazine, a selective herbicide that inhibits photosynthesis.

areas. In North America, the total volume of atrazine was previously greater than all other single herbicides (McEwen and Stephenson, 1979). In the United States, over 40 000 metric tonnes of atrazine were used annually (Hersh and Crumpton, 1989). In 1993, atrazine use on all crops in Ontario totalled 589,852 kg. Atrazine use on field corn decreased from 999 000 kg in 1988 to 585 000 kg in 1993. This was a 41% reduction in atrazine use over a 5 year period (Hunter and McGee, 1994). Atrazine is widely used in corn production in the midwestern United States (Solomon *et al.*, 1996). Atrazine is used in South and Central America for aquatic weed control (Fernández *et al.*, 1993) and in Yugoslavia for weed control in ditchbanks and dry ditches (Murphy *et al.*, 1993). In soils, biodegradation of atrazine is slow and only a small fraction is transformed into metabolites (Johnson and Fuhrmann, 1993; Radosevich *et al.*, 1993).

Due to its high usage rate, a fraction of the agriculturally applied atrazine can contaminate both surface and groundwater (Gaynor *et al.*, 1995; Jones *et al.*, 1982; Wietersen *et al.*, 1993). Approximately 0.1% to 3.0% of atrazine sprayed on fields is lost into aquatic systems (Jones *et al.*, 1982). In one study, the maximum loss of atrazine was 5.8% of the applied pesticide (Sauer and Daniel, 1987). Atrazine can enter groundwater through leaching (Southwick *et al.*, 1990a; 1990b; Wilson *et al.*, 1987) and it has been detected in groundwater removed from wells (Frank *et al.*, 1987a; 1990a). The concentration of atrazine detected in groundwater ranged from 0.01 to 8.29 µg/L (Wehtje *et al.*, 1983), 0.1 to 74 µg/L (Frank *et al.*, 1987b) and 0 to 483 µg/L (Richard *et al.*, 1975).

Atrazine enters surface waters mainly as water phase runoff (Glottfelty *et al.*, 1984; Wu *et al.*, 1983). The amount of atrazine in surface runoff depends upon the total amount applied, the timing of runoff events with respect to application (Glottfelty *et al.*, 1984; Hall *et al.*, 1983; Richard *et al.*, 1975; Triplett *et al.*, 1978), slope, vegetation cover of the treated and untreated areas, and soil texture, type, temperature, moisture content and pH (Merkle and Bovey, 1974; Weber, 1972b). Distance between treated areas and major bodies of surface water influences the amount of atrazine that can contaminate aquatic systems. Within one hour of atrazine application at a rate of 3.36 kg/ha, a 6.3 cm rainstorm caused a 17% loss of the herbicide in runoff. If rain did not occur until 96 hours after application, only 7% of the atrazine was lost (Merkle and Bovey, 1974). Glottfelty *et al.* (1984) determined that if significant runoff occurred within two weeks of application, 2 to 3% of the atrazine moved into the surface waters, reaching a peak

around 300 µg/L. Concentrations rapidly declined and runoff losses effectively ceased after about 6 weeks (Glotfelty *et al.*, 1984). Atrazine concentrations in runoff reached a maximum of 480 µg/L in a study by Triplett *et al.* (1978). Seasonal fluctuations occurred in atrazine concentrations, with the highest levels being detected in the spring shortly after atrazine application (Richard *et al.*, 1975; Wehtje *et al.*, 1983; Wilson *et al.*, 1987; Wu *et al.*, 1983). If atrazine was applied in the autumn to control perennial weeds (e.g. couch grass - *Agropyron repens* (L.) Beauv.), a second but smaller peak of atrazine could be detected in the runoff (Frank and Sirons, 1979). Residual levels of atrazine were also detected in water samples collected in the winter and the spring prior to pesticide application (Wu *et al.*, 1983).

Atrazine residues have been detected in the Great Lakes to a maximum of 110 ng/L in Lake Ontario. Atrazine was detected in all 490 lake water samples collected in a study by Schottler and Eisenreich (1994). Atrazine persists in water and soil long enough for residues to appear throughout the year (Frank *et al.*, 1982). Atrazine residues were detected at twenty-six sites along the Mississippi River and the maximum atrazine concentration was 4735 ng/L (Pereira and Hostettler, 1993).

Atrazine is occasionally used to control aquatic weed problems (Fernández *et al.*, 1993; Murphy *et al.*, 1993). Once the atrazine has entered the aquatic system, it inhibits photosynthesis and thereby decreases growth of non-target aquatic plants and algae. It does not accumulate in higher trophic levels and is relatively non-toxic to fish and invertebrates (Hersh and Crumpton, 1989). Indirectly, it can affect higher trophic levels by reducing habitat and food sources (Dewey, 1986).

Once in an aquatic system, atrazine is not persistent. Half-life values ranged between 10 to 30 days (Glotfelty *et al.*, 1984) with 3 to 12 days for the parent compound. In two estuarine sediments, the half-life of atrazine was 15 and 20 days. Degradation in aquatic systems is much faster than in terrestrial environments. Atrazine removal from the water occurs via sorption to the sediment and degradation in the water to metabolic compounds that may be sorbed onto the sediment. After sorption to sediments, atrazine can be degraded to metabolic compounds and subsequently desorb into the water column. The major short-term metabolite in the Chesapeake Bay system was hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-s-triazine (Jones *et al.*, 1982). Microbial dealkylation of atrazine to desethylatrazine was the major degradation pathway under stream runoff conditions in southern Ontario. Of the 480 water samples

collected from 11 watersheds, 79.0% contained atrazine, 62.2% contained desethylatrazine and 79.8% contained one or both compounds (Frank and Sirons, 1979). Atrazine breakdown products are usually less phytotoxic than the parent compound (Wehtje *et al.*, 1983). Solomon *et al.* (1996) reviewed the scientific literature and included more information on the aquatic fate of atrazine.

#### 5.7.1.2 Methods

In preliminary herbicide trials, formulated atrazine (ATREX 90% WP) was tested in half strength M & S medium (Roshon and Stephenson, 1992), full strength Modified Andrews' medium and Hard Water media (data not presented). Based on the preliminary experiments and the decision to use technical pesticides in the remaining experiments, technical atrazine (99% pure) was purchased from Chem Service<sup>16</sup>. Technical atrazine was not as water soluble as predicted (33 mg/L (Wauchope *et al.*, 1992; WSSA, 1994)). For the MLR (4.48 kg/ha (WSSA, 1989)) and dilution experiments with atrazine, the technical atrazine was dissolved in reagent grade methanol before being added to the modified Andrews' medium. The solubility of atrazine in methanol is 18 000 mg/L (WSSA, 1989) so the technical atrazine readily dissolved in the methanol. Thus, methanol control plants were included with every experiment. At 4.48 kg/ha, the MLR of atrazine would become 2.99 mg/L (Table 27) if this amount was sprayed onto a 15 cm column of water in a wetland situation.

Starting on March 21, 1994, June 30, 1994, March 23, 1995 and May 25, 1995, the EEC experiment was repeated four times. The stock plants used in the first two experiments were cultured in half strength M & S medium and the stock plants utilised in the last two experiments were cultured in modified Andrews' medium. The medium for culturing the stock plants was changed because of problems with the M & S medium (see Section 4.5.2 for further details). Serial dilutions with technical atrazine were conducted twice, starting on February 3, 1995 and May 25, 1995. The concentrations tested were 0.0 (water and methanol controls), 46.7, 93.3, 186.7, 373.3, 746.7, 1493.3, 2987 µg/L in the first dilution series and 0.0 (water and methanol controls), 497.8, 995.6, 1 493.3 , 1 991.1, 2 488.9, 2 987 µg/L in the second dilution series. The stock plants used in both of these experiments were cultured in modified Andrews' medium. After

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<sup>16</sup> Chem Service, Box 3108, West Chester, PA, 19381.

treating the plants with the appropriate atrazine concentrations, the experimental plants were cultured for two weeks under the environmental conditions outlined in Section 4.6. Plant height was measured every second day. At the end of this period, the plants were harvested and measured for shoot length, number of nodes, root number and length, dissolved oxygen in the medium, fresh weight of the total plant and of each section, dry weight of all sections (starting January, 1995), plant area, membrane integrity, chlorophyll *a*, chlorophyll *b*, and carotenoid content. The control data were checked for normality and transformed if necessary. If the data were normal or could be transformed, a t-test was used to compare the water and methanol control data. For non-normal data, the Mann-Whitney U-test was used to compare the two sets of controls for differences. If there was no significant difference between the controls, the data were combined for the rest of the statistical analysis. If the water and methanol controls differed, only the methanol control data were used in the remaining analyses. For the data collected from the endpoint parameters measured during the EEC experiments, the atrazine data were compared to the appropriate control data using a t-test for normal data or the Mann-Whitney U-test for nonparametric data. For the tests conducted with a series of atrazine concentrations, the data collected from each endpoint were compared using a one-way ANOVA (normal data) or the Kruskal Wallis test (nonparametric data). If significant treatment differences were detected, multiple comparison tests were used to determine which treatments differed. NOEC and LOEC were calculated for each endpoint. IC50 values were determined using the weibull method, the cumulative normal distribution model or curve fitting techniques. If stimulation was encountered, SC20 values were calculated (Section 5.6.1).

### 5.7.1.3 Results and Discussion

#### 5.7.1.3.1 Expected Environmental Concentration

Unfortunately, the growth cabinet overheated to 38 °C during the first EEC experiment and most of the data were invalid because of this mechanical failure. The interesting observation from the growth curve data collected during this experiment was that both sets of control plants recovered from the heat stress but the atrazine treated plants were unable to compensate for both the pesticide stress and the additional heat stress. The atrazine treated plants stopped growing soon after the growth cabinet malfunctioned (Figure 48). All the plants survived the second and fourth EEC

experiment but three of the methanol control plants succumbed to contamination in the third atrazine EEC experiment. The data from the last three experiments were analysed and included in this discussion. Visually, the plants from the second atrazine EEC experiment were affected more severely (Figure 49) than the atrazine treated plants from the third and fourth experiments (Figure 50). This difference was most probably caused by the different culturing media prior to experimental initiation; the plants used in the second experiment were cultured in M & S medium and the plants for the third and fourth experiments were cultured in modified Andrews' medium. This re-emphasised the importance of culturing medium on the experimental results.

Representative growth curves from the fourth experiment can be found in Figure 51. Atrazine stimulated an increase in plant height (Figure 49, Figure 50, Figure 51, Table 42 and Table 43). For all experiments, this increase in height did not correspond to an increase in the number of nodes, plant weight, plant area or the ratio between dry and fresh weight (Table 42 and Table 43). The plants treated with 2.99 mg/L of atrazine were significantly lighter than the control plants. This difference in weight was also reflected in the dry/wet weight ratios, which suggested that the atrazine treated plants contained more water and less biomass than the control plants. The number of nodes

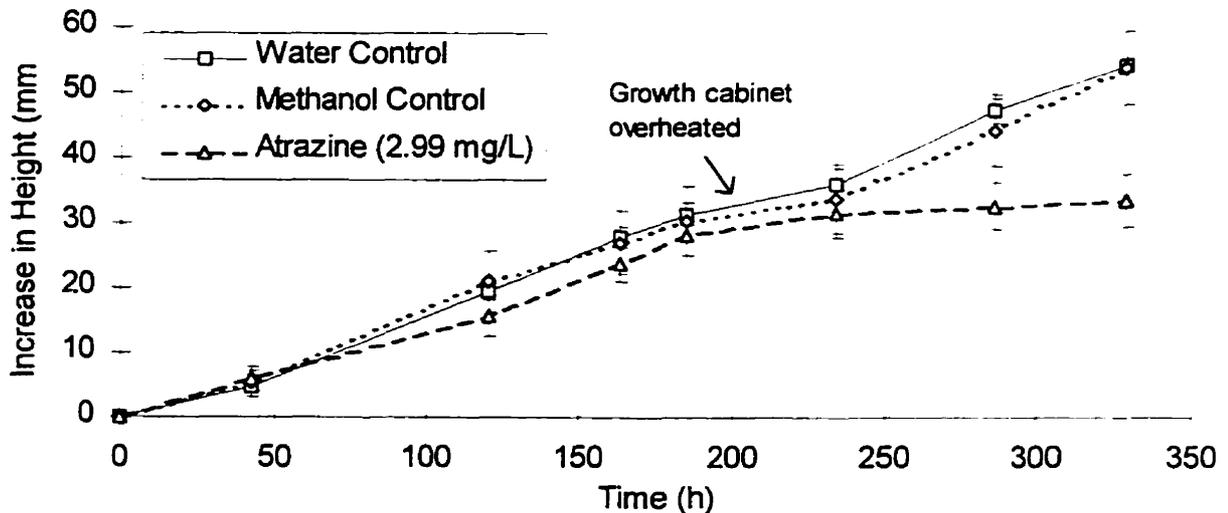
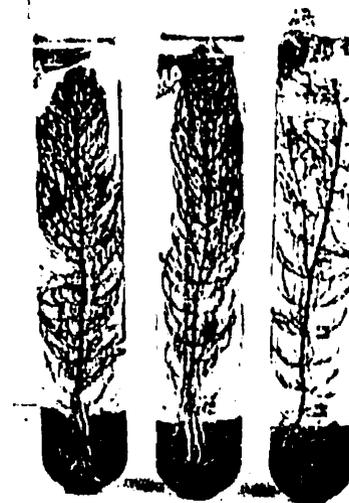


Figure 48: This figure displays the fourteen day growth curves collected from the first experiment that tested the effect of atrazine at a concentration of 2.99 mg/L on the growth of *Myriophyllum sibiricum*. Most of the data from this experiment was invalidated because the growth cabinet overheated to 38 °C between days eight and ten. The control plants managed to recover from the heat stress but the atrazine treated plants were unable to continue growing.



Water	Methanol	EEC
Control	Control	
0.0	0.0	2.99
Atrazine Concentration (mg/L)		

Figure 49: The effect of 2.99 mg/L of atrazine on *Myriophyllum sibiricum* as determined in the second experiment. There were minor differences between the water controls (left) and the methanol controls (middle) but there were numerous differences between the controls and the atrazine treated plants (right). All of these plants were cultured in M & S medium prior to the experiment. For the experiment, the plants were grown in modified Andrews' medium for two weeks.



Water	Methanol	EEC
Control	Control	
0.0	0.0	2.99
Atrazine Concentration (mg/L)		

Figure 50: Three representative *Myriophyllum sibiricum* plants after fourteen days of treatment with 0.4% water (left), 0.4% methanol (centre) or 2.99 mg/L of atrazine (right) added to the medium. The atrazine treated plants from the fourth experiment were visually less affected than those in the second experiment. Atrazine stimulated shoot growth at this concentration. Modified Andrews' medium was used for both culturing and experimentation.

Table 42: *Myriophyllum sibiricum* growth and development were inhibited by the EEC of atrazine when compared with the control plants. All the experimental plants were grown for fourteen days in modified Andrews' medium with 0.4% water, 0.4% methanol or 2.99 mg/L technical atrazine. Negative values indicate stimulation above control levels.

GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)		
	Second Experiment	Third Experiment	Fourth Experiment
Area Under the Growth Curve	-18.5 $\pm$ 12.4 <sup>a</sup>	-33.0 $\pm$ 5.3 <sup>c</sup>	-46.0 $\pm$ 13.8 <sup>a</sup>
Increase in Shoot Length	-17.3 $\pm$ 6.9 <sup>a</sup>	-52.2 $\pm$ 5.6 <sup>c</sup>	-64.5 $\pm$ 9.1 <sup>a</sup>
Increase in Node Number	51.7 $\pm$ 10.9 <sup>a</sup>	42.9 $\pm$ 7.1 <sup>d</sup>	25.4 $\pm$ 4.3 <sup>b</sup>
Nodes per Centimetre	57.0 $\pm$ 10.7 <sup>a</sup>	47.0 $\pm$ 6.4 <sup>c</sup>	30.9 $\pm$ 3.9 <sup>b</sup>
Root Number	68.3 $\pm$ 6.7 <sup>b</sup>	60.6 $\pm$ 16.6 <sup>c</sup>	61.9 $\pm$ 15.5 <sup>a</sup>
Root Length	94.5 $\pm$ 3.3 <sup>b</sup>	83.9 $\pm$ 8.7 <sup>c</sup>	81.4 $\pm$ 1.4 <sup>a</sup>
Dissolved Oxygen	53.1 $\pm$ 25.5 <sup>a</sup>	18.1 $\pm$ 31.7 <sup>c</sup>	58.6 $\pm$ 8.0 <sup>a</sup>
Fresh Weight	73.7 $\pm$ 2.8 <sup>a</sup>	47.4 $\pm$ 6.8 <sup>c</sup>	30.9 $\pm$ 10.0 <sup>a</sup>
Dry to Fresh Weight Ratio	Lack of plant material	32.9 $\pm$ 2.7 <sup>c</sup>	33.2 $\pm$ 1.7 <sup>a</sup>
Membrane Integrity	0.3 $\pm$ 2.9 <sup>b</sup>	6.1 $\pm$ 1.1 <sup>e</sup>	-2.8 $\pm$ 2.6 <sup>a</sup>
Plant Area	88.5 $\pm$ 5.9 <sup>a</sup>	47.0 $\pm$ 9.8 <sup>c</sup>	23.9 $\pm$ 21.6 <sup>a</sup>
Chlorophyll <i>a</i> (fresh)	26.8 $\pm$ 14.3 <sup>b</sup>	-20.8 $\pm$ 10.1 <sup>d</sup>	-15.1 $\pm$ 9.2 <sup>a</sup>
Chlorophyll <i>b</i> (fresh)	16.3 $\pm$ 18.9 <sup>b</sup>	-29.1 $\pm$ 17.1 <sup>d</sup>	-26.1 $\pm$ 19.5 <sup>a</sup>
Carotenoid (fresh)	20.2 $\pm$ 13.2 <sup>b</sup>	-19.4 $\pm$ 9.6 <sup>d</sup>	-22.5 $\pm$ 12.8 <sup>b</sup>
Chlorophyll <i>a</i> (dry)	Not measured	-152.4 $\pm$ 35.5 <sup>c</sup>	-120.1 $\pm$ 30.7 <sup>b</sup>
Chlorophyll <i>b</i> (dry)	Not measured	-168.7 $\pm$ 38.9 <sup>c</sup>	-134.7 $\pm$ 41.8 <sup>b</sup>
Carotenoid (dry)	Not measured	-154.8 $\pm$ 38.0 <sup>c</sup>	-125.5 $\pm$ 32.4 <sup>b</sup>

- a The percent inhibition was calculated based on the mean of all ten control plants.  
b Only the mean of the five methanol control plants was utilised in the calculation of percent inhibition.  
c The percent inhibition was calculated based on the mean of five water control plants plus two methanol control plants.  
d Only the mean of two methanol control plants was utilised in the percent inhibition calculation.  
e The percent inhibition was calculated using the mean of four water controls plus two methanol controls.

Table 43: The effects of 2.99 mg/L of atrazine upon *Myriophyllum sibiricum* growth and development after two weeks of incubation. All experimental plants were grown axenically in modified Andrews' medium with 0.4% water, 0.4% reagent grade methanol or the EEC of technical atrazine.

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Increase in Node Number	Nodes/cm	Root Number	Total Root Length (mm)	D.O. (%)	Fresh Weight (mg)	Dry/Fresh Weight	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
Second Expected Environmental Concentration Experiment											
Combined Controls	8 860.9 ± 988.7 <sup>a</sup>	64.8 ± 4.1 <sup>a</sup>	14.5 ± 1.3 <sup>a</sup>	2.4 ± 0.3 <sup>a</sup>	—	—	36.7 ± 8.8 <sup>a</sup>	512.4 ± 44.5 <sup>a</sup>	11.9 ± 0.4	—	10.9 ± 1.7 <sup>a</sup>
Methanol Control	—	—	—	—	8.2 ± 0.8 <sup>a</sup>	367.6 ± 47.4 <sup>a</sup>	—	—	—	7.7 ± 0.5 <sup>a</sup>	—
Atrazine	10 500.5 ± 1 102.6 <sup>b</sup>	76.1 ± 4.5 <sup>b</sup>	7.0 ± 1.6 <sup>b</sup>	1.0 ± 0.3 <sup>b</sup>	2.6 ± 0.5 <sup>b</sup>	20.1 ± 12.1 <sup>b</sup>	17.2 ± 9.4 <sup>b</sup>	134.8 ± 14.3 <sup>b</sup>	Lack of plant material	7.8 ± 1.5 <sup>a</sup>	1.3 ± 0.6 <sup>b</sup>
Third Expected Environmental Concentration Experiment											
Combined Controls	9 868.8 ± 648.1 <sup>a</sup>	54.7 ± 13.4 <sup>a</sup>	—	2.2 ± 0.1 <sup>a</sup>	9.1 ± 0.9 <sup>a</sup>	446.5 ± 186.5 <sup>a</sup>	29.8 ± 8.6 <sup>a</sup>	773.9 ± 64.8 <sup>a</sup>	14.2 ± 0.7 <sup>a</sup>	9.4 ± 1.5 <sup>a</sup>	12.6 ± 1.3 <sup>a</sup>
Methanol Control	—	—	14.0 ± 0.0 <sup>a</sup>	—	—	—	—	—	—	—	—
Atrazine	13 124.9 ± 527.0 <sup>b</sup>	83.3 ± 3.1 <sup>b</sup>	8.0 ± 1.0 <sup>b</sup>	1.2 ± 0.1 <sup>b</sup>	3.6 ± 1.5 <sup>b</sup>	72.0 ± 38.8 <sup>b</sup>	24.4 ± 9.4 <sup>a</sup>	406.9 ± 52.5 <sup>b</sup>	9.5 ± 0.4 <sup>b</sup>	12.4 ± 0.5 <sup>b</sup>	6.7 ± 1.2 <sup>b</sup>
Fourth Expected Environmental Concentration Experiment											
Combined Controls	9 597.6 ± 588.7 <sup>a</sup>	58.0 ± 3.5 <sup>a</sup>	—	—	8.4 ± 1.2 <sup>a</sup>	434.7 ± 102.6 <sup>a</sup>	67.6 ± 16.2 <sup>a</sup>	783.3 ± 85.9 <sup>a</sup>	13.6 ± 0.6 <sup>a</sup>	8.7 ± 0.9 <sup>a</sup>	12.7 ± 0.8 <sup>a</sup>
Methanol Control	—	—	12.6 ± 1.1 <sup>a</sup>	2.0 ± 0.2 <sup>a</sup>	—	—	—	—	—	—	—
Atrazine	14 011.2 ± 1 325.0 <sup>b</sup>	95.3 ± 5.3 <sup>b</sup>	9.4 ± 0.5 <sup>b</sup>	1.4 ± 0.1 <sup>b</sup>	3.2 ± 1.3 <sup>b</sup>	81.0 ± 6.1 <sup>b</sup>	28.0 ± 5.4 <sup>b</sup>	541.4 ± 78.4 <sup>b</sup>	9.1 ± 0.2 <sup>b</sup>	7.3 ± 1.3 <sup>a</sup>	9.6 ± 2.7 <sup>a</sup>

a, b For each EEC experiment, any means ± s.d. in the same column with the same superscript were not significant different at  $\alpha = 0.05$ . If there was no significant difference between the water and methanol controls, the control data were combined. If there was a significant difference between the control data ( $\alpha = 0.05$ ), the atrazine treatment data were compared with just the methanol control data. For the second and fourth experiments, the combined control consisted of 10 data values and 5 replicates in the atrazine treatments. For the third EEC experiment, 3 methanol control plants were lost due to contamination. Therefore, the combined control consisted of 7 data values, the methanol control consisted of 2 values and the atrazine treatment of 5 plants. For membrane integrity, several plants were lost in the boiling water and thus  $n = 6$  for the combined control and  $n = 2$  for the atrazine treatment.

Table 44: After fourteen days of incubation in sterile modified Andrews' medium, the pigment content of <i>Myriophyllum sibiricum</i> apices treated with the EEC of atrazine (2.99 mg/L) was significantly different from the content in the control apices.						
Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
<b>Second Expected Environmental Concentration Experiment</b>						
Combined Controls	—	—	—	Not Measured	Not Measured	Not Measured
Methanol Control	0.45 ± 0.02 <sup>a</sup>	0.15 ± 0.006 <sup>a</sup>	0.16 ± 0.007 <sup>a</sup>	Not Measured	Not Measured	Not Measured
Atrazine	0.33 ± 0.06 <sup>b</sup>	0.13 ± 0.03 <sup>a</sup>	0.13 ± 0.02 <sup>b</sup>	Not Measured	Not Measured	Not Measured
<b>Third Expected Environmental Concentration Experiment</b>						
Combined Controls	—	—	—	7.3 ± 1.0 <sup>a</sup>	2.8 ± 0.4 <sup>a</sup>	2.4 ± 0.3 <sup>a</sup>
Methanol Control	0.51 ± 0.02 <sup>a</sup>	0.20 ± 0.001 <sup>a</sup>	0.17 ± 0.005 <sup>a</sup>	—	—	—
Atrazine	0.61 ± 0.05 <sup>b</sup>	0.25 ± 0.03 <sup>b</sup>	0.20 ± 0.02 <sup>b</sup>	18.4 ± 2.6 <sup>b</sup>	7.6 ± 1.1 <sup>b</sup>	6.1 ± 0.9 <sup>b</sup>
<b>Fourth Expected Environmental Concentration Experiment</b>						
Combined Controls	0.55 ± 0.04 <sup>a</sup>	0.19 ± 0.02 <sup>a</sup>	—	—	—	—
Methanol Control	—	—	0.18 ± 0.01 <sup>a</sup>	7.4 ± 0.7 <sup>a</sup>	2.6 ± 0.3 <sup>a</sup>	2.5 ± 0.2 <sup>a</sup>
Atrazine	0.63 ± 0.05 <sup>b</sup>	0.24 ± 0.04 <sup>b</sup>	0.22 ± 0.02 <sup>b</sup>	16.2 ± 2.3 <sup>b</sup>	6.1 ± 1.1 <sup>b</sup>	5.6 ± 0.8 <sup>b</sup>

a,b For each EEC experiment, any two means in the same column with the same superscript were not significant different at  $\alpha = 0.05$ , as determined by either the nonparametric Mann-Whitney U-test or the parametric t-test. If there was no significant difference between the methanol and water control ( $\alpha = 0.05$ ), the data were combined for statistical analysis and presentation in this table. If there was a statistically significant difference between the methanol and the water control data ( $\alpha = 0.05$ ), just the methanol control values were used for statistical comparison with the atrazine treatment data and for presentation in this table. For experiments two and four,  $n = 5$  for the atrazine treatment and the methanol controls and  $n = 10$  for the combined water and methanol controls. For the third experiment,  $n = 2$  for the methanol controls,  $n = 7$  for the combined control values and  $n = 5$  for the atrazine treatment. The data presented are the untransformed mean  $\pm$  s.d.

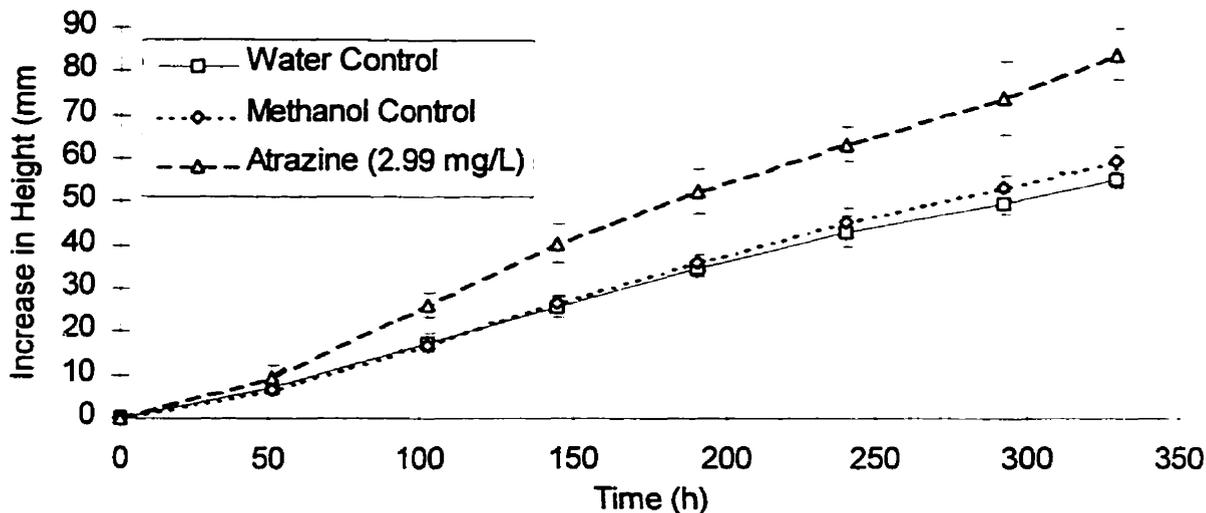


Figure 51: The effect of 2.99 mg/L of atrazine upon *Myriophyllum sibiricum* shoot growth in axenic culture, over fourteen days of exposure. The plants used in this fourth experiment were cultured in modified Andrews' medium before treatment with water, methanol or atrazine spiked modified Andrews' medium. Each data point is the mean of five replicates  $\pm$  the standard deviation.

per plant was also significantly less for the atrazine treated plants than for the control plants, which indicated that the plants treated with atrazine were disproportionately elongated. Membrane integrity was not severely affected by atrazine (Table 42 and Table 43). For *M. sibiricum* plants cultured in M & S medium prior to experimentation, the pigment content in the plant apices was significantly less than the amount in the control apices, but for plants cultured in modified Andrews' medium prior to experimentation, the amount was significantly greater than the pigment concentration extracted from control apices (fresh and dry weight) (Table 44). With triazine herbicide treatment, other experiments have demonstrated an increase in chlorophyll content (Shehata *et al.*, 1993; Cunningham *et al.*, 1984), shoot growth (Ries *et al.*, 1963), shoot to root ratio (Ries and Gast, 1965), nitrogen content (Gramlich and Davis, 1967; Ries and Gast, 1965; Ries *et al.*, 1963) and protein content (Ries *et al.*, 1967). Ries (1976) reviewed the scientific literature and summarised the stimulatory effects of herbicides on plants.

#### 5.7.1.3.2 Dilution Series

Sixteen out of the forty-five experimental plants were lost from the first atrazine dilution series. Based on the results from the first dilution experiment, a narrower range of

concentrations was tested in the second dilution experiment. Only the results from the second experiment are presented in this section. Plant height and area under the growth curve for plants treated with atrazine were stimulated as compared to the control plants (Figure 52, Figure 53 and Table 45). This stimulation was greater than 20% for all treatments so SC20 values could not be calculated (Section 5.6). The increase in height did not correspond to an increase in node number or an increase in weight. In fact, at all atrazine concentrations tested the number of nodes per centimetre of plant length was reduced compared to the controls (Table 45). At atrazine concentrations greater than the NOEC, pigment content of the apices was stimulated. For pigment concentration based on dry weight, twenty percent stimulation in pigment content occurred at atrazine concentrations between 650 and 740  $\mu\text{g}$  (Table 45).

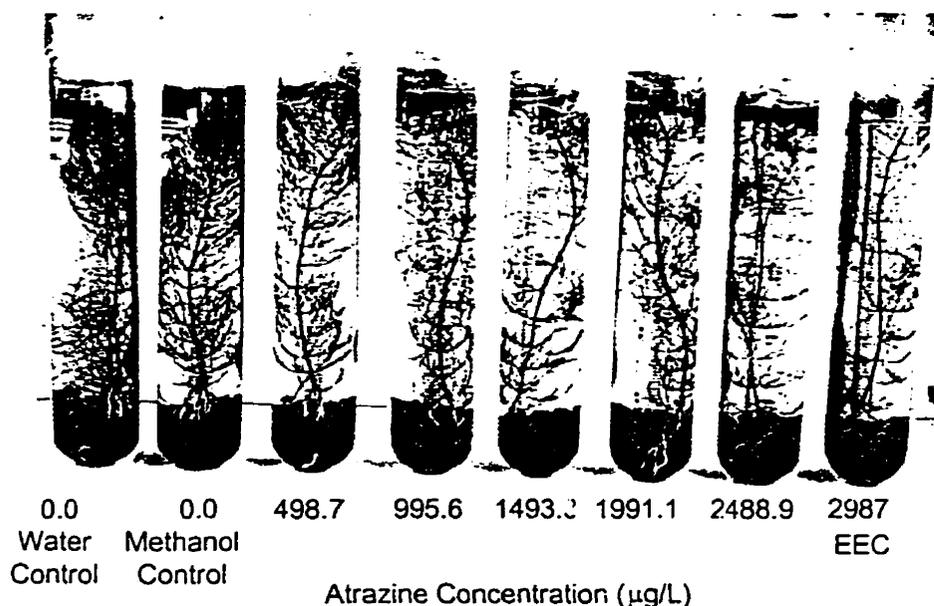


Figure 52: Atrazine, tested at six concentrations, stimulated the growth of *Myriophyllum sibiricum* in axenic culture. The technical atrazine was dissolved in methanol before being added to the modified Andrews' medium. Both water (0.4%) and methanol (0.4%) controls were used.

It is not unusual for phytotoxic compounds to stimulate plant growth at low concentrations. This stimulation of growth (sometimes called hormesis or homologysis (Ries, 1976)) has also been observed in duckweed testing with effluents (Wang, 1991). Algal growth has been stimulated by various herbicides (Cain and Cain, 1983). With two triazine herbicides, chlorophyll a content of algal cells was stimulated at low herbicide concentrations. For one of these herbicides, chlorophyll a content was stimulated by

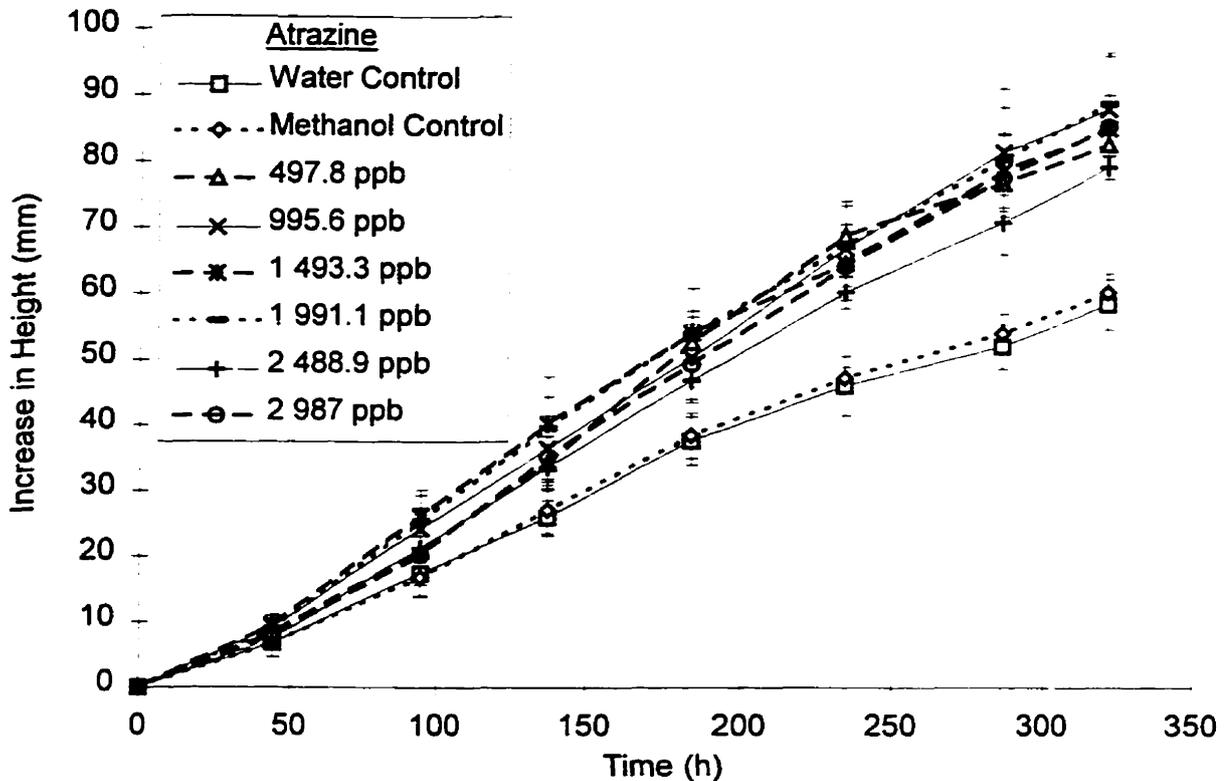


Figure 53: The effect of six concentrations of technical atrazine upon shoot growth of *Myriophyllum sibiricum*. Three centimetre apical segments were exposed, in modified Andrews' medium, to atrazine dissolved in methanol, 0.4% methanol or 0.4% water for fourteen days.

160.87% when compared to the control level (Shehata *et al.*, 1993). Chlorophyll a content of *Potamogeton perfoliatus* apical leaves increased with atrazine treatments (Cunningham *et al.*, 1984). There was stimulation in oxygen production by *Myriophyllum spicatum* at 5 µg/L atrazine (Kemp *et al.*, 1985), the net photosynthesis of *Elodea canadensis* and *M. spicatum* increased at 10 µg/L (Hofmann and Winkler, 1990) and net daily oxygen production of *Potamogeton pectinatus* and *Zostera marina* L. (eelgrass) was stimulated at 75 µg/L (Correll and Wu, 1982). Atrazine stimulated growth in other aquatic macrophyte species, such as *M. spicatum* (Kemp *et al.*, 1985) and *Potamogeton perfoliatus*. With *P. perfoliatus*, the plants increased in height but there was a decrease in weight per unit length. A significant increase (38%) in internodal length was observed at the high atrazine dose (1.2 mg/L) (Cunningham *et al.*, 1984). After 21 days of exposure, shoot length and number of leaves of *Zostera marina* increased at an atrazine concentration of 1 µg/L (Delistraty and Hershner, 1984).

Table 45: The effect of technical atrazine upon *Myriophyllum sibiricum* growth and development endpoints was determined using six concentrations (497.8, 995.6, 1493.3, 1991.1, 2488.9, 2987 µg/L) plus water and methanol controls. The data are presented as the no observable effect concentration, the lowest observed effect concentration, the concentration that inhibited growth by 25 and 50% (upper and lower 95% c.i.) and the concentration that stimulated growth by 20%.

GROWTH PARAMETER	NOEC (µg/L)	LOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	SC20 (µg/L)
Area Under the Growth Curve <sup>a</sup>	Stimulated	Stimulated	Stimulated	Stimulated	Stimulated <sup>e</sup>
Increase in Shoot Length <sup>a</sup>	Stimulated	Stimulated	Stimulated	Stimulated	Stimulated <sup>e</sup>
Increase in Node Number <sup>a</sup>	1 493.3	1 991.1	2 342.3 (1 850.8, 2 964.4) <sup>c</sup>	> 2 987 <sup>a,b</sup>	—
Nodes per Centimetre <sup>a</sup>	All Different	497.8	437.7 (275.2, 696.2) <sup>c</sup>	2 065.8 (1 660.7, 2 569.7) <sup>c</sup>	—
Root Length <sup>a</sup>	497.8	995.6	601.7 (355.8, 1 017.8) <sup>d</sup>	1 130.0 (846.1, 1 509.1) <sup>d</sup>	—
Root Number <sup>a</sup>	995.6	1 493.3	1069.9 (467.4, 2 450.5) <sup>c</sup>	2 118.6 (1 266.7, 3 543.3) <sup>c</sup>	—
Fresh Weight <sup>b</sup>	995.6	1 493.3	1 605.7 (1 083.1, 2 380.9) <sup>c</sup>	> 2 987 <sup>a,b</sup>	—
Dry to Fresh Weight Ratio <sup>b</sup>	497.8	995.6	1293.2 (720.3, 2323.2) <sup>d</sup>	> 2 987 <sup>a,b</sup>	—
Dissolved Oxygen <sup>a</sup>	497.8	995.6	606.4 (317.0, 1 160.7) <sup>c</sup>	1 999.4 (1 438.5, 2 778.8) <sup>c</sup>	—
Membrane Integrity <sup>a</sup>	497.8	995.6 (only)	No Inhibition	No Inhibition	—
Plant Area <sup>a</sup>	1 493.3	1991.1	1 687.0 (1 381.4, 2 060.2) <sup>c</sup>	2 679.4 (2 292.7, 3 131.3) <sup>d</sup>	—
Chlorophyll a (fresh wt) <sup>b</sup>	1 991.1	2 488.9 (only)	Stimulated	Stimulated	2 394.2
Chlorophyll b (fresh wt) <sup>b</sup>	1 493.3	1 991.1	Stimulated	Stimulated	1 898.6
Carotenoid (fresh wt) <sup>b</sup>	995.6	1 493.3	Stimulated	Stimulated	2 456.5
Chlorophyll a (dry wt) <sup>a</sup>	497.8	995.6	Stimulated	Stimulated	738.4
Chlorophyll b (dry wt) <sup>a</sup>	497.8	995.6	Stimulated	Stimulated	650.3
Carotenoid (dry wt) <sup>a</sup>	497.8	995.6	Stimulated	Stimulated	657.7

a The calculations were conducted using combined water and methanol control data.

b The calculations were based on just the methanol control data.

c ICp values and 95% confidence intervals were calculated with the cumulative normal distribution model.

d ICp and 95% confidence values were calculated with the weibull method.

e Stimulation was greater than 20% at all concentrations.

Inhibition was observed for the other endpoint parameters (Table 45). The number of nodes per centimetre, total root length and dissolved oxygen concentration were inhibited by 25% at atrazine concentrations of 437.7 µg/L (c.i. = 275.2 - 696.2 µg/L), 601.7 µg/L (c.i. = 355.8 - 1 017.8 µg/L) and 606.4 µg/L (317.0 - 1 160.7 µg/L), respectively. Oxygen production was also inhibited in the controlled field studies that examined the effect of atrazine on freshwater algae (deNoyelles *et al.*, 1982; Hamilton *et al.*, 1987; Lay *et al.*, 1984). Oxygen evolution of *Thalassia testudinum* Konig, a seagrass, was depressed by 1 mg/L atrazine after 40 hours of exposure (Walsh *et al.*, 1982). After 6 hours of exposure, net productivity of *Zostera marina* was inhibited by 100 µg/L (Delistraty and Hershner, 1984).

Atrazine was not very toxic to *Myriophyllum sibiricum* in axenic culture. The lack of sensitivity of this toxicity test to atrazine may be due to some type of resistance, a solvent and pesticide interaction, the presence of an exogenous carbon source or the fact that the testing was conducted in the laboratory.

The *Myriophyllum sibiricum* plants used in this study were obtained from a lake in southern Ontario (Puslinch Lake), which probably had been contaminated with atrazine. Previous exposure of the population to atrazine may have influenced the results obtained in this study by giving the plants a resistance to atrazine. Other *Myriophyllum* populations without previous exposure to atrazine may have behaved differently. Field exposure to chemicals has produced erroneous results in other cases (Martin, 1973). The field collected native aquatic plant species studied by Powell *et al.* (1996) were less sensitive to the toxicant than domestic rice. They suggested that the exposure to the toxicant at sub-lethal concentrations may have evolved a genetic tolerance to the contaminants. Evolution of tolerance in aquatic macrophytes was discussed further by Lovett-Doust *et al.* (1994b).

Resistance has been shown to develop in algal populations exposed to atrazine in experimental ponds. At 500 µg/L, algal species composition changed between Day 7 and Day 15 with resistant species becoming more dominant. *Cryptomonas marssonii* Skija was removed from ponds that had been treated with 500 µg/L atrazine, cultured in the laboratory without atrazine for several months and was resistant to 500 µg/L atrazine. *Oocystis* sp. isolated from control ponds was completely inhibited by 500 µg/L (deNoyelles *et al.*, 1982). Addition of atrazine to ponds that were previously treated with

100 µg/L showed no immediate decline in <sup>14</sup>C uptake, which indicated that the phytoplankton communities contained resistant species (deNoyelles and Kettle, 1985). Resistance or partial resistance to atrazine was indicated in studies on lake periphyton (Hamilton *et al.*, 1987). A laboratory resistant strain of *Chlamydomonas reinhardtii* and *Chlorella* sp. collected from an atrazine contaminated spring were resistant to atrazine levels of 216 µg/L (Hersh and Crumpton, 1987). The mechanism of resistance in atrazine resistant *Chlamydomonas reinhardtii* appeared to be a modified binding site that has a lesser affinity for atrazine (Hersh and Crumpton, 1989). Alternately, other researchers (Kosinski, 1984; Kosinski and Merkle, 1984) have found no evidence of atrazine resistance in benthic algal species from an agriculturally impacted stream. They were unable to induce resistance by pre-exposing algal populations to 0.01 mg/kg atrazine (Aatrex® 80 WP) for 3 to 4 weeks prior to atrazine exposure (0.1, 1 and 10 mg/kg a.i.) as measured by productivity (Kosinski and Merkle, 1984) and species composition (Kosinski, 1984). An aquatic moss species, *Fontinalis antipyretica* Hedw., demonstrated resistance to formulated atrazine due to binding of the herbicide and morphological changes of the chloroplasts (Hofmann and Winkler, 1990). By 7 weeks post-treatment, recovery of apparent photosynthesis occurred with low doses of atrazine in microcosms containing *Potamogeton perfoliatus*. Detoxification of atrazine was postulated as a possible explanation for the recovery (Cunningham *et al.*, 1984).

The *M. sibiricum* used in this study may have been able to metabolise the herbicide. Numerous terrestrial plant species have been shown to metabolise atrazine (De Prado *et al.*, 1995; Jensen *et al.*, 1977a; 1977b; Shimabukuro, 1967; Thompson, 1972a; 1972b). Other terrestrial plants species are resistant to atrazine due to altered binding sites at the photosystem II complex in the chloroplasts (De Prado *et al.*, 1995; Souza Machado *et al.*, 1978). The exact mechanism of possible resistance in *M. sibiricum* was not determined in this study.

A solvent-pesticide interaction might have reduced the atrazine toxicity. Other researchers (Stratton, 1989; Stratton *et al.*, 1982; Stratton and Smith, 1988) have found that there can be a synergistic, antagonistic and additive effect between solvent carriers and pesticides. For example, Stratton and Smith (1988) determined that acetone and atrazine interacted antagonistically at acetone levels greater than 4% and at atrazine concentrations between 0.1 and 0.3 mg/L. Lower acetone and atrazine concentrations produced an additive toxic effect on the growth of *Chlorella pyrenoidosa* (Stratton and

Smith, 1988). For three species of *Anabaena*, acetone interacted with atrazine synergistically, antagonistically and additively depending upon the solvent and pesticide concentrations and the species (Stratton and Corke, 1981a). During tests with *Scenedesmus quadricauda*, acetone at 0.1% and 0.2% interacted additively with atrazine but above 0.2%, acetone interacted synergistically. With *Chlorella pyrenoidosa*, an additive response was obtained between atrazine and 0.1, 0.2 and 0.6% acetone. Antagonism was seen at 0.4% and synergism at 0.8 and 1.0% acetone (Stratton and Corke, 1981b). Ethanol and atrazine also interacted antagonistically at solvent levels greater than 0.1% for most atrazine concentrations tested (Stratton and Smith, 1988). If there was a methanol-atrazine interaction occurring in the current study with *M. sibiricum*, it would appear to be an antagonistic interaction. No conclusion regarding a possible pesticide-solvent interaction can be made at this point without further research.

Compounds in the medium that the plant utilised as external carbon sources may have promoted the growth of *M. sibiricum*. The stimulation in growth of *Myriophyllum sibiricum* caused by atrazine may be due to the plants using the atrazine as a carbon source or an increase in nitrate reductase activity (Ries *et al.*, 1967). Many organisms adapt their metabolic processes to utilise herbicides as a source of carbon (Averitt and Gangstad, 1976). The axenic toxicity test system was heterotrophic. The presence of 3% sucrose and the high nutrient content of the experimental medium might have allowed the atrazine treated plants to temporarily circumvent photosynthesis. Unfortunately, this cannot be tested because the *M. sibiricum* did not grow in axenic culture without the addition of an external carbon source. Hexazinone, another photosynthetic inhibitor, was more toxic than the atrazine to the *M. sibiricum* in this axenic toxicity test system, which suggested that the sucrose was not influencing the phytotoxicity results (Section 5.7.6). In studies with *Chlamydomonas reinhardtii* comparing heterotrophic to autotrophic medium, the toxicity of atrazine was reduced in the presence of acetate as an external carbon source (Loeppky and Tweedy, 1969). Stimulation in *Potamogeton pectinatus* biomass was detected in a heterotrophic system as compared to an autotrophic system (Fleming *et al.*, 1991). Glucose decreased the toxicity of atrazine to cell production and chlorophyll content of *Chlorella vulgaris* (Ashton *et al.*, 1966). The presence of glucose in the medium reduced the toxic effects of several growth inhibiting herbicides to *C. vulgaris* var. *viridis* (Geoghegan, 1957).

Some research has demonstrated that results collected from field experiments can differ from laboratory experiments. The fact that the testing was conducted in the laboratory may have influenced the toxicity of atrazine. Red pine (*Pinus resinosa* Ait.) field sensitivity was greater than laboratory sensitivity to atrazine (3.22 times more sensitive) but sensitivity was just about equal between the lab and field EC50s for pigweed (*Amaranthus* spp.) (1.07 times more sensitive in the field) (Fletcher *et al.*, 1990). Further research should be conducted to determine which of the above mechanisms affected the toxicity of atrazine to *M. sibiricum*.

For the protection of aquatic life, it was recommended that the maximum concentration of atrazine in marine waters not exceed 0.001 mg/L (NAS/NAE, 1973 as cited in Nowell and Resek, 1994). Based on the results from this toxicity test, a guideline of 0.05 mg/L should be established to protect freshwater aquatic macrophytes. However, *M. sibiricum* may not be a highly sensitive species to the effects of atrazine and therefore this level will probably not be sufficient to protect more sensitive species of algae and macrophytes.

#### 5.7.1.3.3 Aquatic Plant Toxicity Comparisons

The scientific literature contains an enormous amount of data on the effects of atrazine upon algae. Jurgensen and Hoagland (1990), Stratton (1987b) and Solomon *et al.* (1996) summarised these effects. Specific examples are presented and summarised in Table 46. Photosynthetic laboratory bioassays of several algal species yielded a wide range of tolerance to atrazine concentrations. Based on <sup>14</sup>C uptake after 24 hours, *Chlamydomonas reinhardtii* was the most sensitive species (EC50 = 19 µg/L; c.i. = 10 to 28 µg/L) while *Chlorella vulgaris* appeared to be the least sensitive (EC50 = 325 µg/L; c.i. = 298 to 357 µg/L) (Table 46). The more sensitive species would be affected by concentrations found in ground and surface waters, especially after a heavy runoff event (Larsen *et al.*, 1986). The effect of technical atrazine upon photosynthesis of five algal species was examined by Stratton (1981) and it was determined that *Anabaena variabilis* Keutzing was the most sensitive species with an EC50 of 0.07 ± 0.02 mg/L and *Chlorella pyrenoidosa* was the most resistant species with an EC50 of 0.48 ± 0.2 mg/L. For growth yield, *A. inaequalis* was the most sensitive species with an EC50 of 0.03 ± 0.01 mg/L and *A. variabilis* was the least sensitive with an EC50 of 4.0 ± 1.0 mg/L (Stratton, 1981) (Table 46).

When exposed to technical atrazine, *Selenastrum capricornutum* displayed an EC50 of 0.05 mg/L (95% c.i. = 0.042 - 0.067 mg/L), 0.10 mg/L (95% c.i. = 0.059 - 0.160 mg/L) and 0.38 mg/L (95% c.i. not given) as determined in a 4 day population growth test, a 30 minute carbon dioxide fixation test and a 30 minute oxygen generation test, respectively (Table 46) (Versteeg, 1990). In a comparison of static and medium renewal microplate tests with *S. capricornutum*, 72 hour EC50s of  $187.3 \pm 20.9 \mu\text{g/L}$  and  $118.2 \pm 10.7 \mu\text{g/L}$ , respectively, were obtained (Table 46) (Radetski *et al.*, 1995). The toxicity of atrazine to *S. capricornutum* chlorophyll *a* content increased over time. The EC50 was  $283 \mu\text{g/L}$  after 3 days of exposure,  $218 \mu\text{g/L}$  after 5 days of exposure and  $214 \mu\text{g/L}$  after 7 days of incubation (Table 46) (Abou-Waly *et al.*, 1991b).

Four species of marine unicellular algae (*Chlorococcum* sp., *Dunaliella tertiolecta* Butcher, *Isochrysis galbana* Parke and *Phaeodactylum tricornutum* Bohlin) were exposed to technical and formulated atrazine. Oxygen evolution was determined after 90 min of exposure and growth was measured after 10 days of exposure. Growth and oxygen evolution were equally influence by technical atrazine because 0.1 mg/L of technical atrazine inhibited these parameters by 50% in *Chlorococcum* sp. and *I. galbana*. For formulated atrazine (80% W.P.), growth in these two species was inhibited to the 50% level by 0.1 mg/L. Oxygen evolution was not affected as much by formulated atrazine because 0.2 mg/L was required to inhibit oxygen evolution by 50% in *I. galbana* and *P. tricornutum* (Walsh, 1972). The effect of atrazine on oxygen production by 18 species of marine algae was determined with a range in toxicity from  $60 \mu\text{g/L}$  for *Chlamydomonas* sp. to  $460 \mu\text{g/L}$  for *Navicula inserata* F. Hustedt (Hollister and Walsh, 1973).

Another set of photosynthetic bioassays was conducted on algal species removed from a non-contaminated spring and an atrazine contaminated spring that contained year-round atrazine levels between 1.0 to  $5.4 \mu\text{g/L}$ . Algal species collected from these springs were compared with susceptible and atrazine resistant laboratory strains of *Chlamydomonas reinhardtii*. *Chlorella* sp. from the contaminated spring, the non-contaminated spring and the susceptible *Chlamydomonas reinhardtii* exhibited EC50 values that demonstrated a low susceptibility to atrazine ( $42 \mu\text{g/L}$  (c.i. =  $37 - 51 \mu\text{g/L}$ ),  $41 \mu\text{g/L}$  (c.i. =  $26 - 66 \mu\text{g/L}$ ) and  $45 \mu\text{g/L}$  (c.i. =  $37 - 57 \mu\text{g/L}$ ), respectively). The laboratory cultured resistant strain of *Chlamydomonas reinhardtii* demonstrated much

Table 46: Selected from the scientific literature, a summary of the effects of atrazine on various algal species. The values are presented as either EC50 or IC50 values. Test conditions varied between species but general trends in sensitivity can be observed. Sensitivity of the different algal species to this herbicide varied by almost 300 times. Different endpoint measured on the same species varied by almost a factor of 60.

Algal Species	Endpoint	Exposure Duration	EC50/IC50	Reference
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	3 days	58 µg/L	Abou-Waly et al. (1991b)
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	5 days	469 µg/L	Abou-Waly et al. (1991b)
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	7 days	766 µg/L	Abou-Waly et al. (1991b)
<i>Anabaena flos-aquae</i>	Cell Number	5 days	230 µg/L	Hughes et al. (1988)
<i>Anabaena inaequalis</i>	Growth Yield	12 days	30 µg/L	Stratton (1981)
<i>Anabaena variabilis</i>	Growth Yield	12 days	4 000 µg/L	Stratton (1981)
<i>Anabaena variabilis</i>	Photosynthesis	3 hours	70 µg/L	Stratton (1981)
<i>Chlorella pyrenoidosa</i>	Photosynthesis	3 hours	480 µg/L	Stratton (1981)
<i>Chlorella pyrenoidosa</i>	Growth	4 days	60 µg/L	Maule and Wright (1984)
<i>Chlorella vulgaris</i>	<sup>14</sup> C Uptake	24 hours	325 µg/L	Larsen et al. (1986)
<i>Chlorella sp.</i>	Oxygen Evolution	12 hours light + 3 days dark	143 µg/L	Hollister and Walsh (1973)
<i>Chlamydomonas reinhardtii</i>	<sup>14</sup> C Uptake	24 hours	19 µg/L	Larsen et al. (1986)
<i>Chlamydomonas sp.</i>	Oxygen Evolution	12 hours light + 3 days dark	60 µg/L	Hollister and Walsh (1973)
<i>Gloecapsa alpicola</i>	Growth	4 days	5 360 µg/L	Maule and Wright (1984)
<i>Navicula inserta</i>	Oxygen Evolution	12 hours light + 3 days dark	460 µg/L	Hollister and Walsh (1973)
<i>Selenastrum capricornutum</i>	Growth	4 days	50 µg/L	Versteeg (1990)
<i>Selenastrum capricornutum</i>	Oxygen Generation	30 min	380 µg/L	Versteeg (1990)
<i>Selenastrum capricornutum</i>	Carbon Dioxide Fixation	30 min	100 µg/L	Versteeg (1990)
<i>Selenastrum capricornutum</i>	Cell Density (static microplate)	72 hours	187.3 µg/L	Radetski et al. (1995)
<i>Selenastrum capricornutum</i>	Cell Density (semistatic microplate)	72 hours	118.2 µg/L	Radetski et al. (1995)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	3 days	283 µg/L	Abou-Waly et al. (1991b)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	5 days	218 µg/L	Abou-Waly et al. (1991b)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	7 days	214 µg/L	Abou-Waly et al. (1991b)

higher levels of atrazine tolerance (EC50 = 484 µg/L (c.i. = 365 - 660 µg/L)). *Franceia* sp. collected from the contaminated spring showed an atrazine tolerance (EC50 = 720 µg/L (c.i. = 565 - 925 µg/L)) that was even greater than the laboratory produced resistant strain of *C. reinhardii* (Hersh and Crumpton, 1989).

Kratky and Warren (1971) examined the effects of atrazine upon the green alga, *Chlorella pyrenoidosa*, by monitoring chlorophyll and oxygen production. Identical IC50 values (0.08 mg/L) were determined by both methods (Kratky and Warren, 1971). After examining atrazine toxicity to thirteen species of algae, it was determined that *Chlorella pyrenoidosa* was the most sensitive species with an EC50 for chlorophyll *a* content of 0.06 mg/L. *Gloecapsa alpicola* (Lyng.) Bornet. was the least sensitive species with an EC50 of 5.36 mg/L (Table 46). There was no evidence of cell lysis in any of the algal species tested (Maule and Wright, 1984). The exponential growth of *Anabaena variabilis* was reduced to 50% of the control by an atrazine concentration of 0.1 mg/L and growth was completely inhibited at 0.26 mg/L atrazine. Growth of one strain of *Aphanocapsa* was completely inhibited by atrazine concentrations equal to and greater than 2.6 mg/L (Hutber *et al.*, 1979) and exhibited a growth rate equivalent to curve 2 in Figure 4.

Stratton (1984) tested the effect of atrazine upon photosynthesis and growth of five algal species. The effect on photosynthesis varied between species with EC50s ranging from 0.1 µg/mL for *Anabaena variabilis*, 0.3 µg/mL for *A. inaequalis* and *Scenedesmus quadricauda*, and 0.5 µg/mL for *A. cylindrica* and *Chlorella pyrenoidosa*. The range in interspecies variation for cell number and growth rate was more extreme. EC50s for cell number were 0.03 µg/mL for *A. inaequalis*, 0.1 µg/mL for *S. quadricauda*, 0.3 µg/mL for *C. pyrenoidosa*, 1.2 µg/mL for *A. cylindrica* and 4.0 µg/mL for *A. variabilis*. Growth rate was affected by 50% at rates of 0.1 µg/mL for *A. inaequalis*, 0.2 µg/mL for *S. quadricauda*, 1.0 µg/mL for *C. pyrenoidosa*, 3.6 µg/mL for *A. cylindrica* and 5.0 µg/mL for *A. variabilis* (Stratton, 1984). After 3 days of exposure, atrazine affected the chlorophyll *a* content of *Anabaena flos-aquae* at low concentrations (58 µg/L) but as the exposure duration increased the toxicity decreased. After 5 days of exposure, the EC50 was 469 µg/L and after 7 days of exposure, the EC50 was 766 µg/L (Table 46) (Abou-Waly *et al.*, 1991b).

In artificial stream communities, atrazine at both 1 and 10 mg/kg caused inhibition of photosynthesis (Kosinski and Merkle, 1984) and altered species composition (Kosinski 1984). In limnocorral studies with periphyton, net productivity, cell numbers, biomass, number of taxa and chlorophyll a declined with atrazine treatments of 1.56, 0.14 and 0.08 mg/L (Hamilton *et al.*, 1987).

The above studies demonstrate that algal species differ in susceptibility to atrazine. The toxicity of atrazine to algal species within the same genus can vary by more than a factor of 100. Some algal species would be affected by atrazine concentrations that could occur after a heavy runoff event shortly after atrazine application. *Myriophyllum sibiricum* was more sensitive to atrazine compared with some of the tolerant algal species but *M. sibiricum* was twenty times more tolerant of atrazine when compared with the most sensitive algal species.

In studies by Hughes *et al.* (1988), atrazine inoculated cultures of *Anabaena flos-aquae*, *Navicula pelliculosa*, *Dunaliella tertiolecta* and *Lemna gibba* were maintained for 5 days. The EC50 values for cell count were 0.06 mg/L (95% c.i. = 0.002 - 0.21 mg/L) for *Navicula pelliculosa*, 0.17 mg/L (95% c.i. = 0.11 - 0.26 mg/L) and 0.23 mg/L (95% c.i. = 0.12 - 0.38 mg/L) for *Anabaena flos-aquae* (Table 46). For *L. gibba* frond count, the EC50 was 0.17 mg/L (95% c.i. = 0.13 - 0.23) (Table 47). The NOEC was less than 0.1 mg/L for all four species (Hughes *et al.*, 1988).

Supplied as NaH<sup>14</sup>CO<sub>3</sub>, uptake of <sup>14</sup>C was used as an endpoint to determine that atrazine at 2.667 mg/L inhibited eight out of nine algal species (*Cyclotella meneghiana* Kütz., *Nitzschia* sp., *Scenedesmus quadricauda*, *Selenastrum capricornutum*, *Microcystis aeruginosa*, *Oscillatoria* sp., *Pseudoanabaena* sp., *Aphanizomenon flos-aquae* (L.) Ralfs) by greater than 87% when compared to untreated controls. *Anabaena inaequalis* was inhibited by only 65%. Over a 7 day exposure period, *Lemna minor* was severely inhibited by this concentration of atrazine and showed a 95% reduction in frond number when compared to the controls (Peterson *et al.*, 1994). In several static *Lemna minor* tests, with frond count as the endpoint, an IC50 of > 0.1 mg/L was obtained for atrazine (Wang, 1990a). Fifty percent inhibition of the total leaf surface area of *Lemna paucicostata* occurred at a technical atrazine concentration of 3.2·10<sup>-7</sup> M (69 µg/L) after 10 days of incubation (Table 47) (Retzlaff, 1992).

Table 47: A summary of the effects of atrazine on the growth and development of floating, emergent and submersed macrophytes. Test conditions varied for the different species but general trends in sensitivity can be observed.

Macrophyte Species	Endpoint	Exposure Duration	EC50/IC50 (µg/L)	Reference
<i>Elodea canadensis</i>	Length	3 weeks	109	Forney and Davis (1981)
<i>Elodea canadensis</i>	Length	4 weeks	80	Forney and Davis (1981)
<i>Lemna gibba</i>	Frond Count	5 days	170	Hughes <i>et al.</i> (1988)
<i>Lemna paucicostata</i>	Leaf Surface Area	10 days	69	Retzlaff (1992)
<i>Potamogeton perfoliatus</i>	Oxygen Production	4 weeks	55	Kemp <i>et al.</i> (1985)
<i>Potamogeton perfoliatus</i>	Stem Dry Weight	4 weeks	30	Kemp <i>et al.</i> (1985)
<i>Potamogeton perfoliatus</i>	Photosynthesis	24 hours	80	Jones <i>et al.</i> (1986)
<i>Potamogeton perfoliatus</i>	Photosynthesis	24 hours	77	Jones and Winchell (1984)
<i>Potamogeton perfoliatus</i>	Length	3 weeks	474	Forney and Davis (1981)
<i>Potamogeton perfoliatus</i>	Dry Weight	3 weeks	907	Forney and Davis (1981)
<i>Potamogeton perfoliatus</i>	Percent Kill	3 weeks	53	Forney and Davis (1981)
<i>Ruppia maritima</i>	Photosynthesis	24 hours	102	Jones and Winchell (1984)
<i>Vallisneria americana</i>	Leaf Length	6 weeks	532	Forney and Davis (1981)
<i>Zannichellia palustris</i>	Photosynthesis	24 hours	91	Jones and Winchell (1984)
<i>Myriophyllum spicatum</i>	Oxygen Production	4 weeks	117	Kemp <i>et al.</i> (1985)
<i>Myriophyllum spicatum</i>	Stem Dry Weight	4 weeks	91	Kemp <i>et al.</i> (1985)
<i>Myriophyllum spicatum</i>	Photosynthesis	24 hours	104	Jones and Winchell (1984)
<i>Myriophyllum spicatum</i>	Length	4 weeks	1 104	Forney and Davis (1981)
<i>Myriophyllum spicatum</i>	Branch Number	5 days	3 700	Bird (1993)
<i>Myriophyllum sibiricum</i>	Root Length	14 days	1 130.0	This Document
<i>Myriophyllum sibiricum</i>	Dissolved Oxygen	14 days	1 999.4	This Document
<i>Myriophyllum sibiricum</i>	Nodes per Centimetre	14 days	2 065.8	This Document

Aquatic macrophytes also exhibit varied sensitivity to atrazine (Table 47). The review by Solomon *et al.* (1996) gave examples of atrazine toxicity to freshwater and marine macrophytes. The results obtained varied with the endpoint measured. For atrazine, photosynthetic inhibition gave more consistent results within species than growth measurements.

Four aquatic macrophyte species were examined by Jones and Winchell (1984) and they determined that *Potamogeton perfoliatus* was slightly more sensitive than *Myriophyllum spicatum*, *Ruppia maritima* and *Zannichellia palustris* to the phytotoxic effects of atrazine. Based on photosynthesis, atrazine IC<sub>50</sub> values were determined to be  $77 \pm 3.3 \mu\text{g/L}$ ,  $91 \pm 3.2 \mu\text{g/L}$ ,  $102 \pm 3.4 \mu\text{g/L}$  and  $104 \pm 3.3 \mu\text{g/L}$  for *P. perfoliatus*, *Z. palustris*, *R. maritima* and *Myriophyllum spicatum*, respectively (Table 47) (Jones and Winchell, 1984). Atrazine was not very toxic to *M. spicatum*, as determined by an IC<sub>50</sub> of  $1\ 104 \mu\text{g/L}$  for effect on length. *Vallisneria americana* and *Elodea canadensis* were intermediate in susceptibility to atrazine, while *P. perfoliatus* was sensitive (IC<sub>50</sub> =  $53 \mu\text{g/L}$  for percent kill) (Table 47) (Forney and Davis, 1981).

Jones and Estes (1984) conducted a study on detached *P. perfoliatus* leaves to determine if soil adsorbed herbicide affected photosynthesis. The soil adsorbed atrazine was not readily taken up by the leaves but atrazine in solution was absorbed by the plant, which reduced the rate of photosynthesis (Jones and Estes, 1984). In microcosm experiments, atrazine concentrations greater than  $50 \mu\text{g/L}$  decreased *P. perfoliatus* oxygen production over an eleven week study period. After four weeks of treatment, apparent photosynthesis was inhibited by 50% at atrazine concentrations of  $55$  and  $117 \mu\text{g/L}$  for *P. perfoliatus* and *M. spicatum*, respectively and final biomass was inhibited by 50% at  $30$  and  $91 \mu\text{g/L}$  (Table 47) (Kemp *et al.*, 1985). In the study by Kemp *et al.* (1985), regression coefficients for each line were given in place of confidence intervals (Kemp *et al.*, 1985). Photosynthesis was reduced by 50% in *P. perfoliatus* at an atrazine concentration of  $80 \mu\text{g/L}$  (Table 47) (Jones *et al.*, 1986). After 30 days of incubation, 100% mortality of *Vallisneria americana* plants was observed in microcosm studies at atrazine concentrations of  $120 \mu\text{g/L}$ . At a concentration of  $12 \mu\text{g/L}$ , mortality reached approximately 50% after 47 days of exposure (Correll and Wu, 1982).

Leaves of *Thalassia testudinum* were exposed to technical atrazine for 40 hours and the EC50 for oxygen evolution was estimated to be 320 µg/L (Walsh *et al.*, 1982). *Zostera marina* was exposed to technical grade atrazine for 21 days in a flow-through system and these experiments were repeated four times. Except for the first experiment, 100% mortality occurred at 1 000 µg/L in all replicates. LC50 values ranged from 100 µg/L (95% c.i. = 45 - 221 µg/L) in the second experiment to 510 µg/L (95% c.i. = 229 - 1 274 µg/L) in the first experiment. The results from the last two experiments were very similar with LC50 = 365 µg/L (95% c.i. = 220 - 606 µg/L) and LC50 = 367 µg/L (95% c.i. = 221 - 609 µg/L) for the third and fourth experiments, respectively. The authors suggested that the difference between the first experiment and the other three tests was due to cooler temperatures during the first experiment (Delistraty and Hershner, 1984). After 48 hours of exposure to 3 and 5 mg/L of technical atrazine, chloroplasts in *Elodea canadensis* cells migrated to the centre of the cells and chlorophyll leaked into the cytoplasm (Dabydeen and Leavitt, 1981).

The phytotoxic effects of technical grade atrazine on oxygen production of *Myriophyllum spicatum* cultured in modified Andrews' medium was detectable within 10 min at a minimum concentration of  $1 \cdot 10^{-7}$  M (28.4 µg/L). Aqueous extracts from soil spiked with formulated atrazine (Aatrex® 4L) were inhibitory to the *M. spicatum* at a minimum concentration of 0.07 kg/ha or 0.02 mg/250 g of soil (Selim *et al.*, 1989).

Fleming *et al.* (1991) examined the effect of technical atrazine on the biomass production of *Potamogeton pectinatus* in three different toxicity testing systems. In the heterotrophic system, biomass was stimulated above control levels by 0.01 mg/L. Ten milligrams inhibited biomass by  $33 \pm 9\%$ , when compared to control plants grown in the heterotrophic system. In both the autotrophic and microcosm systems, atrazine concentrations greater than or equal to 0.1 mg/L inhibited growth below control levels (Fleming *et al.*, 1991).

The effect of atrazine upon the production of new axillary buds, leaves, roots and branches by *Myriophyllum spicatum* was examined in tissue culture (Bird, 1993; Christopher and Bird, 1992). Formulated atrazine at a concentration of 3.7 mg/L caused a 50% reduction in the number of branches produced after 5 days of treatment (Bird, 1993). The number of branches was affected by atrazine concentrations greater than 30 mg/L. Visually, at atrazine concentrations of 100 mg/L, the plants appeared

chlorotic. There was no significant effect on root or bud production by atrazine concentrations up to 100 mg/L (Christopher and Bird, 1992). One of the most sensitive endpoints determined in the *M. sibiricum* axenic toxicity test was root length, which was not affected in the study by Christopher and Bird (1992) with *M. spicatum*. The differences observed between the bioassay conducted by Bird (1993) and Christopher and Bird (1992) and the *M. sibiricum* toxicity test may also be a result of species differences between the two species of *Myriophyllum*.

#### 5.7.1.3.4 Conclusions

If the EEC (2.99 mg/L) for atrazine were to enter an aquatic system, *Myriophyllum sibiricum* would probably not be affected. There might be minor effects to total root length or oxygen production but if the exposure was short term the *M. sibiricum* population should be able to recover. *M. sibiricum* would not be affected if only one percent of the MLR entered the aquatic system.

Based on EC50 and IC50 values presented in the scientific literature, *Chlamydomonas reinhardtii* is the most sensitive aquatic plant to the effects of atrazine (Table 46). The susceptibility to atrazine is closely followed by numerous algal and macrophyte species (Table 46 and Table 47). This culture of *Myriophyllum sibiricum* was not very sensitive to atrazine. It was almost 60 times less sensitive than *C. reinhardtii*. If the toxicity results from the recognised aquatic plant bioassays (*Selenastrum capricornutum* and *Lemna gibba*) were compared with the *M. sibiricum* toxicity test, the *M. sibiricum* toxicity test would be the least sensitive test (Table 46 and Table 47). Inclusion of the *M. sibiricum* toxicity test with the other aquatic plant toxicity tests may not be beneficial in studies with atrazine.

## 5.7.2 2,4-D

### 5.7.2.1 Introduction

The herbicide 2,4-D [(2,4-dichlorophenoxy)acetic acid] (Figure 54) is systemic and used to control broadleaf weeds in cereal crops, turfgrass, pastures and non-crop land. This herbicide is used for aquatic weed control in Canada (OMAF, 1994a; OMAF, 1994b), the United States (Anderson, 1993, Steward, 1993), Asia (Gopal, 1993), Central and South America (Fernández *et al.*, 1993) and European countries permitting aquatic herbicide use (Murphy *et al.*, 1993). The ester formulation is registered for aerial

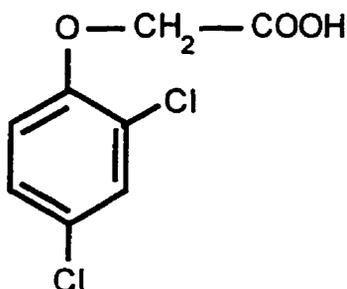


Figure 54: The molecular structure of the growth regulating compound: 2,4-D.

application in Canada (Canadian Pulp and Paper Association and Forestry Canada, 1992). It is capable of restraining the growth of *Myriophyllum spicatum*, water hyacinth, bulrush (*Scirpus* spp.), bladderwort (*Utricularia* spp.), water lily (WSSA, 1994), coontail (OMAF, 1994b) and sedges (Gopal, 1993). The exact mode of phytotoxic action is unknown but 2,4-D mimics the auxin hormonal action in plants and causes epinasty or swelling and twisting of stems and cupping of leaves. Chlorosis of the growing tips, growth inhibition and necrosis follow (WSSA, 1994). This herbicide also affects DNA, RNA and protein synthesis (Chen *et al.*, 1972; 1973).

Sixty-six percent of all the 2,4-D sold in Canada was used in the prairie provinces (Government of Canada, 1991). On agricultural crops in Ontario during 1993, 134,869 kg a.i. 2,4-D amine was sprayed with an additional 225 052 kg a.i. 2,4-D being applied by licensed applicators (Hunter and McGee, 1994). The use of 2,4-D in agriculture and urban areas has resulted in 2,4-D residues being detected in surface waters (Frank *et al.*, 1982; Government of Canada, 1991), farm ponds (Frank *et al.*, 1990b) and drinking water wells (Frank *et al.*, 1987a). For the protection of aquatic organisms, the National Academy of Sciences and the National Academy of Engineering (NAS/NAE) recommended that the concentration of 2,4-D not exceed 0.003 mg/L in ambient surface water (NAS/NAE, 1973 as cited in Nowell and Resek, 1994). In water, 2,4-D dissipates readily as was seen in a limnocorral study, where within 15 days less than 5% of the applied compound was present. Half-lives were determined to be 4.5 days for the lower rate of 40 µg/L and 7.8 days for the higher application rate of 100 µg/L (Solomon *et al.*, 1988). In a series of studies with plastic pools, swimming pools and field sites, an application of 2,4-D at 4.48 kg ae/ha, dissipated with the following trends: a decrease of 58 µg/L for each 0.61 m increase in water depth; a 115 µg/L drop in residues for each 7 °C increase in temperature above 15.6 °C; and a decrease of 53 µg/L for each 7 day interval after treatment (Averitt and Gangstad, 1976). Because of the additional impact of photolysis, 2,4-D is usually less persistent in water than in soil. Organic matter strongly adsorbs 2,4-D but it is readily leached out of mineral soil. It

persists from 2 to 4 weeks. In water, degradation is slow but rapid in hydrosol (Reinert and Rodgers, 1987; Horowitz, 1976).

2,4-D has been used to control excessive aquatic plant growth. In turbid water, 2,4-D ester is effective at controlling growth of some *Myriophyllum* species at a rate of 3 kg a.i./ha (Mitchell and Orr, 1985). Along with controlling *Myriophyllum* species, 2,4-D is also effective for managing other problem aquatic plants, such as water hyacinth, bulrush, bladderwort and water lily. A single spring application of 2,4-D (2.24 to 4.48 kg ae/ha) has been used to control water hyacinths while more frequent applications at higher rates have been used for alligator weed (*Alternanthera philoxeroides* (Mart.) Griseb.) control (Averitt and Gangstad, 1976). Recommended rates usually do not exceed 2.2 kg ae/ha for control of broadleaf weeds in turfgrass, small grain crops and field corn (WSSA, 1994).

#### 5.7.2.2 Methods

Preliminary experiments were conducted utilising three concentrations of formulated 2,4-D in half strength M & S medium (Roshon and Stephenson, 1992) and three concentrations of both technical and formulated 2,4-D in Hard Water medium and modified Andrews' medium (data not presented). Technical 2,4-D was used in the remaining experiments. The maximum recommended label rate for 2,4-D was 2.2 kg/ha (WSSA, 1989) and this converted to an expected environmental concentration of 1.47 mg/L. Water solubility of technical 2,4-D is 900 mg/L (WSSA, 1989) (Table 27) so the technical herbicide was dissolved in sterile water prior to being added to the liquid growth medium. The EEC experiment was conducted twice with initiation dates of February 21, 1994 and February 9, 1995. The stock plants that were used in these experiments were cultured in M & S medium for the first EEC experiment and in modified Andrews' medium for the second EEC experiment.

Based on the experiments with the EEC of 2,4-D, a dilution series containing seven concentrations (2.0, 6.0, 18.1, 54.3, 163, 489 and 1467 µg/L) of technical 2,4-D plus control plants was conducted once starting on February 9, 1995. The stock plants for this dilution series were cultured in modified Andrews' medium. A more detailed description of the methods may be found in Section 4.6.

### 5.7.2.3 Results and Discussion

#### 5.7.2.3.1 Expected Environmental Concentration

Within two days of initiating both EEC experiments, the apices treated with 2,4-D showed signs of epinasty including leaves folding downwards, stem rupture and reduction in pigmentation. These phytotoxic effects continued until the fourteenth day when the experiment was terminated (Figure 56). 2,4-D affected all the endpoints that were measured. Area under the growth curve and final plant height were inhibited (Figure 55, Table 48 and Table 49). The percent inhibition of these two parameters was slightly less for the second EEC experiment than it was for the first (Table 48). Even though these two experiments were conducted a year apart, weight was the only endpoint parameter where the results from the two experiments differed. In the second experiment, the weight of the 2,4-D treated plants was not significantly different from the weight of the control plants ( $\alpha = 0.05$ ) (Table 49). The difference in weight of the treated plants between experiments could be an artefact of changing the culture medium from M & S medium to modified Andrews' medium. Stock plants grown in modified Andrews' medium were slightly heavier than those cultured in M & S medium. Even the control plants grown from the stock plants cultured in modified Andrews' medium were heavier than those cultured in M & S medium (Table 49). For both experiments, root length and

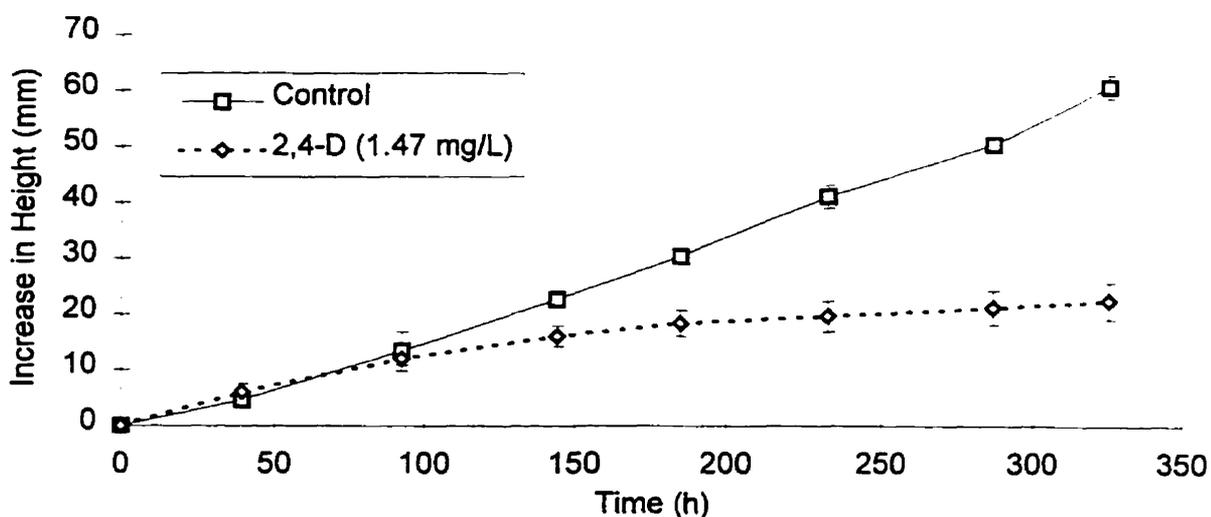


Figure 55: Growth curves for *Myriophyllum sibiricum* plants cultured for 14 days in either modified Andrews' medium or medium spiked with 1.47 mg/L of 2,4-D. Data presented are from the first EEC experiment with 2,4-D.



root number were inhibited by 100% when compared to the control (Table 48 and Table 49). Pigment concentration of the apices, expressed on a fresh and dry weight basis, was also severely inhibited (Table 48).

Table 48: <i>Myriophyllum sibiricum</i> growth and development was inhibited by the EEC of 2,4-D (1.47 mg/L) when compared with control plants.		
GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)	
	First Experiment	Second Experiment
Area Under the Growth Curve	45.3 $\pm$ 6.4	29.4 $\pm$ 5.9
Increase in Shoot Length	56.8 $\pm$ 4.6	36.7 $\pm$ 2.1
Root Number	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
Root Length	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
Fresh Weight	44.8 $\pm$ 9.2	-5.8 $\pm$ 8.4
Membrane Integrity	29.7 $\pm$ 7.0	47.0 $\pm$ 7.6
Plant Area	79.4 $\pm$ 5.5	37.2 $\pm$ 7.8
Chlorophyll <i>a</i> (fresh)	90.7 $\pm$ 1.3	92.4 $\pm$ 1.5
Chlorophyll <i>b</i> (fresh)	85.6 $\pm$ 3.2	94.9 $\pm$ 3.0
Carotenoid (fresh)	87.9 $\pm$ 0.8	89.4 $\pm$ 1.3
Chlorophyll <i>a</i> (dry)	Not measured	94.2 $\pm$ 1.2
Chlorophyll <i>b</i> (dry)	Not measured	96.1 $\pm$ 2.2
Carotenoid (dry)	Not measured	91.8 $\pm$ 1.2

Table 49: The effect of 2,4-D (1.47 mg/L) upon the growth and development of *Myriophyllum sibiricum* after fourteen days of exposure.

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
First Expected Environmental Concentration Experiment										
Control	8 939.3 ± 283.2 <sup>a</sup>	62.4 ± 2.1 <sup>a</sup>	6.3 ± 1.5 <sup>a</sup>	377.6 ± 60.1 <sup>a</sup>	541.9 ± 60.8 <sup>a</sup>	0.48 ± 0.02 <sup>a</sup>	0.18 ± 0.007 <sup>a</sup>	0.16 ± 0.005 <sup>a</sup>	8.0 ± 0.9 <sup>a</sup>	10.7 ± 2.1 <sup>a</sup>
2,4-D	4 888.5 ± 569.3 <sup>b</sup>	26.9 ± 2.9 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	299.0 ± 49.8 <sup>b</sup>	0.04 ± 0.006 <sup>b</sup>	0.03 ± 0.006 <sup>b</sup>	0.02 ± 0.001 <sup>b</sup>	22.9 ± 3.5 <sup>b</sup>	2.2 ± 0.6 <sup>b</sup>
Second Expected Environmental Concentration Experiment										
Control	8 816.2 ± 542.2 <sup>a</sup>	54.7 ± 1.2 <sup>a</sup>	8.0 ± 1.2 <sup>a</sup>	455.3 ± 72.0 <sup>a</sup>	796.8 ± 89.8 <sup>a</sup>	0.59 ± 0.05 <sup>a</sup>	0.22 ± 0.02 <sup>a</sup>	0.20 ± 0.02 <sup>a</sup>	8.0 ± 0.8 <sup>a</sup>	14.2 ± 1.5 <sup>a</sup>
2,4-D	6 223.2 ± 517.8 <sup>b</sup>	34.6 ± 1.2 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	842.8 ± 66.9 <sup>a</sup>	0.04 ± 0.009 <sup>b</sup>	0.01 ± 0.007 <sup>b</sup>	0.02 ± 0.003 <sup>b</sup>	31.6 ± 3.8 <sup>b</sup>	8.9 ± 1.1 <sup>b</sup>

a, b For each EEC experiment, any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$ . Data presented are the untransformed mean  $\pm$  s.d. for 5 replicates. The control treatments for the first EEC experiment lost two replicates due to contamination.

#### 5.7.2.3.2 Dilution Series

Plants treated with the 2,4-D dilution series demonstrated visual epinasty at 18.1  $\mu\text{g/L}$  and higher (Figure 57). The epinasty started within 48 hours of treatment and continued throughout the fourteen days of treatment. The epidermis became separated from the vascular tissue. Growth curves for the dilution experiment are shown in Figure 58. The higher rates of 2,4-D demonstrated delayed inhibition (refer to Section 4.3.2.2) in terms of height and area under the growth curve. It was not until approximately eight days after the experiment was initiated that these parameters differed from the control. If the experiment lasted only seven days (length of the *Lemna* growth test), the treated plants would not be distinguishable from the control plants on the basis of plant height or area under the growth curve (Figure 58). The lag period before pesticide toxicity, as was noticeable in the *Myriophyllum* toxicity test with 2,4-D, might have been due to the fact that some pesticides tend to accumulate slowly in an organism (Martin, 1973). After fourteen days of exposure to 2,4-D, *M. sibiricum* was severely affected (Table 50). Root length and root number were most severely affected since they both had a NOEC of 2  $\mu\text{g/L}$  and IC50s of 13.1 and 17.7  $\mu\text{g/L}$ , respectively. The concentration of chlorophyll *a*, chlorophyll *b* and carotenoids were the next most severely affected parameters, especially when expressed in terms of the dry weight of the apex (Table 50). Chlorophyll *b* content was very variable (Table 50), especially at the NOEC. The fresh weight of the fourteen day old control plants ( $813.0 \pm 75.4$  mg) was greater than the average weight of plants subjected to 163  $\mu\text{g/L}$  of 2,4-D ( $561.3 \pm 60.0$  mg). At the maximum 2,4-D concentration of 1467  $\mu\text{g/L}$ , plant weight ( $800.7 \pm 44.0$  mg) was close to the average control plant weight. This fluctuation in weight with changing 2,4-D concentration could be an artefact of the mode of action of this chemical. Since 2,4-D mimics the plant growth hormone, auxin, the high concentration of 2,4-D stimulated excessive shoot growth that was not beneficial to the survival of the plants. Even though the treated plants were as heavy as the control plants, they lacked roots and contained low concentrations of photosynthetic pigments and therefore would be unlikely to survive a long-term exposure to high concentrations of this herbicide.

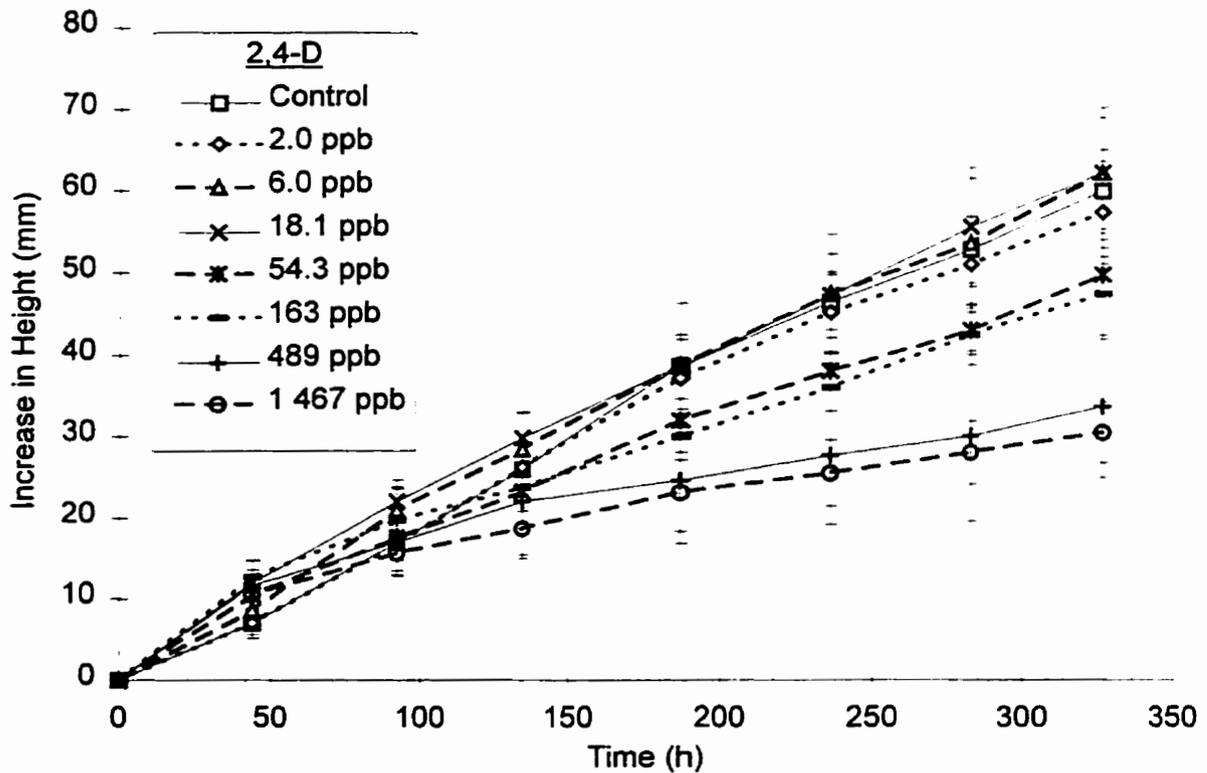


Figure 58: Fourteen day growth curves for the dilution series of 2,4-D. A total of five plants were lost due to fungal contamination so the values plotted are the average of five replicates  $\pm$  s.d. (control, 2.0, 6.0 and 18.1  $\mu\text{g/L}$ ), the average of four replicates  $\pm$  s.d. (54.3, 163 and 1 467  $\mu\text{g/L}$ ) and the average of three replicates  $\pm$  s.d. (489  $\mu\text{g/L}$ ).

The results reported here, based on a maximum application rate of 2.2 kg/ha, may greatly underestimate the effects of 2,4-D on aquatic vegetation because the formulation POST<sup>®</sup> may be applied at rates of 22.4 kg ae/ha in aquatic situations (WSSA, 1994) and researchers report the common usage of 2,4-D rates up to 4.48 kg ae/ha (Averitt and Gangstad, 1976). If the 2,4-D residues in freshwater remain below the NAS/NAE recommended limit of 0.003 mg/L (NAS/NAE, 1973 as cited in Nowell and Resek, 1994), *M. sibiricum* populations should not be severely affected. Other studies have shown that results from 2,4-D lab studies are comparable to field studies (Fletcher *et al.*, 1990), so theoretically these laboratory studies may adequately mimic field situations.

Table 50: No observable effect concentrations, the average percent inhibition ( $\pm$  s.d.) at the NOEC, IC25 and IC50 values (upper and lower 95% c.i.) for the effect of 2,4-D upon *Myriophyllum sibiricum* growth and development endpoint parameters.

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	% I at NOEC	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	18.1	-8.3 $\pm$ 8.8	355.7 (145.9; 867.2) <sup>b</sup>	> 1 467 <sup>b,c</sup>
Increase in Shoot Length	6.0	-5.1 $\pm$ 4.7	409.9 (210.6; 797.8) <sup>b</sup>	> 1 467 <sup>b,c</sup>
Root Length	2.0	4.3 $\pm$ 13.0	5.1 (2.6; 10.0) <sup>c</sup>	13.1 (8.3; 20.6) <sup>c</sup>
Root Number	2.0	10.0 $\pm$ 11.1	7.8 (3.7; 16.17) <sup>b</sup>	17.7 (10.4; 30.1) <sup>b</sup>
Fresh Weight <sup>a</sup>	Fluctuated	Fluctuated	> 1 467 <sup>c</sup>	> 1 467 <sup>c</sup>
Membrane Integrity	18.1	-9.2 $\pm$ 11.1	228.3 (198.5; 262.5) <sup>c</sup>	801.3 (725.3; 885.2) <sup>c</sup>
Plant Area	18.1	-0.9 $\pm$ 10.5	73.7 (12.5; 436.1) <sup>c</sup>	957.4 (221.5; 4 138.0) <sup>c</sup>
Chlorophyll a (fresh wt)	54.3	-6.3 $\pm$ 21.5	166.2 (109.8; 251.5) <sup>b</sup>	313.7 (238.2; 413.1) <sup>b</sup>
Chlorophyll b (fresh wt)	54.3	-33.3 $\pm$ 97.4	152.3 (75.8; 306.0) <sup>b</sup>	253.7 (112.5; 572.1) <sup>b</sup>
Carotenoid (fresh wt)	54.3	6.7 $\pm$ 10.1	146.1 (111.1; 192.1) <sup>c</sup>	308.5 (256.2; 371.4) <sup>c</sup>
Chlorophyll a (dry wt)	18.1	7.2 $\pm$ 16.5	42.6 (30.1; 60.5) <sup>b</sup>	123.8 (96.2; 159.2) <sup>b</sup>
Chlorophyll b (dry wt)	54.3	12.5 $\pm$ 63.3	67.2 (38.9; 116.2) <sup>b</sup>	141.3 (101.8; 196.1) <sup>b</sup>
Carotenoid (dry wt)	18.1	6.0 $\pm$ 13.9	34.5 (21.2; 56.3) <sup>b</sup>	109.6 (75.7; 158.8) <sup>b</sup>

a The total plant fresh fluctuated from an average control weight of 813.0  $\pm$  75.4 mg down to an average low of 561.3  $\pm$  60.0 mg at a 2,4-D concentration of 163  $\mu\text{g/L}$  and then increased to an average of 800.7  $\pm$  44.0 mg at the EEC. No NOEC could be calculated and the cumulative normal program estimated the IC25 and IC50 to be greater than 1467  $\mu\text{g/L}$ .

b IC<sub>p</sub> values and 95% confidence intervals calculated with the weibull method.

c IC<sub>p</sub> and 95% confidence values calculated with the cumulative normal distribution model.

### 5.7.2.3.3 Aquatic Plant Toxicity Comparisons

Table 51 and Table 52 contain summaries of the effects of 2,4-D on aquatic plants. At high concentrations, 2,4-D adversely affected most algal species in both laboratory and field studies (Wright, 1978). This herbicide was not very toxic to *Selenastrum capricornutum* when tested in either the traditional flask assay or the microplate assay. Ninety-six hour cell count EC50s of 25.9 mg/L (c.i. = 23.8 - 28.3 mg/L) and 24.2 mg/L (c.i. = 23.7 - 24.7 mg/L) were obtained for the flask and microplate assays, respectively (Table 51) (St. Laurent *et al.*, 1992). Growth of *Chlorella pyrenoidosa*, *Chlorococcum* sp., *Anabaena variabilis* and *Lyngbya* sp. was not affected by 2,4-D concentrations up to 10 µM (2.26 mg/L). Photosynthesis was slightly inhibited in *Lyngbya* sp. at 2,4-D concentrations less than the lowest concentration tested (<1 µM (0.226 mg/L)), in *Chlorococcum* at 10 µM, in *Anabaena* at both 0.1 (0.023 mg/L) and 1.0 µM and in *Chlorella* at 0.1 and 10.0 µM. EC50 values for photosynthesis could not be calculated for any of these four algal species. Respiration of *Chlorococcum* was slightly stimulated by a 2,4-D concentration of 10 µM. 2,4-D had no effect on respiration of the other algal species (Hawxby *et al.*, 1977). One hundred mg/L of 2,4-D reduced the growth of *Anabaena variabilis* to 50% of control growth (Table 51) and 500 mg/L of 2,4-D was required to completely inhibit the growth of this species (Hutber *et al.*, 1979). At a 2,4-D concentration of 5 mg/L, *Chlorella pyrenoidosa* showed no substantial toxicity symptoms. 2,4-D produced significant inhibition in chlorophyll content of *C. pyrenoidosa* cells at a concentration of 50 mg/L and chlorophyll production was halted at 440 mg/L (Huang and Gloyna, 1968). In a microplate test, EC50 values for cell count were determined at 2,4-D concentrations of 19 mg/L for *Chlorella vulgaris* and 56 mg/L for *C. pyrenoidosa* (Table 51) (Baarschers *et al.*, 1988). Gramlich and Frans (1964) also found that 2,4-D was not inhibitory to *Chlorella pyrenoidosa* growth at low concentrations but a concentration of approximately  $1.5 \cdot 10^{-4}$  M (33.9 mg/L) inhibited growth by fifty percent (Table 51) (Gramlich and Frans, 1964). After 4 days of treatment, chlorophyll production of *Chlamydomonas reinhardtii* was inhibited 50% by a technical 2,4-D concentration of < 0.1 mM (< 22.1 mg/L) (Fedtke, 1992). At concentrations between 1.0 and 80.0 µM (0.22 and 17.7 mg/L), reagent grade 2,4-D did not significantly affect zygospore germination or growth of *Chlamydomonas moewusii* Gerloff (Cain and Cain, 1983).

Table 51: Selected from the scientific literature, a summary of IC50 values for aquatic plant species exposed to 2,4-D. Test conditions differed between the species but general trends in sensitivity can be observed. The submersed macrophytes are more sensitive to the effects of 2,4-D followed closely by the floating macrophytes. The algal species can tolerate high concentrations of 2,4-D.

Species	Endpoint	Exposure Duration	EC50 or IC50	Reference
<b>Algae</b>				
<i>Anabaena variabilis</i>	Growth	48 hours	100.0 mg/L	Hutber <i>et al.</i> (1979)
<i>Chlorella pyrenoidosa</i>	Growth	5 days	33.9 mg/L	Gramlich and Frans (1964)
<i>Chlorella pyrenoidosa</i>	Cell Count	10 - 14 days	56 mg/L	Baarschers <i>et al.</i> (1988)
<i>Chlorella vulgaris</i>	Cell Count	10 - 14 days	19 mg/L	Baarschers <i>et al.</i> (1988)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	96 hours	25.9 mg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	24.2 mg/L	St. Laurent <i>et al.</i> (1992)
<b>Floating Macrophytes</b>				
<i>Lemna paucicostata</i>	Leaf Surface Area	10 days	0.75 mg/L	Retzlaff (1992)
<i>Salvinia natans</i> (L.) All.	Wet Weight	4 weeks	6.5 mg/L	Gönca and Sencic (1994)
<i>Salvinia natans</i>	Chlorophyll	4 weeks	0.3 mg/L	Gönca and Sencic (1994)
<b>Macrophytes</b>				
<i>Oryza sativa</i>	Root Length	96 hours	0.1 mg/L	Hardcastle and Wilkinson (1970)
<i>Myriophyllum spicatum</i>	Branch Number	5 days	0.04 mg/L	Bird (1993)
<i>Myriophyllum sibiricum</i>	Root Length	14 days	0.01 mg/L	This Document
<i>Myriophyllum sibiricum</i>	Carotenoid (dry)	14 days	0.11 mg/L	This Document

**Table 52: No observable effect concentrations for 2,4-D upon macrophytes. Based on NOEC, *Myriophyllum sibiricum* was forty times as sensitive to 2,4-D as *M. spicatum*.**

Species	Endpoint	Exposure Duration	NOEC	Reference
<b>Macrophytes</b>				
<i>Myriophyllum spicatum</i>	New Buds	5 days	80 µg/L	Christopher and Bird (1992)
<i>Myriophyllum spicatum</i>	Root Number	5 days	20 µg/L	Christopher and Bird (1992)
<i>Myriophyllum spicatum</i>	Number of Branches	5 days	20 µg/L	Christopher and Bird (1992)
<i>Myriophyllum spicatum</i>	Total Number of New Structures	5 days	20 µg/L	Christopher and Bird (1992)
<i>Myriophyllum sibiricum</i>	Root Length	14 days	2.0 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Root Number	14 days	2.0 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Increase in Shoot Height	14 days	6.0 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	18.1 µg/L	This Document

Four species of marine unicellular algae (*Chlorococcum* sp., *Dunaliella tertiolecta*, *Isochrysis galbana* and *Phaeodactylum tricornutum*) were exposed to technical and formulated 2,4-D. Growth of *Chlorococcum* sp., *I. galbana* and *P. tricornutum*, measured after 10 days of exposure, was inhibited 50% by 50 mg/L of technical 2,4-D. Formulated 2,4-D (butoxyethanol ester) was less toxic than the technical product to the growth of *Chlorococcum* sp., *D. tertiolecta* and *I. galbana* because 75 mg/L was required to inhibit the growth of these species by 50%. *D. tertiolecta* was the most sensitive species to the effects of technical 2,4-D, as measured with oxygen evolution after 90 min of exposure, because the EC50 was 50 mg/L, while the EC50 for the other three species was 60 mg/L. One hundred mg/L was required to reduce oxygen evolution by 50% in *Chlorococcum* sp., *D. tertiolecta* and *I. galbana* (Walsh, 1972). The phytotoxicity of 2,4-D towards algal species was summarised by Stratton (1987b).

In seven algal species, uptake of  $^{14}\text{C}$ , supplied as  $\text{NaH}^{14}\text{CO}_3$ , was not significantly affected by 2,4-D at a rate of 2.917 mg/L. There was a 34% reduction in *Lemna minor* frond production at this 2,4-D concentration (Peterson *et al.*, 1994). Since the 2,4-D rate of 2.917 mg/L is about double the rate used in the axenic *Myriophyllum* toxicity test, 2,4-D is definitely more toxic to the submersed rooted macrophyte than it was to any of the aquatic plant species studied by Peterson *et al.* (1994). The effect of temperature and various light intensities on the toxicity of 2,4-D to *Lemna minor* was examined by Blackman and Robertson-Cuninghame (1955). These authors determined that light intensity during herbicidal treatment did not affect toxicity but that light intensity prior to treatment was influential. A toxic effect was caused by lower 2,4-D concentrations when the plants were exposed to low light intensities before herbicidal treatment. A similar pattern was observed for increasing temperature (Blackman and Robertson-Cuninghame, 1955). The effects of light intensity and temperature on the toxicity of this herbicide were not examined with the axenic *M. sibiricum* toxicity test.

The cell membrane permeability of *Lemna minor* plants was increased by 2,4-D concentrations of  $10^{-2}$  mM (2.2 mg/L) after 12 to 72 hours and  $10^{-3}$  mM (0.22 mg/L) after 96 hours (O'Brien and Prendeville, 1979). In contrast, the membrane permeability of *M. sibiricum* plants was affected by 2,4-D at concentrations greater than 18.1  $\mu\text{g/L}$  after fourteen days of treatment (Table 50).

Fifty percent inhibition of the total leaf surface area of *Lemna paucicostata* occurred at a 2,4-D concentration of  $3.4 \cdot 10^{-6}$  M (0.75 mg/L) after ten days of incubation (Table 51) (Retzlaff, 1992). In bioassays with the free-floating water fern, *Salvinia natans* (L.) All., the EC50 for 2,4-D was determined to be 6, 6.5, 6.5, 0.3 and 0.3 mg/L for the growth of leaves, wet weight, length of stems and the amount of chlorophyll *a* and chlorophyll *b*, respectively (Table 51). Chlorophyll content from *S. natans* was also variable. Visual epinasty was observed after the first week of 2,4-D exposure (Gönca and Sencic, 1994). The EC50 results for pigment content of *S. natans* were very similar to those obtained for pigment content on a fresh weight basis of *M. sibiricum*. Pigment content on a dry weight basis and root development of *M. sibiricum* were more sensitive to the effects of 2,4-D than this water fern.

2,4-D at a rate of 0.1 mg/L produced a 50% reduction in root elongation in domestic rice seedlings (*Oryza sativa* L. var. Bluebonnet) (Table 51) (Hardcastle and Wilkinson, 1970). *Myriophyllum sibiricum* in axenic culture was more sensitive to the phytotoxic effects of 2,4-D upon root growth than were rice seedlings. Even the pigment content of *M. sibiricum* apices was inhibited at levels below those that affected rice.

Most of the studies discussing 2,4-D and its relationship to rooted aquatic macrophytes deal with rates to control nuisance aquatic vegetation under different environmental conditions. Murphy and Barrett (1993) summarised the types of aquatic plants that are controlled at different rates of 2,4-D. Case studies examining the effectiveness of this herbicide at controlling aquatic plant populations in field situations are presented by Anderson (1993) and Fernández *et al.* (1993). Water hyacinth absorbed 2,4-D from the water column treated with either 0.01 or 0.001 mg/L via the roots and then transported a fraction of it into the shoot. Over the 14 day study period, 2,4-D remained in the water column at detectable levels (Wang *et al.*, 1994a).

Using a prairie wetland system in Saskatchewan, it was determined that visual injury of *M. sibiricum* occurred at 2,4-D (Formula 40<sup>®</sup> - 500 g/L of 2,4-D dimethylamine salt) concentrations of 0.01 and 0.1 mg a.i./L, while *Potamogeton pectinatus* was visually affected at 0.1 mg/L of 2,4-D. Sixty days after 2,4-D treatment at a rate of 0.1 mg/L, *M. sibiricum* plant weight was reduced. Plant weight and tuber production of *P. pectinatus* were stimulated by 0.01 mg/L of 2,4-D. Flower production in *M. sibiricum* was stimulated by 0.01 mg/L 2,4-D. In the prairie wetland enclosures, shoots were

more frequently injured than were the roots of either plant species (Forsyth *et al.*, 1997). There are numerous similarities between the results obtained in the field study by Forsyth *et al.* (1997) and the axenic laboratory study with *M. sibiricum*. Visual injury was observed at similar concentrations. Plant weight in both studies decreased at 2,4-D concentrations approximating 0.1 mg/L. The field study utilised only two 2,4-D concentrations so it cannot be determined whether or not the plant weight would have fluctuated as it did in the current study. Reproductive parameters were not examined in the current study.

The effect of 2,4-D upon the production of new axillary buds, leaves, roots and branches of *Myriophyllum spicatum* was examined in tissue culture. After five days of incubation, 2,4-D caused a fifty percent reduction in the number of branches produced at 0.04 mg/L (Table 51) (Bird, 1993). Production of leaves by *M. spicatum* was not affected by 2,4-D at concentrations between 0.02 and 0.1 mg/L. The NOECs were 0.08, 0.02, 0.02 and 0.02 mg/L for the production of new buds, roots, branch and total number of new structures, respectively (Christopher and Bird, 1992). In the *Myriophyllum sibiricum* axenic toxicity test, the root growth was affected at concentrations 10 times lower (Table 50) than that observed by Christopher and Bird (1992). Most other parameters measured on *M. sibiricum* were affected at levels similar to those obtained in the study on *M. spicatum* (Table 50) (Christopher and Bird, 1992). With the production of new buds and roots, the toxic effect of 2,4-D was not consistent between concentrations. For example, the effect observed at a low concentration could be inhibited more than at a higher level (Christopher and Bird, 1992). This variation in toxic effect was observed with the total fresh weight in the *Myriophyllum sibiricum* toxicity test. Visual effects, such as swelling at the nodes, yellowing of tissue and epidermal separation from the vascular tissue were observed in *M. spicatum* treated with 2,4-D (Christopher and Bird, 1992). These visual effects were also noted in the current study.

#### 5.7.2.3.4 Conclusions

If 2,4-D were to enter an aquatic system at the expected environmental concentration of 1.47 mg/L, there would be detrimental effects on the *Myriophyllum sibiricum* population. Plant root length and pigment content would be severely affected. If the protection of non-target aquatic plants was desired, caution should be exercised to ensure that not even 1% of the EEC reach the aquatic system. Less than 1% of the

expected environmental concentration would inhibit root growth by more than 50%. The use of buffer zones between the terrestrial spray location and non-target wetland areas would be advisable.

*Myriophyllum sibiricum*, as tested in the axenic toxicity test, was the most sensitive aquatic macrophyte to the phytotoxic effects of 2,4-D (Table 51 and Table 52). The most sensitive *M. sibiricum* endpoint parameter to 2,4-D was total root length. Based upon IC50/EC50 values for 2,4-D, *M. sibiricum* was 4 times more sensitive than *M. spicatum* branch number, 10 times more sensitive than root elongation of *Oryza sativa*, 30 times more sensitive than stem length and chlorophyll content of *Salvinia natans* and 75 times more sensitive than leaf area of *Lemna paucicostata*. To the toxic effects of 2,4-D, *M. sibiricum* was 1 900 and 2 400 times more sensitive than *Chlorella vulgaris* and *Selenastrum capricornutum* population growth, respectively (Table 51). Based on NOECs presented in the literature, *M. sibiricum* was 40 times more sensitive to 2,4-D than *M. spicatum* (Table 52). Therefore, it would be beneficial to the registration process of auxin mimicing herbicides to include the *M. sibiricum* toxicity test in order to protect non-target aquatic environments.

### 5.7.3 Diquat

#### 5.7.3.1 Introduction

Diquat, a bipyridylium herbicide (Figure 59), is a nonselective membrane toxin (Ashton and Crafts, 1981), which is registered for use in Canada for both terrestrial and aquatic use. In Ontario during 1993, 7 247 kg a.i. of diquat was applied on field crops, fruits and vegetables (Hunter and McGee, 1994). Diquat is used as a preharvest desiccant for terrestrial crops, such as clover (*Trifolium* spp.), potatoes (*Solanum tuberosum* L.), alfalfa (*Medicago sativa* L.) (Thomson, 1993), soybean, canola (*Brassica*

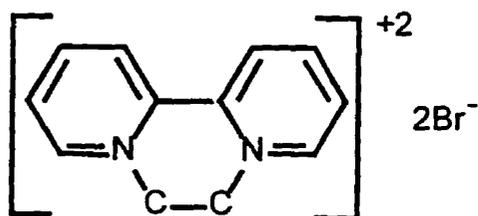


Figure 59: The chemical structure of diquat, a bipyridylium herbicide.

spp.), flax (*Linum usitatissimum* L.) and mustard (*Brassica* spp.) (OMAF, 1994a).

Diquat effectively controls aquatic macrophyte growth at concentrations of 0.5 to 1.0 mg dm<sup>-3</sup> in clear standing water (Mitchell and Orr, 1985). Recommended rates of application range from 2.24 to 4.48 kg/surface ha to control aquatic weeds

(WSSA, 1989). Diquat successfully regulates growth of cattails, filamentous algae, bladderwort, coontail, *Elodea*, pennywort (*Hydrocotyle* spp.), *Salvinia*, water hyacinth (WSSA, 1994; OMAF, 1994b; Saskatchewan Environment and Public Safety, 1986), *Chara* and *Potamogeton* in ponds, lakes and drainage ditches (OMAF, 1994a; Saskatchewan Environment and Public Safety, 1986).

Diquat's contact phytotoxicity is due to a disruption of membrane integrity via a reduction of the diquat cation and subsequent production of a diquat free radical and other free radicals. The reducing potential comes mainly from photosynthesis and to a minor extent from respiration. In the presence of oxygen and water, the free radical is oxidised and hydrogen peroxide is produced. This hydrogen peroxide is responsible for the destruction of cellular and chloroplast membranes (Ashton and Crafts, 1981; Calderbank and Slade, 1976; Kunert and Dodge, 1989; Simsiman *et al.*, 1976). Some evidence suggests that continuous light increases the speed of this reaction. Secondary effects include the disruption of the photosynthetic process and a subsequent reduction in chlorophyll content (Kunert and Dodge, 1989) and reduction or stimulation of respiration (Melendez *et al.*, 1993).

If used in terrestrial applications, diquat is not expected to reach aquatic systems because it is rapidly adsorbed onto soil particles, particularly clay. Once bound to the soil, only wind blown soil bound diquat can enter an aquatic system. When the herbicide is sprayed onto a body of water, the diquat is rapidly adsorbed onto sediment, suspended particulate matter and plant tissue. Once adsorbed to sediment, the diquat tends to remain tightly bound and is unavailable for plant uptake and is only slightly available for microbial degradation (Calderbank and Slade, 1976; Reinert and Rodgers, 1987; Simsiman and Chesters, 1976; Simsiman *et al.*, 1976). It can persist in a bound state for several months (Frank and Comes, 1967; Horowitz, 1976).

#### 5.7.3.2 Methods

Diquat dibromide monohydrate that was 99% pure was purchased from Chem Service<sup>16</sup>. Since technical diquat is water soluble (Table 27) only a water control was required. For aquatic weed control, the maximum recommended label rate is 4.48 kg/ha of surface water (WSSA, 1989). If this was sprayed onto a 15 cm column of water, the MLR converts to an EEC of 2.99 mg/L (Table 27). The EEC experiment was repeated three times (start dates = March 21, 1994; July 11, 1994 and April 13, 1995). Stock

plants used in the first and second EEC experiments were cultured in M & S medium while stock plants used for the last experiment were cultured in modified Andrews' medium. All other experimental procedures followed those described in Chapter 4.6. Percent inhibition of the EEC as compared to the control was calculated.

Three dilution series were conducted. The first dilution series (46.7 to 2987 µg/L) did not go low enough to reach a NOEC or IC50 so two lower dilution series (0.73 to 2 987 µg/L and 4.1 to 2 987 µg/L) were conducted. These experiments were initiated on June 20, 1994, July 11, 1994 and April 24, 1995. As with the EEC experiments, the stock plants for the first two dilution series were cultured in M & S medium and stock plants used in the last dilution series experiment were cultured in modified Andrews' medium. Methods followed those outlined in Chapter 4.6. The data were checked for normality. Normal data were analysed using a one-way ANOVA and non-normal data were analysed using the Kruskal-Wallis test to determine NOEC. IC50 and 95% confidence intervals were determined using either the weibull or cumulative normal distributions.

### 5.7.3.3 Results and Discussion

#### 5.7.3.3.1 Expected Environmental Concentration

At the EEC (2.99 mg/L), diquat severely inhibited plant growth and development. The effects can be visually seen in Figure 61. Figure 60 displays the growth curves from the second EEC experiment. For all three EEC experiments, Table 53 lists the percent inhibition of the treated plants as compared to the control plants and Table 54 presents the means and standard deviations for both the control and diquat treated plants. As seen in Figure 60, Table 53 and Table 54, the area under the growth curve and increase in shoot height were severely inhibited by the EEC of diquat. In the first two EEC experiments, root number and root length were totally inhibited by the EEC of diquat. In the last EEC experiment, one plant produced a few small roots. Membrane permeability was slightly affected by diquat (% inhibition between 10 and 55%) (Table 53). The disruption of plant membranes in the diquat treatments was significant when compared to the controls (Table 54). Of all the toxicants tested in the axenic *M. sibiricum* toxicity test, the second highest level of membrane damage was observed with diquat. During the second diquat EEC experiment, the highest level of percent membrane damage was 58.3% with an average of  $35.9 \pm 15.0\%$ . As compared with the

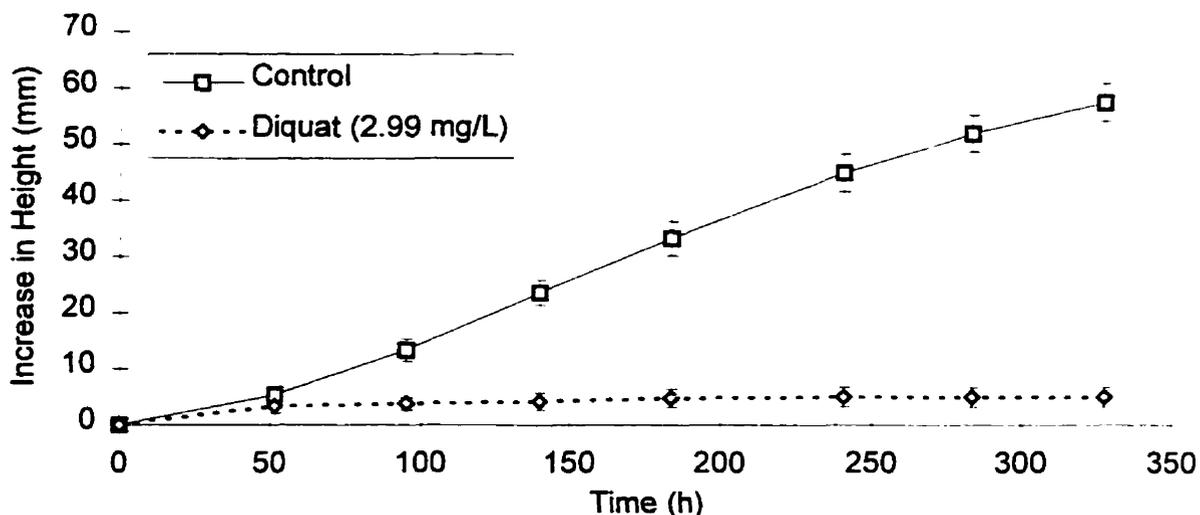


Figure 60: The effect of the expected environmental concentration (2.99 mg/L) of diquat on the growth of *M. sibiricum* over fourteen days of axenic culture in modified Andrews' medium.

control values, the percent inhibition for membrane damage was  $54.6 \pm 30.4\%$  (Table 53). The other growth parameters measured were severely inhibited by diquat with the lowest being 80% inhibition. Percent inhibition of the pigment content of plants from the first experiment was slightly lower than for the other two experiments. This might have occurred because the growth cabinet over heated to 38 °C on Day 9 of the first experiment. This over heating does not seem to have affected the percent inhibition for the other endpoint parameters (Table 53) but it might have slightly reduced the plant height (Table 54). In the third experiment, the fresh weight of the control plants might be slightly greater than the control plant weight from the first and second experiments (Table 54) because of the change in stock plant culturing medium from M & S to modified Andrews'. Even though the three experiments were conducted over a year apart, there was good correlation between the percent inhibition for endpoints other than pigment content based on fresh apical weight and total plant weight. Expressing pigment content on either a fresh or dry weight basis did not change the percent inhibition (Table 53).

#### 5.7.3.3.2 Dilution Series

For the first dilution series with diquat (46.7 to 2987 µg/L), the no observable effect level was either equal to or less than the lowest dilution (Table 55). IC50 values



Table 53: The percent inhibition of the EEC of diquat upon <i>Myriophyllum sibiricum</i> growth and development.			
GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)		
	First Experiment	Second Experiment	Third Experiment
Area Under the Growth Curve	87.4 $\pm$ 4.4	85.7 $\pm$ 4.8	81.6 $\pm$ 10.0
Increase in Shoot Length	86.2 $\pm$ 1.4	89.8 $\pm$ 3.2	80.7 $\pm$ 7.4
Root Number	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	93.9 $\pm$ 13.7
Root Length	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	99.6 $\pm$ 0.8
Fresh Weight	79.2 $\pm$ 5.9	81.9 $\pm$ 3.2	80.6 $\pm$ 3.9
Membrane Integrity	33.1 $\pm$ 16.3	54.6 $\pm$ 30.4	10.4 $\pm$ 3.0
Plant Area	81.5 $\pm$ 7.5	82.6 $\pm$ 7.4	76.8 $\pm$ 8.3
Chlorophyll <i>a</i> (fresh)	75.1 $\pm$ 4.9	84.6 $\pm$ 11.6	89.6 $\pm$ 4.3
Chlorophyll <i>b</i> (fresh)	80.4 $\pm$ 3.0	94.7 $\pm$ 5.7	98.0 $\pm$ 3.2
Carotenoid (fresh)	70.3 $\pm$ 6.6	80.6 $\pm$ 15.4	86.5 $\pm$ 4.9
Chlorophyll <i>a</i> (dry)	Not measured	Not measured	91.1 $\pm$ 3.1
Chlorophyll <i>b</i> (dry)	Not measured	Not measured	98.3 $\pm$ 2.7
Carotenoid (dry)	Not measured	Not measured	88.3 $\pm$ 3.5

and 95% confidence intervals for final shoot height and area under the growth curve were calculated as 346.1  $\mu\text{g/L}$  (153.4; 781.5) and 271.3  $\mu\text{g/L}$  (196.9; 373.7), respectively (Table 56). For the other endpoint parameters, the IC50 value could not be correctly estimated because they were less than the lowest concentration tested.

The effects of the second diquat dilution series upon *Myriophyllum sibiricum* may be visually assessed in Figure 62 while the growth curves for the seven diquat concentrations plus the control are in Figure 63. The NOEC for this dilution series was 11.7  $\mu\text{g/L}$  for most of the endpoints. Area under the growth curve and chlorophyll *b* content had NOEC of 0.73 and less than 0.73, respectively (Table 55). Melendez *et al.* (1993) stated that chlorophyll content could not be measured due to interference by diquat (Melendez *et al.*, 1993), so caution should be used when interpreting the low chlorophyll results. For most of the parameters, IC50 values could be calculated

Table 54: Untransformed means and standard deviations for the three experiments conducted to determine the effect of the EEC of diquat upon the growth and development of *Myriophyllum sibiricum* in axenic culture. For all endpoint parameters, the control plants were significantly different from the diquat treated plants ( $\alpha = 0.05$ ).

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	Fresh Weight (mg)	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
First Expected Environmental Concentration Experiment										
Control	8 129.2 ± 1 158.4 <sup>a</sup>	53.7 ± 5.9 <sup>a</sup>	8.4 ± 0.9 <sup>a</sup>	213.0 ± 26.7 <sup>a</sup>	479.6 ± 38.6 <sup>a</sup>	0.52 ± 0.09 <sup>a</sup>	0.20 ± 0.04 <sup>a</sup>	0.17 ± 0.03 <sup>a</sup>	9.6 ± 1.9 <sup>a</sup>	5.4 ± 1.0 <sup>a</sup>
Diquat	1 024.9 ± 358.2 <sup>b</sup>	7.4 ± 0.7 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	99.9 ± 28.1 <sup>b</sup>	0.13 ± 0.03 <sup>b</sup>	0.04 ± 0.006 <sup>b</sup>	0.05 ± 0.01 <sup>b</sup>	25.7 ± 7.9 <sup>b</sup>	1.0 ± 0.4 <sup>b</sup>
Second Expected Environmental Concentration Experiment										
Control	9 296.3 ± 664.1 <sup>a</sup>	60.9 ± 3.9 <sup>a</sup>	8.2 ± 1.3 <sup>a</sup>	384.6 ± 44.0 <sup>a</sup>	500.5 ± 26.3 <sup>a</sup>	0.53 ± 0.04 <sup>a</sup>	0.19 ± 0.02 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>	9.0 ± 0.6 <sup>a</sup>	13.5 ± 2.1 <sup>a</sup>
Diquat	1 330.4 ± 447.6 <sup>b</sup>	6.2 ± 1.9 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	90.7 ± 15.9 <sup>b</sup>	0.10 ± 0.05 <sup>b</sup>	0.013 ± 0.01 <sup>b</sup>	0.04 ± 0.02 <sup>b</sup>	35.9 ± 15.0 <sup>b</sup>	2.4 ± 1.0 <sup>b</sup>
Third Expected Environmental Concentration Experiment										
Control	9 860.2 ± 479.9 <sup>a</sup>	63.4 ± 4.1 <sup>a</sup>	9.8 ± 1.5 <sup>a</sup>	543.9 ± 205.0 <sup>a</sup>	741.9 ± 54.9 <sup>a</sup>	0.58 ± 0.06 <sup>a</sup>	0.20 ± 0.03 <sup>a</sup>	0.21 ± 0.02 <sup>a</sup>	8.8 ± 0.3 <sup>a</sup>	14.2 ± 0.5 <sup>a</sup>
Diquat	1 667.3 ± 1 065.7 <sup>b</sup>	12.2 ± 4.7 <sup>b</sup>	0.6 ± 1.3 <sup>b</sup>	2.0 ± 4.4 <sup>b</sup>	143.8 ± 29.1 <sup>b</sup>	0.06 ± 0.02 <sup>b</sup>	0.004 ± 0.006 <sup>b</sup>	0.03 ± 0.01 <sup>b</sup>	14.0 ± 1.5 <sup>b</sup>	3.3 ± 1.2 <sup>b</sup>

a,b For each experiment, any two values in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as determined with the nonparametric Mann-Whitney U-test.  $n = 5$  for all endpoints and experiments except for the pigment content determined in the second experiment where  $n = 4$ .

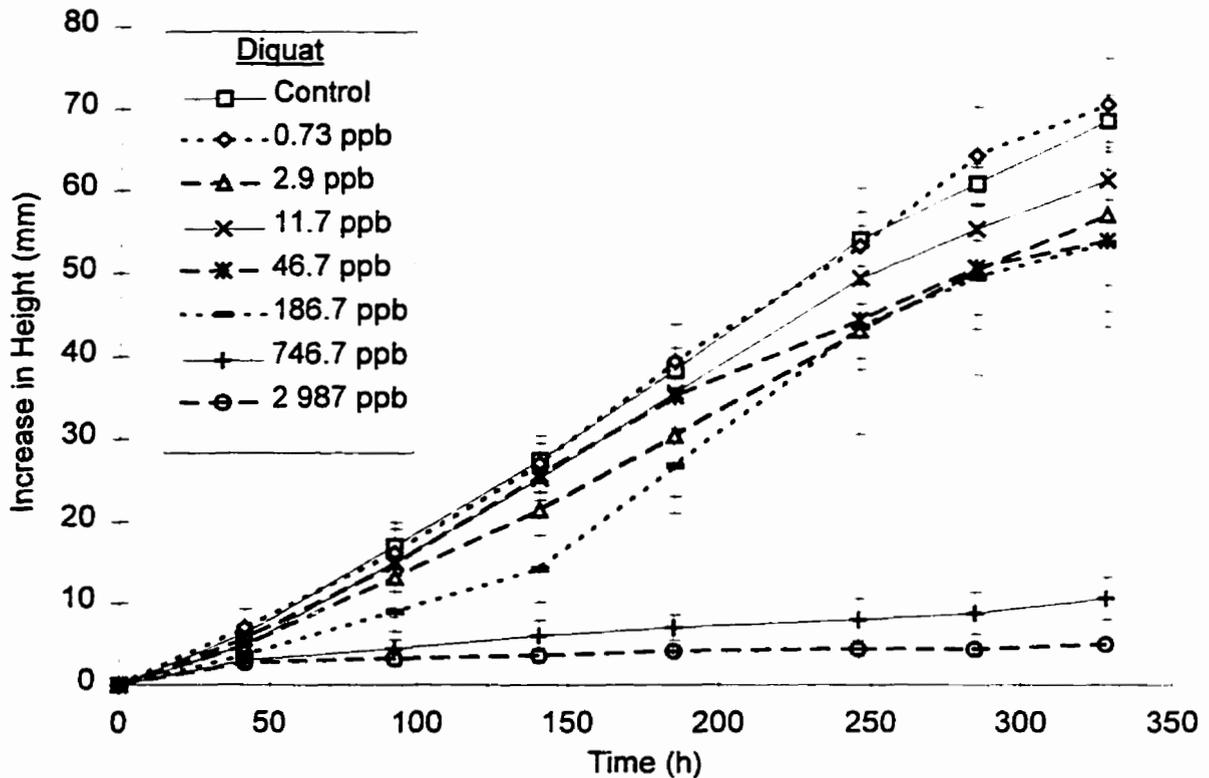


Figure 63: Fourteen day growth curves for *Myriophyllum sibiricum* plants exposed to diquat concentrations between 0.73 and 2987  $\mu\text{g/L}$  (second diquat dilution series) added to modified Andrews' medium.

(Table 56). The 95% confidence ranges overlap with the two IC50 values calculated in the first dilution series. Stimulation was observed for root length at low concentrations so the weibull and cumulative normal distribution programs could not interpret IC50 values from these data. Using curve fitting techniques, an IC50 value of 79.7 mg/L was estimated for root length but confidence limits could not be determined using these techniques. For the other growth parameters, all the 95% confidence intervals overlapped 46.7  $\mu\text{g/L}$  (Table 56) as would be expected from the results of the first dilution series.

The endpoint measurements from the third dilution series indicated reduced diquat toxicity as compared to the results from the previous two experiments (Table 55 and Table 56). The NOEC ranged from 4.1  $\mu\text{g/L}$  for carotenoid content to 110.6  $\mu\text{g/L}$  for the area under the growth curve and increase in plant height (Table 55). Based on the IC50, the most sensitive endpoint was root length with an IC50 of 105.7  $\mu\text{g/L}$  and 95 % c.i. of 84.0 to 133.0  $\mu\text{g/L}$  (Table 56). The decrease in *Myriophyllum sibiricum* sensitivity

between the first two dilution series and the third dilution series may have been caused by the change in medium for culturing the stock plants. It was observed that plants grown in modified Andrews' medium before experimentation had a greater initial fresh weight. This greater fresh weight might provide more resources for the apex to utilise.

Based on the NAS/NAE guidelines for the protection of aquatic life, diquat levels in freshwater should not exceed 0.0005 mg/L and diquat levels in marine systems should not exceed 0.15 mg/L (NAS/NAE, 1973 as cited in Nowell and Resek, 1994). The freshwater recommendation would just barely protect the *M. sibiricum* from the phytotoxic effects of diquat.

#### 5.7.3.3.3 Aquatic Plant Toxicity Comparison

Based on the results from the diquat dilution series, *M. sibiricum* in this toxicity test system appears to be fairly sensitive to the toxic effects of diquat when compared to other aquatic plant species (Table 57). Philips *et al.* (1992) compared inhibition of chlorophyll *a* content and cell number when ten species of algae were exposed to diquat (0.0 to 2.94 mg/L) for three and seven days. *Anabaena flos-aquae*, *Lyngbya wollei* (Farlow in Gomont) Speziale & Dyck and *Microcystis aeruginosa* (blue-green algae) were sensitive to diquat with 7 day IC<sub>50</sub> values for chlorophyll *a* of 130, 175 and 53 µg/L, respectively (Table 57). *Chlorella vulgaris* (green alga) was insensitive to the MLR of diquat after three days but by day seven the toxicity had increased to produce an IC<sub>50</sub> on chlorophyll *a* of 410 µg/L and an IC<sub>50</sub> on cell number of 307 µg/L (Table 57). Another green algal species, *Selenastrum capricornutum*, was sensitive to diquat as indicated by the inhibition of chlorophyll *a* content by 50% at 26 µg/L and the inhibition of cell number by 50% at 47 µg/L (Table 57). *Navicula* sp. (a freshwater diatom) demonstrated an IC<sub>50</sub> of 106 for chlorophyll *a* content. A marine diatom (*Skeletonema costatum*) and *Euglena gracilis* (Euglenophyceae) were unaffected by the MLR (Philips *et al.* 1992). After incubation for up to 48 h, diquat at a concentration of 3·10<sup>-5</sup> M (10 mg/L) did not affect oxygen production and chlorophyll content of *Chlorella pyrenoidosa* in flask culture. At this concentration of diquat, there was a slight decrease in cell number but no cell disruption (Zweig *et al.*, 1968).

Table 55: No observable effect concentrations for the effect of diquat upon the *Myriophyllum sibiricum* growth and development endpoint parameters.

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )		
	First Dilution Series	Second Dilution Series	Third Dilution Series
Area Under the Growth Curve	< 46.7	0.73	110.6
Increase in Shoot Length	< 46.7	11.7	110.6
Root Length	< 46.7	11.7	36.9
Root Number	< 46.7	11.7	36.9
Fresh Weight	46.7	11.7	36.9
Membrane Integrity	46.7	11.7	Treatments not different from control
Plant Area	< 46.7	11.7	12.3
Chlorophyll <i>a</i>	< 46.7	11.7	12.3
Chlorophyll <i>b</i>	46.7	< 0.73	12.3
Carotenoid	< 46.7	11.7	4.1

The effect of diquat upon *S. capricornutum* was examined by St. Laurent *et al.* (1992). They determined that the test system also influenced diquat toxicity during a comparison of the flask and microplate assays. With the flask assay system, 96 hour EC50s of 34.2  $\mu\text{g/L}$  (c.i. = 25.0 - 43.6  $\mu\text{g/L}$ ) were obtained, while much lower EC50s of 4.9  $\mu\text{g/L}$  (2.4 - 7.3  $\mu\text{g/L}$ ) were obtained in the microplate system (Table 57). They pointed out that the results from the microplate bioassay were not very reliable for this herbicide (St. Laurent *et al.*; 1992). The diquat EC50 for growth of *Chlamydomonas moewusii* was 1.8  $\mu\text{M}$  (0.3 mg/L). Zygospore germination was inhibited by 50% at a concentration of 83  $\mu\text{M}$  (15.3 mg/L) (Table 57) (Cain and Cain, 1983). The results from numerous other studies suggest that *M. sibiricum* in axenic culture was as sensitive to diquat as some of the algal species but much more sensitive than other species. It also demonstrated the need to test the effects of a chemical upon more than one aquatic plant species.

Table 56: IC50 values and 95% confidence intervals for the three dilution series conducted with diquat. The effect of diquat upon selected morphological and physiological characteristics of <i>Myriophyllum sibiricum</i> .			
GROWTH PARAMETER	IC50 (µg/L)		
	First Dilution Series	Second Dilution Series	Third Dilution Series
Area Under the Growth Curve	271.3 (196.9; 373.7) <sup>a</sup>	365.7 (241.1; 554.6) <sup>b</sup>	982.8 (769.8; 1254.8) <sup>a</sup>
Increase in Shoot Length	346.2 (152.4; 781.5) <sup>a</sup>	403.8 (259.8; 627.7) <sup>b</sup>	1610.9 (1370.4; 1893.6) <sup>a</sup>
Root Length	< 46.7 <sup>a,b</sup>	79.7 <sup>c</sup>	105.7 (84.0; 133.0) <sup>a</sup>
Root Number	< 46.7 <sup>a,b</sup>	57.0 (47.7; 68.0) <sup>b</sup>	155.1 (118.2; 203.5) <sup>a</sup>
Fresh Weight	< 46.7 <sup>a,b</sup>	78.2 (35.4; 172.7) <sup>b</sup>	184.0 (106.9; 317.1) <sup>a</sup>
Membrane Integrity	> 2987 <sup>a,b</sup>	> 2987 <sup>b</sup>	> 2987 <sup>a</sup>
Plant Area	< 46.7 <sup>a,b</sup>	56.2 (23.1; 137.0) <sup>b</sup>	127.7 (76.5; 213.1) <sup>a</sup>
Chlorophyll <i>a</i>	< 46.7 <sup>a,b</sup>	71.5 (18.4; 278.0) <sup>b</sup>	201.3 (141.2; 287.0) <sup>a</sup>
Chlorophyll <i>b</i>	< 46.7 <sup>a,b</sup>	36.9 (23.1; 59.0) <sup>b</sup>	206.8 (134.8; 317.1) <sup>a</sup>
Carotenoid	< 46.7 <sup>a,b</sup>	139.2 (35.1; 552.4) <sup>b</sup>	249.6 (160.8; 387.4) <sup>a</sup>

a IC50 values and 95% confidence intervals were calculated using the weibull model.

b IC50 and 95% confidence values were calculated with the cumulative normal distribution model.

c Stimulation occurred in this endpoint parameter so the weibull and cumulative normal distribution models were unable to calculate IC50 values. Using curve fitting techniques, IC50 values were estimated.

Four species of marine unicellular algae (*Chlorococcum* sp., *Dunaliella tertiolecta*, *Isochrysis galbana* and *Phaeodactylum tricornutum*) were exposed to diquat. For all four species, the EC50 for oxygen evolution after 90 min of exposure was greater than 5 000 mg/L. Growth was a more sensitive parameter for *I. galbana* and *P. tricornutum* because the ten day EC50 values were 15 mg/L diquat. Ten day growth of *D. tertiolecta* was inhibited by 50% at 30 mg/L of diquat and growth of *Chlorococcum* sp. was inhibited by 50% at 300 mg/L (Table 57) (Walsh, 1972).

An aquatic microcosm was used to examine the effects of 0.3, 1, 3, 10 and 30 mg/L diquat on a mixed algal population (green algae, diatoms and blue-greens). After treatment with diquat (10 and 30 mg/L) for 14 and 21 days, there was an increase in unicellular green algal density. The unicellular green alga, primarily *Oocystis*, *Characium*, *Ankistrodesmus*, *Cosmarium* and *Staurastrum*, were resistant to these high

Table 57: A summary of IC50 and EC50 values selected from the literature. This allows a comparison of diquat's effects on aquatic plant species even though the test conditions differed between species. *Myriophyllum sibiricum* was very slightly less sensitive to the effects of diquat than *Selenastrum capricornutum*. *Chlamydomonas moewusii* and *Chlorella vulgaris* were less sensitive to diquat.

Species	Endpoint	Exposure Duration	IC50/EC50 (µg/L)	Reference
<b>Algae</b>				
<i>Anabaena flos-aquae</i>	Chlorophyll a Growth	7 days	130	Philips <i>et al.</i> (1992)
<i>Chlamydomonas moewusii</i>	Zygosporer Germination	7 days	300	Cain and Cain (1983)
<i>Chlamydomonas moewusii</i>	Cell Number	7 days	15 300	Cain and Cain (1983)
<i>Chlorella vulgaris</i>	Chlorophyll a	7 days	307	Philips <i>et al.</i> (1992)
<i>Chlorella vulgaris</i>	Growth	7 days	410	Philips <i>et al.</i> (1992)
<i>Chlorococcum sp.</i>	Growth	10 days	200 000	Walsh (1972)
<i>Dunaliella tertiolecta</i>	Growth	10 days	30 000	Walsh (1972)
<i>Isochrysis galbana</i>	Growth	10 days	15 000	Walsh (1972)
<i>Phaeodactylum tricornutum</i>	Growth	10 days	15 000	Walsh (1972)
<i>Lyngbya wollei</i>	Chlorophyll a	7 days	175	Philips <i>et al.</i> (1992)
<i>Microcystis aeruginosa</i>	Chlorophyll a	7 days	53	Philips <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Cell Number	7 days	47	Philips <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Chlorophyll a	7 days	26	Philips <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	96 hours	34.2	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	4.9	St. Laurent <i>et al.</i> (1992)
<b>Submersed Macrophytes</b>				
<i>Myriophyllum sibiricum</i>	Chlorophyll b (dry)	14 days	36.9	This Document
<i>Myriophyllum sibiricum</i>	Root Number	14 days	57.0	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	56.2	This Document

concentrations of diquat. By day 14, diquat concentrations of 0.3, 10 and 30 mg/L had decreased diatom densities by 54 to 74% when compared to control levels.

Cyanobacteria were completely absent from all treatments by day 14. The most sensitive endpoint was the electron transport system where LOECs were measured between 0.18 and 0.33 mg/L diquat (Melendez *et al.*, 1993). The axenic *Myriophyllum sibiricum* toxicity test was more sensitive to the phytotoxic effects of diquat than the mixed algal microcosm.

Diquat was extremely inhibitory (99% to 100% of control values) to *Lemna minor* frond production and  $\text{NaH}^{14}\text{CO}_3$  uptake in seven different species of algae at a level of 2.99 mg/L. The other two species of algae studied (*Scenedesmus quadricauda* and *Selenastrum capricornutum*) were inhibited by 53% and 69% of the control (Peterson *et al.*, 1994). This was the same level of diquat (2.99 mg/L) used in the MLR experiments with *Myriophyllum sibiricum*. *M. sibiricum* was also severely inhibited at this level. The lower level of inhibition in two of the algal species studied by Peterson *et al.* (1994) reinforces the need for multi-species testing. *Selenastrum capricornutum* is one of the most commonly tested algal species and if *S. capricornutum* were the only species tested, environmental risk would be underestimated.

Birmingham and Coleman (1983) examined the effects of soil-bound diquat (Reglone<sup>®</sup> formulation) upon *Anaebana flos-aquae* and *Lemna* sp. Chlorophyll content of *A. flos-aquae* was strongly inhibited by 33.3 mg/L and growth ceased at 334 mg/L Reglone<sup>®</sup>. *Lemna* sp. was unaffected by soil-bound Reglone<sup>®</sup> at 0.3 and 3.3 mg/L but growth ceased at 33.4 and 334 mg/L (Birmingham and Coleman, 1983). *Lemna minor* was rapidly bleached by diquat at 1 mg/L after one day. By the eleventh day of treatment, toxic effects at 0.5  $\mu\text{g/L}$  were noticeable (Funderburk and Lawrence, 1963). Membrane leakage from *Lemna minor* fronds occurred at diquat concentrations of 1.8  $\mu\text{g/mL}$  after 3 h and 0.00018  $\mu\text{g/mL}$  after 72 h. At the lowest concentration, visible injury was not evident (O'Brien and Prendeville, 1978). Light influenced the effect of diquat on membrane permeability (MacDonald *et al.*, 1993a; O'Brien and Prendeville, 1978). Light intensity and quality have been shown to affect the toxicity of diquat to giant duckweed (*Spirodela polyrrhyza* (L.) Schleid (giant duckweed)) (Blackburn and Weldon, 1965) but the effect of light was not examined in the current study. Leakage of cellular content was observed from a microcosm containing a mixed algal population at concentrations greater than or equal to 3 mg/L (Melendez *et al.*, 1993). As determined by the NOEC

results in the first two *Myriophyllum sibiricum* dilution series, membrane permeability of this species (Table 55) was affected to a greater degree than that of *Lemna minor* or the mixed algal population.

The impact of diquat upon *Hydrilla verticillata* (a rooted aquatic macrophyte) was examined by Cassidy and Rodgers (1989). They calculated a lethal concentration of 600 mg/L dry weight and found that membrane disruption was more sensitive than chlorophyll *a* content (Cassidy and Rodgers, 1989). Membrane disruption and chlorophyll *a* content were equally sensitive endpoint parameters in the *M. sibiricum* toxicity test.

Sutton (1985) determined that older *M. aquaticum* appeared to be more susceptible to diquat than younger plants. A rate of 0.02 mg/L resulted in a dry weight decrease when the treatment was compared to the control. Since these authors did not state the percentage of decrease in the treatment, it is difficult to compare the results to those obtained in the current study.

*Myriophyllum exalbescens*, *Potamogeton crispus*, *P. foliosus* Raf. (leafy pondweed), *P. pectinatus*, *P. pusillus* L. (small pondweed) and *Ranunculus trichophyllus* Chaix (white buttercup) were eliminated from a study area by 0.5 mg/L diquat (Hiltibrant, 1965). Yeo (1967) also found that 500 µg/L completely controlled American milfoil in a reservoir study. That author found that *Chara* was tolerant to diquat at rates up to 1 000 µg/L while American elodea and southern naiad (*Najas guadalupensis* (Spreng.) Mangus) were controlled for longer than 8 weeks by a concentration of 250 µg/L (Yeo, 1967). Both the study by Hiltibrant (1965) and Yeo (1967) were conducted to determine diquat concentrations to control nuisance aquatic vegetation. If aquatic vegetation control was the purpose of a study, the *Myriophyllum sibiricum* data from the axenic toxicity test would provide a rough estimate of toxic concentrations.

The amount of diquat absorbed by three algal species differed. *Navicula pelliculosa* absorbed the largest amount (mg/g dry weight) of diquat while *Chlorella vulgaris* absorbed the least. The authors determined that between 40 and 70% of the diquat was adsorbed to organic binding sites on the plant's surface via ion exchange (Birmingham and Colman, 1983). This adsorbed herbicide may become detrimental to other stages in the food web. It would be interesting to quantify pesticide absorption with the *Myriophyllum sibiricum* toxicity test.

#### 5.7.3.3.4 Conclusions

If the expected environmental concentration of diquat (2.99 mg/L) was to enter an aquatic system, both the algal and macrophyte populations would be affected. Pigment content, root length and plant area of *M. sibiricum* plants would be reduced. One percent of the EEC could cause inhibitory effects to plant area and pigment content. Long term diquat exposure effects have not been determined but caution should be exercised to limit the amount of diquat that enters non-target wetland areas.

Unfortunately, the research that has been conducted on aquatic macrophytes determined herbicide efficacy or physiological properties so a comparison with *M. sibiricum* toxicity could not be made. In a comparison of the toxicological effects of diquat on algae and *M. sibiricum*, *Selenastrum capricornutum* was the most sensitive species (Table 57). If the microplate toxicity test results were suspect as suggested by St. Laurent *et al.* (1992), *M. sibiricum* was just slightly less sensitive than *S. capricornutum*. Based on diquat toxicity data in the scientific literature, most other algal species were quite tolerant to diquat. They were 3.5 to 5 400 times more tolerant to diquat than chlorophyll *b* content of *M. sibiricum* (Table 57).

#### 5.7.4 Fluridone

##### 5.7.4.1 Introduction

Fluridone was first introduced in 1976 to control annual grasses and broadleaf weeds (Ashton and Crafts, 1981). In the United States, fluridone (Figure 64) is used to control aquatic weed problems and it can be applied to water bodies as a dispersible liquid, wettable powder or granular formulation (Murphy and Barrett, 1993; Wauchope *et al.*, 1992). This systemic, aquatic herbicide controls mainly submersed and floating species, such as bladderwort, coontail, elodea, watermilfoil, naiad, pondweed, hydrilla

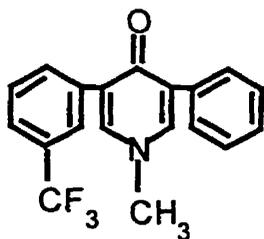


Figure 64: Fluridone's molecular structure.

and paragrass (Anderson, 1993; Murphy and Barrett, 1993; Steward, 1993; WSSA, 1994). Fluridone is absorbed into the plant shoots from the water and into the roots from the hydrosol. The herbicidal mode of action of fluridone is the blockage of carotenoid biosynthesis by inhibiting phytoene desaturase (Bartels and Watson, 1978; Sandmann and Böger, 1989; WSSA, 1994). Phytoene and phytofluene, carotenoid precursors, accumulate in treated plants. The

phytotoxic action of fluridone is modified by light intensity (Anderson, 1981b; Bartels and Watson, 1978; Drexler and Fletcher, 1981). Without the carotenoid pigments, triplet state chlorophyll and singlet oxygen are not quenched, which leads to the destruction of chlorophyll and cellular damage (Kunert and Dodge, 1989) including lipid peroxidation and protein and membrane destruction (WSSA, 1994).

The primary degradation pathway of fluridone in aquatic systems is photolysis, while volatilisation and biodegradation play minor roles (Muir and Grift, 1982; Reinert and Rodgers, 1987). In laboratory studies, there was a lack of fluridone degradation in autoclaved sediments (Muir and Grift, 1982), thus degradation might be microbial. Fluridone degradation is also influenced by the size of the area treated in relationship to the size of the water body, water depth, dissolved oxygen, turbidity (West *et al.*, 1983), formulation (Mossler *et al.*, 1993; West *et al.*, 1983), timing of application (West *et al.*, 1983; 1990) and organic matter content (Loh *et al.*, 1979; Muir *et al.*, 1980; Shea and Weber, 1983). The half-life of fluridone in water appears to be site specific with half-lives ranging from 2 (Muir and Grift, 1982; Reinert and Rodgers, 1987) to 60 days (Reinert and Rodgers, 1987; West *et al.*, 1983). In a dissipation study using 40 ponds and lakes, the average half-life was 20 days in water and 3 months in the hydrosol (West *et al.*, 1983). Only 4% of the initial fluridone application was detected 110 days after application (West and Parka, 1981). In another study, the fluridone concentration in the water decreased to nondetectable levels by 324 days after treatment (West *et al.*, 1990). Once in an aquatic environment, 10% of fluridone would partition into the water and 43% would partition into the sediment (Reinert, 1989). Different soil components have the ability to bind different amounts of fluridone (Mossler *et al.*, 1993; Shea and Weber, 1983) but once adsorbed to the sediment, fluridone is tightly bound (Reinert, 1989). Under laboratory conditions, the average half-life of fluridone in three sediment types was 12 months, while under field conditions the fluridone half-life in sediment was about 17 weeks (Muir and Grift, 1982). The half-life of fluridone in the hydrosol was greater than one year in three ponds on the Canadian prairie (Muir *et al.*, 1980). In summary, fluridone persistence in aquatic environments could range from 2 days to 12 months (Reinert and Rodgers, 1987).

#### 5.7.4.2 Methods

Technical fluridone at a purity of 99.2% was used in this study. It was not possible to dissolve the fluridone in solution at the predicted solubility level of 10 mg/L (Wauchope *et al.*, 1992) or 12 mg/L (WSSA, 1994) (Table 27). This may have been caused by interference from other ions in solution (Conder *et al.*, 1995). Thus, the technical fluridone was dissolved in methanol before addition to the liquid plant medium. Water controls and methanol controls were used for all the experiments. Stock solutions of technical fluridone dissolved in methanol were prepared on the day prior to experimental initiation.

For fluridone, the recommended maximum label rate (MLR) is 4.5 kg/ha (WSSA, 1989). If this amount was sprayed onto a 15 cm column of water, it would be equivalent to an expected environmental concentration (EEC) of 3.0 mg/L (Table 27). Starting on June 9, 1994 and April 3, 1995, two EEC experiments were conducted. Plants used in the first experiment were cultured in M & S medium, while plants used in the second EEC experiment were cultured in modified Andrews' medium. For both experiments, modified Andrews' medium was used as the experimental medium.

Two dilution series experiments with fluridone were conducted starting on April 3, 1995 and May 4, 1995. The first dilution series contained fluridone concentrations of 46.9, 93.8, 187.5, 375, 750, 1 500 and 3 000 µg/L plus the water and methanol controls. Fluridone concentrations of 4.1, 12.3, 37, 111, 333, 1 000 and 3 000 µg/L were used in the second dilution experiment. All concentrations and controls were replicated five times. Modified Andrews' medium was used for both plant culturing and experimentation. The methods outlined in Section 4.6 were followed.

#### 5.7.4.3 Results and Discussion

##### 5.7.4.3.1 Expected Environmental Concentration

In plants treated with the EEC (3.0 mg/L) of fluridone, all new growth, including the main shoot and lateral buds, was pink in colour (Figure 66). This pink coloration probably indicated that only the anthocyanins remained. After fourteen days of treatment, chlorophyll *a*, chlorophyll *b* and carotenoid concentration, as measured spectrophotometrically, were the most severely affected endpoints. (Table 58). Pigment content of treated apices decreased by almost 100% when compared to the control plants (Table 59). Fluridone inhibited the production of carotenoid pigments, which

allowed for the photodegradation of the chlorophyll pigments. This decrease in photosynthetic pigments also caused a decrease in the amount of oxygen dissolved in the liquid growth medium (Table 58 and Table 59). In fluridone treated plants, the root length, fresh weight and plant area differed from the controls. Fluridone had a minimal effect upon area under the growth curve (Figure 65) and plant height (Figure 66). Even though plant height was not highly inhibited by fluridone, the increase in the number of nodes over the fourteen day experimental period was less in fluridone treated plants. This suggested that the plants were spindly and elongating rapidly without a normal amount of cellular division. Only in the second EEC experiment was there a toxic effect on membrane permeability of *M. sibiricum* (Table 60). In the literature, leakage from membranes has been commonly cited because fluridone initiates the destruction of membrane lipids and proteins (WSSA, 1994). Even though the EEC experiments were conducted ten months apart, the results were similar between the two experiments (Table 59 and Table 60).

For most of the endpoint parameters examined, there was no significant difference between the methanol and water controls (Table 59 and Table 60). These results, as well as those determined earlier in the solvent experiments, supported the

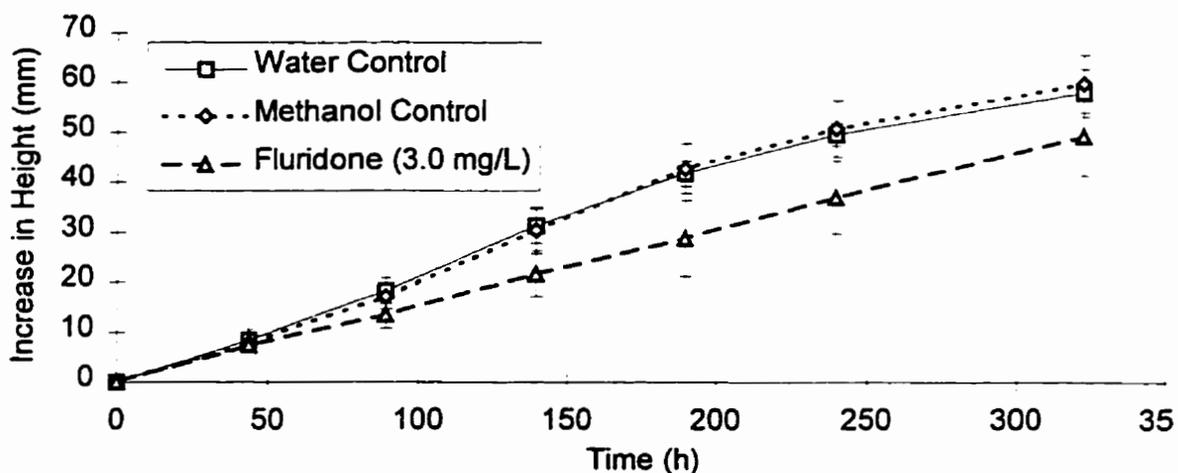


Figure 65: The influence of fluridone, at the EEC of 3.0 mg/L, on the growth of *Myriophyllum sibiricum* in axenic culture. Fluridone was dissolved in methanol before being mixed into the modified Andrews' medium. Data are the mean of five replicates with standard deviation bars for the fluridone treatment, as well as the water and methanol controls.

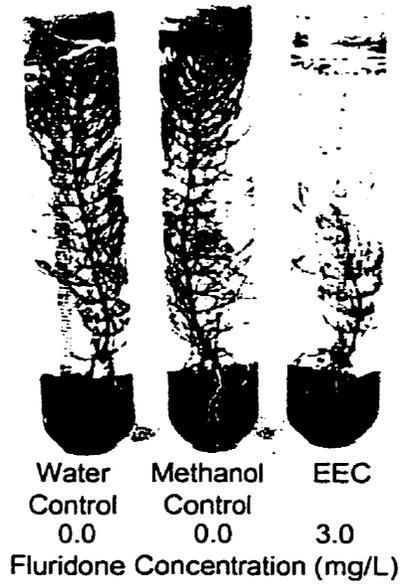


Figure 66: Test tubes containing *Myriophyllum sibiricum*. From left to right: a water control plant, a methanol control plant and a plant treated with the EEC (3.0 mg/L) of fluridone. These plants had been cultured in sterile modified Andrews' medium for two weeks.

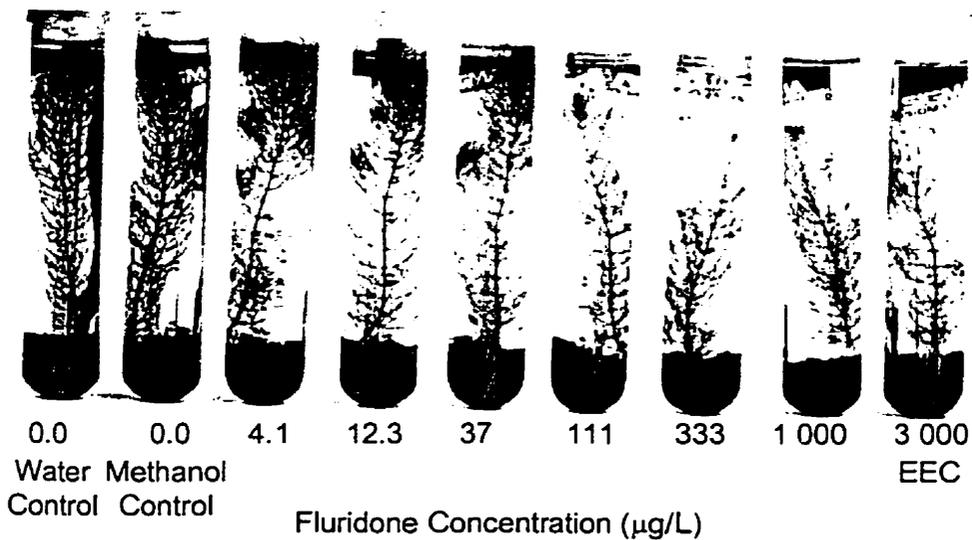


Figure 67: The effect of fluridone upon the growth of *Myriophyllum sibiricum* after fourteen days of incubation in modified Andrews' medium spiked with different herbicide concentrations.

Table 58: Influence of the EEC of fluridone (3.0 mg/L) on the pigment content of *Myriophyllum sibiricum* apices. After 14 days of incubation in sterile modified Andrews' medium, pigment content of treated plants was inhibited, as statistically compared with either the methanol control or the combined water and methanol controls.

Treatment	Chlorophyll <i>a</i> Content (mg/g fresh weight)	Chlorophyll <i>b</i> Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll <i>a</i> Content (mg/g dry weight)	Chlorophyll <i>b</i> Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
<b>First Expected Environmental Concentration Experiment</b>						
Combined Controls	0.50 ± 0.18 <sup>a</sup>	0.18 ± 0.07 <sup>a</sup>	0.17 ± 0.06 <sup>a</sup>	Not Measured	Not Measured	Not Measured
Methanol Control	—	—	—	Not Measured	Not Measured	Not Measured
Fluridone	0.003 ± 0.001 <sup>b</sup>	0.003 ± 0.003 <sup>b</sup>	0.003 ± 0.002 <sup>b</sup>	Not Measured	Not Measured	Not Measured
<b>Second Expected Environmental Concentration Experiment</b>						
Combined Controls	—	—	—	7.49 ± 2.46 <sup>a</sup>	2.82 ± 0.93 <sup>a</sup>	2.54 ± 0.81 <sup>a</sup>
Methanol Control	0.54 ± 0.04 <sup>a</sup>	0.20 ± 0.02 <sup>a</sup>	0.19 ± 0.01	—	—	—
Fluridone	0.0003 ± 0.0006 <sup>b</sup>	0.0004 ± 0.001 <sup>b</sup>	0.003 ± 0.001 <sup>b</sup>	0.006 ± 0.0003 <sup>b</sup>	0.008 ± 0.01 <sup>b</sup>	0.07 ± 0.02 <sup>b</sup>

a,b For each EEC experiment, any means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test or the parametric t-test. For all parameters,  $n = 4$  for the methanol controls in the first EEC experiment and  $n = 5$  for the water control and the fluridone treatment. If there was no significant difference between the methanol and water control ( $\alpha = 0.05$ ), the data were combined for statistical analysis and presentation in this table. The data presented are the untransformed mean  $\pm$  s.d.

hypothesis that methanol did not affect the growth and development of *M. sibiricum* in axenic culture.

Table 59: Inhibition of the growth and development of *Myriophyllum sibiricum* plants by fluridone at a concentration of 3.0 mg/L when compared with the control plants. All experimental plants were incubated for two weeks in test tubes containing sterile modified Andrews' liquid growth medium. The stock plants used in the first experiment were cultured in M & S medium, while the stock plants utilised in the second experiment were cultured in modified Andrews' medium.

GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)	
	First Experiment	Second Experiment
Area Under the Growth Curve	7.4 $\pm$ 12.3 <sup>a</sup>	24.9 $\pm$ 13.0 <sup>a</sup>
Increase in Shoot Length	10.8 $\pm$ 8.6 <sup>a</sup>	13.8 $\pm$ 10.1 <sup>a</sup>
Increase in Number of Nodes	44.7 $\pm$ 9.3 <sup>a</sup>	24.8 $\pm$ 7.2 <sup>a</sup>
Root Number	10.0 $\pm$ 26.4 <sup>a</sup>	8.0 $\pm$ 19.2 <sup>a</sup>
Root Length	70.6 $\pm$ 7.8 <sup>a</sup>	62.7 $\pm$ 5.8 <sup>a</sup>
Dissolved Oxygen	70.0 $\pm$ 3.8 <sup>a</sup>	61.3 $\pm$ 11.6 <sup>a</sup>
Fresh Weight	49.3 $\pm$ 10.6 <sup>a</sup>	51.9 $\pm$ 10.2 <sup>a</sup>
Membrane Integrity	1.4 $\pm$ 3.2 <sup>a</sup>	4.2 $\pm$ 2.5 <sup>a</sup>
Plant Area	76.0 $\pm$ 15.3 <sup>a</sup>	62.4 $\pm$ 7.0 <sup>a</sup>
Chlorophyll <i>a</i> (fresh)	99.4 $\pm$ 0.2 <sup>a</sup>	99.9 $\pm$ 0.1 <sup>b</sup>
Chlorophyll <i>b</i> (fresh)	98.5 $\pm$ 1.5 <sup>a</sup>	99.8 $\pm$ 0.5 <sup>b</sup>
Carotenoid (fresh)	98.3 $\pm$ 1.1 <sup>a</sup>	98.2 $\pm$ 0.6 <sup>b</sup>
Chlorophyll <i>a</i> (dry)	Not measured	99.9 $\pm$ 0.2 <sup>a</sup>
Chlorophyll <i>b</i> (dry)	Not measured	99.7 $\pm$ 0.6 <sup>a</sup>
Carotenoid (dry)	Not measured	97.4 $\pm$ 0.7 <sup>a</sup>

a Percent inhibition was calculated based on the combined mean of the water and methanol controls.

b For the calculation of percent inhibition, the mean of five methanol control plants was utilised in analysis of the second experiment.

Table 60: Morphological and physiological effects of fluridone (3.0 mg/L) upon the growth and development of *Myriophyllum sibiricum* after two weeks of incubation. All experimental plants were cultured axenically in modified Andrews' medium with the addition of water, methanol or the EEC of fluridone.

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Increase in Number of Nodes	Root Number	Total Root Length (mm)	D.O. (%)	Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
<b>First Expected Environmental Concentration Experiment</b>									
Combined Controls	9 484.7 ± 638.1 <sup>a</sup>	65.9 ± 2.6 <sup>a</sup>	14.1 ± 2.2 <sup>a</sup>	8.9 ± 1.2 <sup>a</sup>	416. ± 110.4 <sup>a</sup>	76.7 ± 20.0 <sup>a</sup>	511.9 ± 78.3 <sup>a</sup>	8.1 ± 1.2 <sup>a</sup>	10.2 ± 2.7 <sup>a</sup>
Fluridone	8 779.5 ± 1 170.7 <sup>a</sup>	58.8 ± 5.7 <sup>b</sup>	7.8 ± 1.3 <sup>b</sup>	8.0 ± 2.3 <sup>a</sup>	122.8 ± 32.4 <sup>b</sup>	23.0 ± 2.9 <sup>b</sup>	259.6 ± 54.3 <sup>b</sup>	8.8 ± 1.6 <sup>a</sup>	2.4 ± 1.6 <sup>b</sup>
<b>Second Expected Environmental Concentration Experiment</b>									
Combined Controls	10 639.4 ± 866.1 <sup>a</sup>	61.4 ± 3.3 <sup>a</sup>	12.5 ± 1.6 <sup>a</sup>	10.0 ± 2.0 <sup>a</sup>	495.1 ± 154.5 <sup>a</sup>	49.1 ± 14.4 <sup>a</sup>	765.0 ± 122.2 <sup>a</sup>	9.9 ± 1.4 <sup>a</sup>	12.6 ± 2.6 <sup>a</sup>
Fluridone	7 991.4 ± 1 379.1 <sup>b</sup>	53.0 ± 6.2 <sup>b</sup>	9.4 ± 0.9 <sup>b</sup>	9.2 ± 1.9 <sup>a</sup>	184.5 ± 28.6 <sup>b</sup>	17.4 ± 5.2 <sup>b</sup>	367.9 ± 78.3 <sup>b</sup>	12.0 ± 1.2 <sup>b</sup>	4.7 ± 0.9 <sup>b</sup>

a,b For each EEC experiment, any means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test and parametric t-test. The control data were combined for all these endpoint parameters because there was no significant difference between the water and methanol controls. For all parameters, the combined control consisted of 9 data values in the first EEC experiment, the combined control consisted of 10 values in the second experiment and there were 5 replicates in the both fluridone treatments. The data presented are the untransformed mean  $\pm$  s.d.

#### 5.7.4.3.2 Dilution Series

The effect of a series of fluridone concentrations upon *Myriophyllum sibiricum* growth was conducted because the expected environmental concentration was phytotoxic. Ten out of the forty-five plants used in the first fluridone dilution series became contaminated. A hundred percent survival was obtained with the second fluridone dilution series so those results are presented. General comparison of the two experiments suggested that the results were similar where the concentrations overlapped. In the first experiment, pigment content at all fluridone concentrations differed from the control. Therefore, the minimum concentration tested was lowered from 46.9 µg/L in the first experiment to 4.1 µg/L in the second experiment. The effect of fluridone upon the growth of *Myriophyllum sibiricum*, as obtained from the second dilution series, is shown in Figure 67 and Figure 68. The pink coloration of new apical and lateral growth is most striking in Figure 67. This change in coloration was caused by an inhibition of carotenoid synthesis and the photodestruction of chlorophyll content (Doong *et al.*, 1993). Statistical analysis of the dilution series data demonstrated that the pigment content of the apices was the most sensitive endpoint parameter (Table 61). This corresponded with fluridone's mode of action as an inhibitor of carotenoid synthesis (Table 27). The IC<sub>50</sub> values were all less than 88 µg/L (Table 61). Based on the NOEC analysis, all the treatments were significantly less than the controls for chlorophyll *a* and carotenoid content (fresh weight). Thus, fluridone was toxic to *M. sibiricum* at concentrations less than 1/729 or 0.14% of the EEC. For chlorophyll *b* content based on dry weight, the treatments also differed from the controls. At 4.1, 12.3 and 37 µg/L, the chlorophyll *b* content was slightly stimulated but it was stimulated less than 20%. The chlorophyll *b* content at 111, 333, 1 000, 3 000 µg/L was reduced.

This sudden change from stimulation to inhibition could be the reason that there were large confidence intervals around the IC<sub>25</sub> and IC<sub>50</sub> values (Table 61). In other plant species, pigment content differed from the controls at fluridone concentrations greater than 10 µg/L (Drexler and Fletcher, 1981; Westerdahl and Hall, 1987). For all the fluridone treatments, the amount of dissolved oxygen in the medium was significantly different from the control oxygen levels. Because there was significant inhibition in dissolved oxygen at all treatments, no IC<sub>25</sub> or IC<sub>50</sub> could be calculated. To determine the lowest concentration at which fluridone does not inhibit pigment content

as compared to the control, the dilution series could be repeated using concentrations lower than 100  $\mu\text{g/L}$ . For plant length and fresh weight, the treatment values did not differ from the control at fluridone concentrations of 4.1  $\mu\text{g/L}$ . Statistically significant stimulation in plant length and fresh weight of *M. sibiricum* plants was observed at 12.3  $\mu\text{g/L}$ . No significant difference from the controls was observed at fluridone concentrations of 37  $\mu\text{g/L}$  for fresh weight or at concentrations of 37 and 111  $\mu\text{g/L}$  for plant length. Thus, the LOEC values were 111  $\mu\text{g/L}$  and 333  $\mu\text{g/L}$  for fresh weight and plant length, respectively (Table 61). The stimulation was not enough to calculate SC20 values. Even though there was stimulation in plant height, there was inhibition of node number. Some of the LOECs obtained from this experiment were higher than the IC25 and IC50 values (Table 61). The weibull and cumulative normal models used to

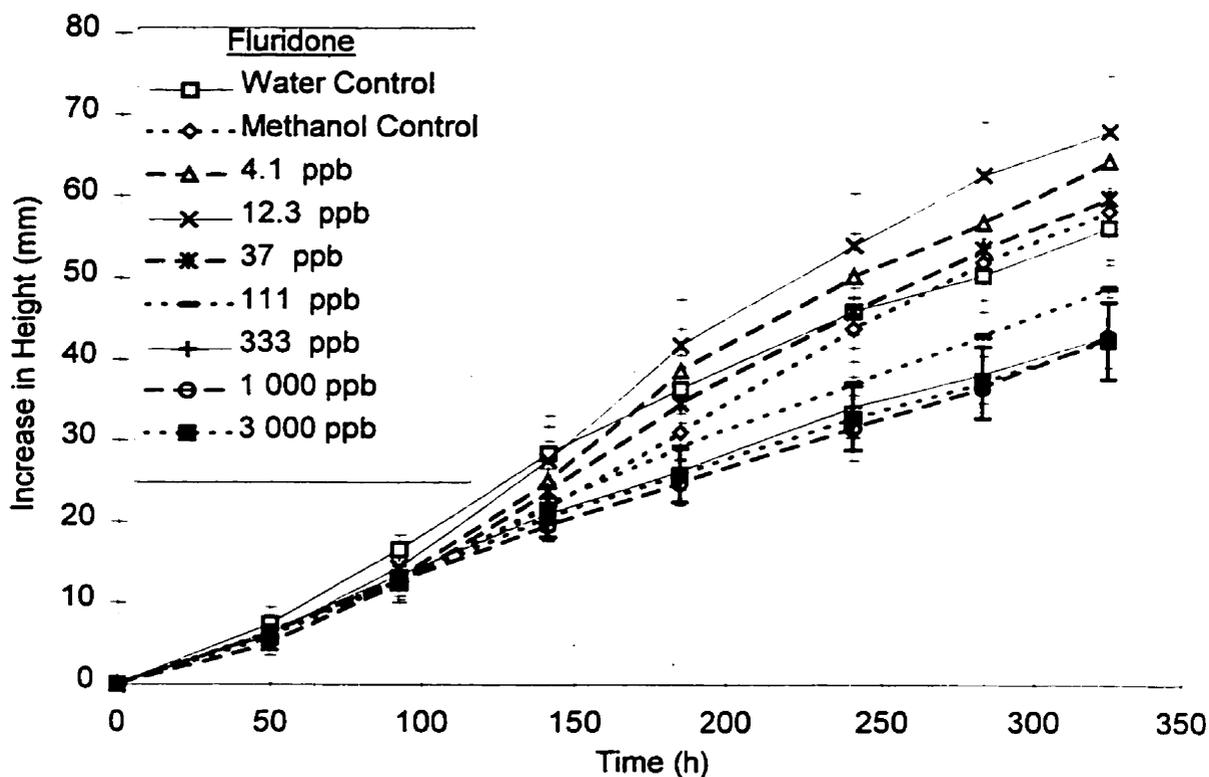


Figure 68: Growth of *Myriophyllum sibiricum* plants in axenic culture with various fluridone concentrations and modified Andrews' medium for fourteen days. Both a water control and methanol control were used since the fluridone was dissolved in methanol before addition to the growth medium. For both parameters displayed in this graph, increase in plant length and area under the growth curve, there was no statistical difference between the two controls ( $\alpha = 0.05$ ).

Table 61: The effect of fluridone upon *Myriophyllum sibiricum* growth and development endpoint parameters. The data from the second dilution series are presented as the no observable effect concentration, the average percent inhibition ( $\pm$  s.d.) at the NOEC, LOEC, IC25 and IC50 values (upper and lower 95% c.i.).

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	% I at NOEC	LOEC ( $\mu\text{g/L}$ )	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	111	15.1 $\pm$ 13.9	333	780.9 (105.0, 5 810.4) <sup>a</sup>	> 3 000 <sup>a,b</sup>
Increase in Shoot Length	4.1	-12.6 $\pm$ 7.4	333	1 159.2 (108.1, 12 442.7) <sup>a</sup>	> 3 000 <sup>a,b</sup>
Increase in Number of Nodes	37	-0.9 $\pm$ 7.2	111	368.9 (59.8, 2277.1) <sup>b</sup>	> 3 000 <sup>b</sup>
Root Length	37	16.9 $\pm$ 15.8	111	38.9 (6.7, 226.8) <sup>a</sup>	650.3 (122.3, 3 459.0) <sup>a</sup>
Root Number	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Fresh Weight	4.1	-8.5 $\pm$ 13.0	111	81.7 (19.8, 337.7) <sup>a</sup>	784.2 (211.1, 2 913.2) <sup>a</sup>
Membrane Integrity	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Plant Area	12.3	1.8 $\pm$ 11.8	37	36.7 (10.2, 132.8) <sup>a</sup>	262.3 (85.2, 807.6) <sup>a</sup>
Chlorophyll <i>a</i> (fresh weight)	All Different	All Different	All Different	38.0 (33.7, 42.9) <sup>b</sup>	50.4 (42.7, 59.5) <sup>b</sup>
Chlorophyll <i>b</i> (fresh weight)	4.1	5.5 $\pm$ 10.9	12.3	37.0 (33.9, 40.4) <sup>b</sup>	51.2 (46.8, 56.1) <sup>b</sup>
Carotenoid (fresh weight)	All Different	All Different	All Different	29.1 (20.6, 41.0) <sup>b</sup>	44.6 (36.6, 54.2) <sup>b</sup>
Chlorophyll <i>a</i> (dry weight)	37	-10.5 $\pm$ 14.1	111	76.7 (4.9, 1 203.5) <sup>b</sup>	85.1 (11.8, 612.9) <sup>b</sup>
Chlorophyll <i>b</i> (dry weight)	All Different	All Different	All Different	77.5 (0.4, 13 794.9) <sup>b</sup>	87.2 (2.7, 2 834.2) <sup>b</sup>
Carotenoid (dry weight)	37	11.8 $\pm$ 17.8	111	45.6 (40.9;50.8) <sup>b</sup>	60.3 (54.4, 66.9) <sup>b</sup>

a IC<sub>p</sub> and 95% confidence values were calculated with the weibull method.

b IC<sub>p</sub> values and 95% confidence intervals were calculated with the cumulative normal distribution model.

Data from both control treatments were used in the data analysis of area under the curve, plant length, root number, fresh weight, membrane integrity, plant area and chlorophyll *b* (dry weight). Only the methanol control values were used in the analysis of root length, chlorophyll *a* (fresh and dry weight), chlorophyll *b* (fresh weight) and carotenoid content (fresh and dry weight).

determine the IC25 and IC50 values could extrapolate between the concentrations tested, while the NOECs and LOECs were based upon actual fluridone concentrations tested. Refer to Section 5.6 for a further explanation of LOEC, as used in this document. Plant area was also severely affected by fluridone since a NOEC of 12.3 µg/L was obtained in this study. Whether this was a true inhibition or an artefact of the photocopying system was not known. The pink coloured plants did not photocopy well so this parameter might underestimate the true plant area. Fluridone did not affect the membrane permeability of *M. sibiricum* (Table 61) even though other authors have noted the destruction of membrane lipids and proteins (WSSA, 1994).

The MLR of 4.5 kg a.i. fluridone per hectare of surface area treated used in this study was recommended in an early edition of the WSSA Herbicide Handbook (WSSA, 1989). This recommended value has since been lowered to 2.24 kg a.i./ha (WSSA, 1994). If this amount was sprayed into a 15 cm column of water, an EEC of 1.49 mg/L would result. This is half of the EEC used in the current study. As seen in the results from the dilution series, toxic effects would be noted at this lower rate. Fluridone does not degrade rapidly under sterile conditions (Muir and Grift, 1982) so it was assumed that the nominal fluridone concentrations would remain in the test tube system for the two week period.

As of 1994, there were no standards or guidelines in the United States for the protection of aquatic life from the toxic effects of fluridone (Nowell and Resek, 1994). Based on the results from this study with *M. sibiricum*, guidelines should be set at less than 25 mg/L for the complete protection of aquatic plants from the phytotoxic effects of fluridone.

#### 5.7.4.3.3 Aquatic Plant Toxicity Comparison

Numerous reports in the scientific literature consider the efficacy of fluridone for the control of aquatic plants (Fox *et al.*, 1994; Kay, 1991; Netherland *et al.*, 1993; Struve *et al.*, 1991; Westerdahl and Hall, 1987) but there are very few studies that provide aquatic plant toxicity data in terms of EC50 or NOEC (Table 62). This discussion will focus on the phytotoxicity of fluridone to aquatic plants.

After 4 days of treatment, 50% inhibition of chlorophyll production in *Chlamydomonas reinhardtii* was obtained by a technical fluridone concentration of  $6.3 \cdot 10^{-6}$  M (2.1 mg/L) (Table 62) (Fedtke, 1992). Twenty-four hours after inoculation

with 6.1  $\mu\text{M}$  (2.0 mg/L) of technical fluridone, cells of *C. reinhardtii* were bleached, growth and cell division were inhibited and this concentration was the IC50 for growth after 48 hours. The IC50 for flagella regeneration in *C. reinhardtii* was determined to be 121  $\mu\text{M}$  (39.85 mg/L) after 96 to 120 hours (Table 62) (Fedtke, 1982). In a field study, two sequential applications of fluridone at a rate of 125  $\mu\text{g a.i./L}$  changed algal species composition. In one of the two ponds, phytoplankton number and algal chlorophyll content were significantly decreased in treated enclosures (Struve *et al.*, 1991).

Over a four day period, *Lemna minor* plants were exposed to C-14 radiolabelled pesticides in a static flask experiment. The amount of C-14 that accumulated after 4, 24, 48 and 96 hours was determined. Fluridone had a bioconcentration factor (BCF) of 6 in distilled water and a BCF of 4 in river water, which indicated that it was not bioconcentrated to any significant amount. At fluridone concentrations between 1 and 0.01 mg/L, the 20 day growth curves for *L. minor* based on mean frond number (Lockhart *et al.*, 1983) were similar in shape to *M. sibiricum* growth curves (Figure 68). In a laboratory toxicity test, *Lemna minor* plants exposed to sediment samples containing 0.91 to 6.92 mg/L of fluridone showed signs of chlorosis (Muir *et al.*, 1980).

Twenty-one day old rice (*Oryza sativa* L.) seedlings were treated with technical fluridone at a concentration of  $6.07 \cdot 10^{-7}$  M (0.2 mg/L). Phytotoxic symptoms developed after three to five days of exposure. When compared with fluridone tolerant plant species, the fluridone was translocated to the shoots more rapidly in the rice (Berard *et al.*, 1978). Chromatograms of *M. sibiricum* extracts showed two distinct anthocyanins (Ceska and Ceska, 1985). When fluridone inhibits the synthesis of carotenoids and light degrades chlorophyll, the remaining pigment is most likely anthocyanin and this pigment gives all new tissue a pink coloration (Doong *et al.*, 1993). Seven to ten days after fluridone treatment, most species of plants show white or pink growing tips (WSSA, 1994). Apical tips of *Hydrilla verticillata* became bleached and purple in colour within four days of treatment with fluridone (Sonar<sup>®</sup> AS) at rates greater than 15  $\mu\text{g/L}$  (Netherland and Getsinger, 1995b) and treatment with 5 or 50  $\mu\text{g/L}$  fluridone (Doong *et al.*, 1993). These growing tips continued to elongate. Some treated apical shoots of *Myriophyllum spicatum* became albescent but others turned brown and necrotic within seven days of treatment. Seven days after treatment with 5 to 100  $\mu\text{g/L}$ , plants of both species contained significantly reduced chlorophyll content and the net photosynthetic

Table 62: The effects of fluridone on aquatic plant species are presented as IC50/EC50 values. Experimental conditions differ between the species but general trends in sensitivity can be observed. Pigment content of *Myriophyllum sibiricum* was more 40 times more sensitive to fluridone than the one algal species for which IC50 values were available.

Species	Endpoint	Exposure Duration	IC50	Reference
<b>Algae</b>				
<i>Chlamydomonas reinhardtii</i>	Flagella Regeneration	96 - 120 hours	39.85 mg/L	Fedtke (1982)
<i>Chlamydomonas reinhardtii</i>	Growth	48 hours	2.0 mg/L	Fedtke (1982)
<i>Chlamydomonas reinhardtii</i>	Chlorophyll Production	4 days	2.1 mg/L	Fedtke (1992)
<b>Submersed Macrophytes</b>				
<i>Myriophyllum sibiricum</i>	Chlorophyll a (fresh)	14 days	50.4 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Chlorophyll b (fresh)	14 days	51.2 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Carotenoid (fresh)	14 days	44.6 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	262.3 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Root Length	14 days	650.3 µg/L	This Document

rate of *M. spicatum* plants was reduced significantly when compared to the controls (Netherland and Getsinger, 1995b).

The effect of fluridone upon peroxidase activity in *Myriophyllum spicatum* and *Hydrilla verticillata* was examined by Sprecher *et al.* (1993). It was determined that exposure to fluridone concentrations of 12, 24 and 48  $\mu\text{g/L}$  for 30 days caused a three-fold increase in peroxidase activity in *M. spicatum* shoots. *H. verticillata* plants treated with 48  $\mu\text{g/L}$  contained significantly more peroxidase than untreated plants (Sprecher *et al.*, 1993). The chlorophyll content of *M. sibiricum* plants exposed to fluridone was slightly more sensitive over a shorter time span than the peroxidase levels examined by Sprecher *et al.* (1993).

After seven and fourteen days of treatment with 1 mg/L of fluridone, *Potamogeton pectinatus* and *P. richarsonii* showed visible injury including a reduction in shoot growth and the production of albescence near the shoot tips. These leaves became necrotic after fourteen days. The fluridone was not transported from the treated shoots into untreated roots. Approximately three percent of the fluridone was transported from treated roots into untreated shoots and this caused visible injury. After fourteen days of exposure to radiolabelled fluridone, there was no evidence of fluridone metabolism by these aquatic plant species (Marquis *et al.*, 1981). After 24 hours of exposure to 1.0 mg/L of fluridone, chlorophyll *a* and chlorophyll *b* content was reduced in *P. nodosus* and *P. pectinatus* by 75% and 40%, respectively (Anderson, 1981b). In aquaria, growth of *Hydrilla verticillata* and *Myriophyllum spicatum*, measured at 30, 60 and 90 days post-treatment, was inhibited by fluridone (Sonar<sup>®</sup> AS) concentrations of 1.0, 2.0, 3.0, 4.0 and 25.0  $\mu\text{g/L}$ . As compared with control plants, fluridone concentrations of 0.25 and 0.5  $\mu\text{g/L}$  did not affect hydrilla growth. After thirty days of treatment, a fluridone concentration of 0.5  $\mu\text{g/L}$  significantly increased the chlorophyll content, expressed in terms of fresh weight, of treated hydrilla shoot apices. Sixty and ninety days after treatment, chlorophyll content of hydrilla plants treated with 0.75 to 25.0  $\mu\text{g/L}$  was significantly reduced compared to the control. Chlorophyll content of shoot apices, net photosynthesis and growth of *M. spicatum* was reduced by fluridone treatments of 3.0, 4.0 and 25.0  $\mu\text{g/L}$  (Netherland and Getsinger, 1995a). In mature *H. verticillata* plants, total chlorophyll and total carotenoid content were significantly reduced compared to controls after two to twelve weeks of treatment with 5 and 50  $\mu\text{g/L}$  fluridone. As

compared to control levels, anthocyanin content was significantly increased in plants treated with 50 µg/L for two to twelve weeks. Young *H. verticillata* plants were not as sensitive to the effects of fluridone (Doong *et al.*, 1993). Fluridone concentrations of 5 and 50 µg/L reduced shoot dry weight of *H. verticillata* plants significantly when compared to the weight of control plants (MacDonald *et al.*, 1993b). Fluridone phytotoxicity to *H. verticillata* plants was influenced by the iron concentration in the plant tissue prior to treatment (Spencer and Ksander, 1989). Any possible interactions between iron in the modified Andrews' medium and the toxicity of fluridone to *Myriophyllum sibiricum* were not examined in the current study.

#### 5.7.4.3.4 Conclusions

If 3.0 mg/L, the EEC, of fluridone entered an aquatic system, populations of *Myriophyllum sibiricum* would be severely affected. Pigment content, plant area and root length would be most severely affected. Fluridone concentrations as low as 1% of the EEC could cause approximately 25% inhibition in pigment content and area of *M. sibiricum* plants. The effects of long term exposure to inhibitory concentrations of fluridone have not been determined but high concentrations of fluridone should not be allowed to reach non-target aquatic areas.

To this author's knowledge, very little toxicological data exist in the scientific literature for the effects of fluridone upon aquatic plants (Table 62). Based on IC50 values, *Myriophyllum sibiricum* was 45 times more sensitive to fluridone than *Chlamydomonas reinhardtii* (Table 62). In general, the phytotoxic effects of fluridone upon *M. sibiricum* in axenic culture were comparable to the efficacy results cited in the literature. Toxicological studies with algae (*Selenastrum capricornutum*) and floating macrophytes (*Lemna gibba* or *L. minor*) need to be conducted with fluridone to determine their relative sensitivity in comparison with *M. sibiricum*.

## 5.7.5 Glyphosate

### 5.7.5.1 Introduction

Glyphosate (Figure 69) is a non-selective broad-spectrum herbicide used in crop,

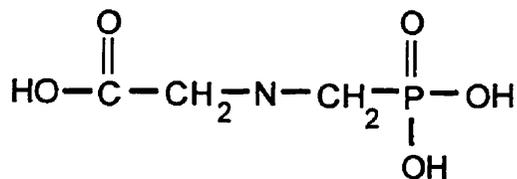


Figure 69: Glyphosate's structural formula.

non-crop and aquatic weed control (Murphy and Barrett, 1993; WSSA, 1989). Glyphosate has been registered for use in Canada since 1976 (Trotter *et al.*, 1990). In Ontario, glyphosate is used to control annual and perennial broad-leaved weeds and grasses before planting all kinds of crops. It is also used as a directed

spray under fruit trees and in silvicultural site preparation. It is the major herbicide used for conifer release in forestry and has a restricted registration for aerial spraying (Canadian Pulp and Paper Association and Forestry Canada, 1992; 1993a). Between 1988 and 1993, the use of glyphosate in Ontario for corn and soybeans increased by 300%. The amount of glyphosate used on all crops in Ontario totalled 414,821 kg a.i. in 1993. Licensed applicators used an additional 18,556 kg a.i. of glyphosate. Two hundred forty-two kg a.i. of glyphosate were sprayed on provincial highway roadsides in 1993 (Hunter and McGee, 1994). Between 1985 and 1988, more than 195 400 kg of glyphosate active ingredient were aerially applied in New Brunswick. In forestry, glyphosate accounted for 81% of the total national herbicide use, with Ontario being the largest user (Trotter *et al.*, 1990).

In Canada, glyphosate is not federally registered for use in or around irrigation ditches, but it can be used in dry drainage ditches to control cattails (Saskatchewan Environment and Public Safety, 1986). Glyphosate is used for aquatic weed control in the United States (Steward, 1993; Anderson, 1993), Central and South America (Fernández *et al.*, 1993), Asia (Gopal, 1993), Australasia (Mitchell and Bowmer, 1993) and certain European countries (United Kingdom, Yugoslavia and Belgium) (Murphy *et al.*, 1993). This herbicide is efficient at controlling emergent and floating aquatic plant species but is ineffective against submersed macrophytes (Barrett, 1985; Evans, 1978; Murphy and Barrett, 1993; Welker and Riemer, 1982). Perennial aquatic plants are poorly controlled by glyphosate due to poor translocation to underground storage organs

(Bowmer and Eberbach, 1993; Bowmer *et al.*, 1993). Glyphosate is also used in rice production (WHO, 1994a).

Glyphosate is absorbed through the foliage and translocated throughout the plant (Marquis *et al.*, 1981; OMAF, 1994a). It inhibits aromatic amino acid biosynthesis between shikimate and chorismate (Amrhein *et al.*, 1980) at the level of 5-enolpyruvylshikimate 3-phosphate synthase (Amrhein, 1992; Amrhein and Roy, 1992; Carlisle and Trevors, 1988; Duke, 1988; Rubin *et al.*, 1984). Glyphosate also inhibits  $\delta$ -aminolevulinic acid synthesis (Duke, 1988; Kitchen *et al.*, 1981b). It ultimately affects protein synthesis, nucleic acid synthesis, respiration (Hoagland and Duke, 1982) and indirectly promotes destruction of photosynthetic pigments in foliage (Carlisle and Trevors, 1988; Duke, 1988; Trotter *et al.*, 1990). Glyphosate is translocated basipetally in emergent and floating aquatic plants (Barrett, 1985). There is no known degradation pathway for glyphosate in plants (Duke, 1988).

Glyphosate residues enter aquatic environments through spills from terrestrial applications (Trotter *et al.*, 1990) or overspray from forestry applications (Feng *et al.*, 1990; Payne, 1992; Payne and Thompson, 1992; Trotter *et al.*, 1990). If recommended application rates are applied, runoff and leaching of glyphosate after a terrestrial application will be minimal (Edwards *et al.*, 1980; Rueppel *et al.*, 1977). Glyphosate residues have been detected in surface water of prairie grasslands (Government of Canada, 1991). This herbicide is adsorbed onto particulate matter in water and sediments (Bowmer *et al.*, 1986; Brønstad and Friestad, 1985; Murphy and Barrett, 1993) and is degraded by microorganisms (Brønstad and Friestad, 1985; Duke, 1988; Moshier and Penner, 1978; Murphy and Barrett, 1993; Reinert and Rodgers, 1987; Rueppel *et al.*, 1977; Smith and Aubin, 1993; Wang *et al.*, 1994b). Photodecomposition in water is a minor degradation pathway (Antón *et al.*, 1993; Brønstad and Friestad, 1985; Duke, 1988; Reinert and Rodgers, 1987; Rueppel *et al.*, 1977). Glyphosate is more persistent in sediment than in water (MacKenzie, 1996; Newton *et al.*, 1984; Paveglio *et al.*, 1996). On average, glyphosate persists in aquatic systems for a month or less (Reinert and Rodgers, 1987). If more background information on glyphosate is required, reviews exist on general glyphosate chemistry and toxicology (Carlisle and Trevors, 1988; Duke, 1988; Hoagland and Duke, 1982; Smith and Oehme, 1992; Trotter *et al.*, 1990; WHO, 1994a), the fate of glyphosate in aquatic systems (Brønstad and Friestad, 1985; Reinert, 1989; Reinert and Rodgers, 1987.; Rueppel *et al.*, 1977; Tooby,

1985; WHO, 1994a) and phytotoxic effects (Smith and Oehme, 1992; Trotter *et al.*, 1990; Zwiazek and Blake, 1990).

#### 5.7.5.2 Methods

For glyphosate, a recommended maximum label rate of 4.48 kg/ha (Carlisle and Trevors, 1988; Rueppel *et al.*, 1977; WSSA, 1989) was used in this study. If this MLR was sprayed onto a 15 cm deep column of water, an expected environmental concentration of 2.99 mg/L would be obtained. Technical glyphosate is extremely soluble in water (Table 27) so this compound was dissolved in sterile nanopure water before being added to the modified Andrews' medium. The technical glyphosate acid (97%) was obtained from Monsanto Company. For the EEC experiment, 1 mL of concentrated glyphosate stock solution was added to 250 mL of modified Andrews' growth medium. Forty mL of this solution was dispensed into 5 replicate test tubes. For the control test tubes in this experiment, 1 mL of sterile water was added to 250 mL of sterile modified Andrews' medium. This mixture was vortexed and then 40 mL of this solution was pipetted into prepared test tubes. The EEC of technical glyphosate was used in the comparison of technical pesticides and formulated products (Section 5.3) and in a study of the interactions between glyphosate and iron (Section 5.4) so the glyphosate EEC experiment was conducted one further time. This EEC experiment utilised stock plants that were cultured in modified Andrews' medium and was initiated on March 13, 1995. Other procedures followed those outlined in Section 4.6.

A glyphosate dilution series (0.0, 4.1, 12.3, 36.9, 110.6, 331.9, 995.6 and 2 987 µg/L) was conducted with stock plants cultured in modified Andrews' medium. The dilution series was initiated on March 13, 1995. A concentrated glyphosate stock solution was made 16 h before experiment initiation to ensure that the solution was completely mixed. Five mL of this concentrated stock solution were added to 250 mL of modified Andrews' medium, vortexed and dispensed into test tubes. Appropriate amounts of concentrated glyphosate stock solution and sterile water were added to 250 mL of modified Andrews' medium to make the various dilutions ending with 5 mL of sterile water being added to the modified Andrews' medium for the control medium. The statistical analysis was conducted based on the nominal glyphosate concentrations since glyphosate residues usually do not decline in sterile water (Brønstad and Friestad, 1985;

Rueppel *et al.*, 1977; Tooby, 1985). The remainder of the methods follow those outlined in Section 4.6.

### 5.7.5.3 Results and Discussion

#### 5.7.5.3.1 Expected Environmental Concentration

One control plant was lost from this experiment due to a broken test tube. The EEC of technical glyphosate inhibited *Myriophyllum sibiricum* growth (Figure 70 and Figure 71). However, inhibition with this herbicide was not as severe as with some of the other herbicides. The most severely affected parameter was root length (Table 63). In other plant species, root and rhizome growth have been shown to be strongly inhibited by glyphosate (Claus and Behrens, 1976; Hoagland and Duke, 1982). The untransformed means and standard deviations are listed in Table 64 and Table 65. Comparing the values displayed in Table 63, Table 64 and Table 65 to those displayed in Section 5.3 (Comparison of Technical and Formulated Pesticides) reveals numerous similarities. Shoot height was extremely similar in both experiments. Percent inhibition of area under the growth curve, shoot height, root length and membrane integrity were very similar. The difference in weight between these two experiments was probably due to the fact that the stock plants used in the experiment discussed in Section 5.3 were cultured in M & S medium while those used in the current chapter were cultured in modified Andrews' medium. Also shown in Section 5.3 were data indicating pigment concentrations (fresh weight basis) of apices treated with glyphosate were significantly greater than the amount of pigment determined from control apices. In this experiment, pigment concentration based on fresh weight was not significantly different between the control and treated plants. When the pigment concentration results were expressed on an apical dry weight basis, the concentration in the control plants was significantly greater than the concentration found in apices from treated plants (Table 65).

The recommended MLR used in this study, 4.48 kg/ha, (WSSA, 1989) was recently reduced to 4.2 kg/ha (WSSA, 1994) and thus the determined toxicity to aquatic macrophytes might be slightly overestimated. This change in the glyphosate regulation lowers the EEC from 2.987 mg/L ( $1.77 \cdot 10^{-5}$  M) to 2.8 mg/L ( $1.66 \cdot 10^{-5}$  M).

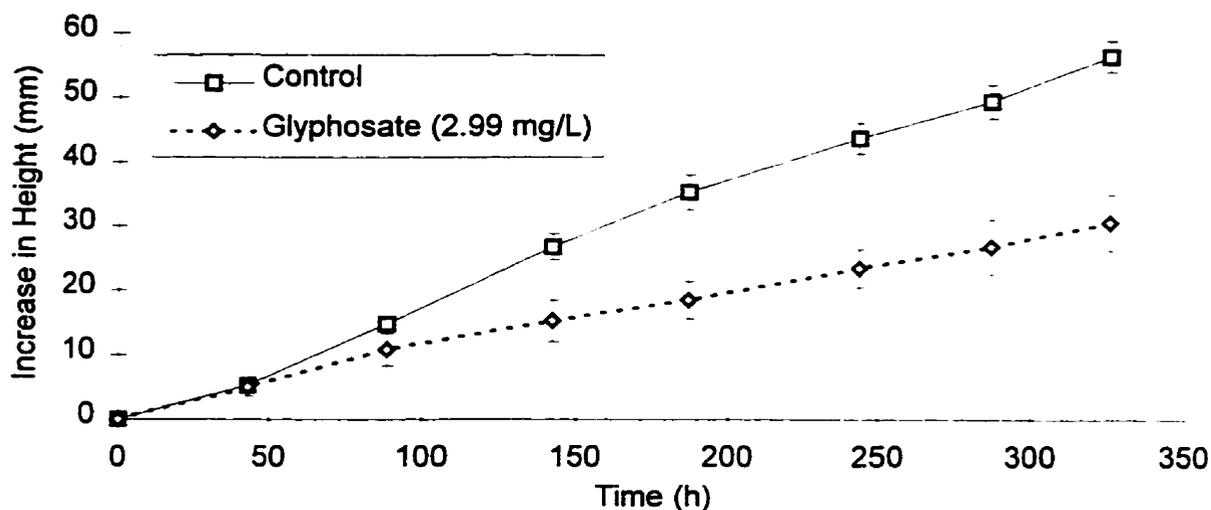


Figure 70: Growth curves for *Myriophyllum sibiricum* plants treated with the EEC (2.99 mg/L) of technical glyphosate and control plants. Plants were cultured for 14 days in modified Andrews' medium spiked with either glyphosate or sterile water.

Table 63: When compared with the four control plants, <i>Myriophyllum sibiricum</i> growth and development was inhibited by the EEC (2.99 mg/L) of glyphosate.	
GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)
Area Under the Growth Curve	43.5 $\pm$ 9.1
Increase in Shoot Length	39.6 $\pm$ 2.0
Root Number	63.5 $\pm$ 10.9
Root Length	95.9 $\pm$ 3.3
Fresh Weight	65.7 $\pm$ 5.6
Membrane Integrity	2.1 $\pm$ 2.5
Plant Area	55.7 $\pm$ 8.6
Chlorophyll a (fresh)	11.6 $\pm$ 7.5
Chlorophyll b (fresh)	13.9 $\pm$ 9.9
Carotenoid (fresh)	6.4 $\pm$ 6.5
Chlorophyll a (dry)	53.6 $\pm$ 6.8
Chlorophyll b (dry)	54.6 $\pm$ 7.6
Carotenoid (dry)	50.9 $\pm$ 7.1



**Table 64: Untransformed means  $\pm$  s.d. for numerous endpoint parameters collected from *Myriophyllum sibiricum* plants cultured for fourteen days in the presence or absence of 2.99 mg/L glyphosate.**

Treatment	Area under the growth curve	Shoot Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant area (cm <sup>2</sup> )
Control	9 356.9 $\pm$ 452.0 <sup>a</sup>	56.3 $\pm$ 0.6 <sup>a</sup>	7.7 $\pm$ 1.2 <sup>a</sup>	456.4 $\pm$ 84.6 <sup>a</sup>	786.9 $\pm$ 31.1 <sup>a</sup>	7.5 $\pm$ 0.9 <sup>a</sup>	13.1 $\pm$ 0.7 <sup>a</sup>
Glyphosate	5 287.6 $\pm$ 851.9 <sup>b</sup>	34.0 $\pm$ 1.1 <sup>b</sup>	2.8 $\pm$ 0.8 <sup>b</sup>	18.8 $\pm$ 15.2 <sup>b</sup>	270.3 $\pm$ 43.7 <sup>b</sup>	8.6 $\pm$ 1.3 <sup>a</sup>	5.8 $\pm$ 1.1 <sup>b</sup>

a,b Any two means in the same column with the same superscript were not significantly different based on the results from either a t-test or the Mann-Whitney U-test. n = 4 for control and n = 5 for glyphosate.

**Table 65: The effect of the EEC of technical glyphosate on pigment content of the apex of *Myriophyllum sibiricum* plants expressed on a fresh and dry weight basis.**

Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
Control	0.59 $\pm$ 0.09 <sup>a</sup>	0.22 $\pm$ 0.04 <sup>a</sup>	0.19 $\pm$ 0.03 <sup>a</sup>	8.2 $\pm$ 1.3 <sup>a</sup>	3.1 $\pm$ 0.5 <sup>a</sup>	2.7 $\pm$ 0.4 <sup>a</sup>
Glyphosate	0.52 $\pm$ 0.04 <sup>a</sup>	0.19 $\pm$ 0.02 <sup>a</sup>	0.18 $\pm$ 0.01 <sup>a</sup>	3.8 $\pm$ 0.6 <sup>b</sup>	1.4 $\pm$ 0.2 <sup>b</sup>	1.3 $\pm$ 0.2 <sup>b</sup>

a,b Any two means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as determined with either the parametric t-test or the nonparametric Mann-Whitney U-test. Values displayed are the untransformed means and s.d. of four control replicates and five treatment replicates.

### 5.7.5.3.2 Dilution Series

*Myriophyllum sibiricum* growth in response to a series of glyphosate concentrations is illustrated in Figure 72 and Figure 73. Visually, there was no difference between the control plants and plants treated with concentrations equal to or less than 331.9  $\mu\text{g/L}$ . Using statistical analysis ( $\alpha = 0.05$ ), it was verified that there was no significant difference between the control plants and those treated with 331.9  $\mu\text{g/L}$  for area under the growth curve, increase in shoot length, root length, chlorophyll a and carotenoid content (Table 66). Pigment content, expressed as mg pigment/g dry weight of the apex, was used for the statistical analysis because of the fluctuation in plant weight, which was possibly one of the phytotoxic effects of glyphosate. Cooley and Foy (1992) found that technical glyphosate decreased fresh weight but had no effect on dry weight of *Lemna gibba* (Cooley and Foy, 1992). These fluctuations in pigment content due to weight were also seen in the technical glyphosate EEC results in Section 5.3.

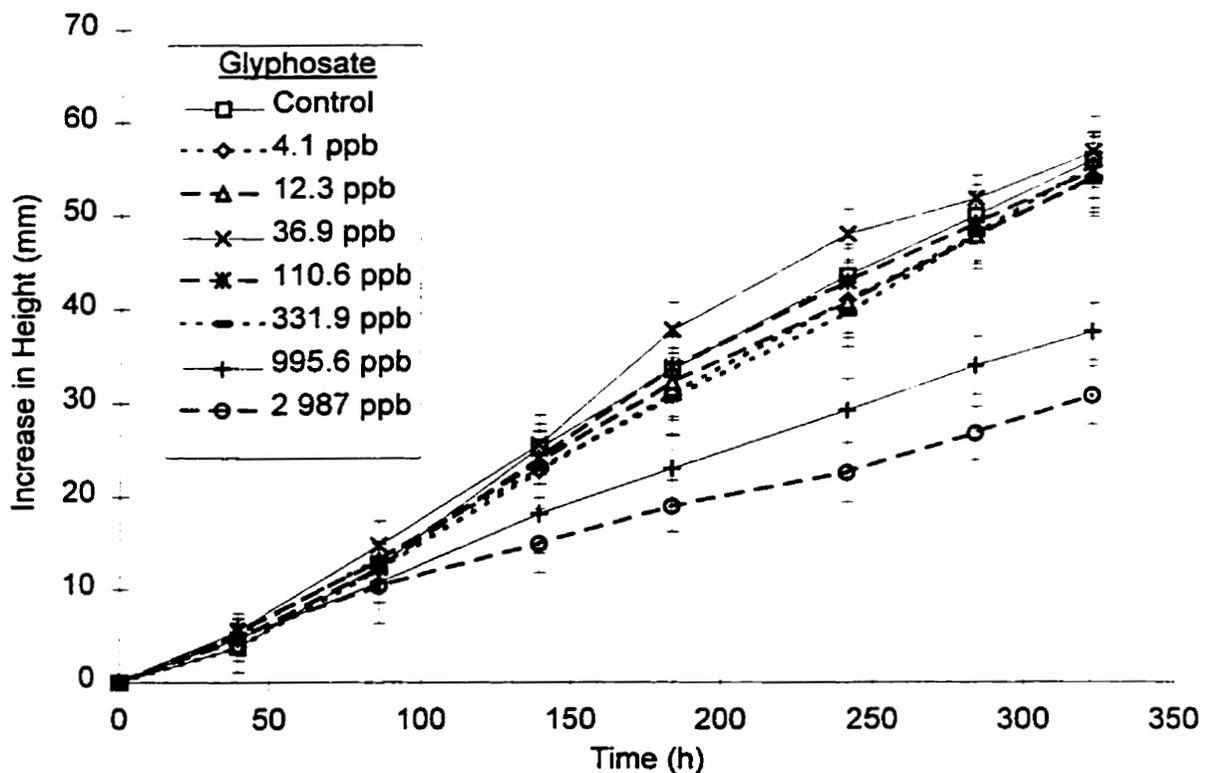


Figure 73: Growth curves of *Myriophyllum sibiricum* plants treated with a dilution series of technical glyphosate for 14 days. The control plants did not differ from plants treated with 331.9  $\mu\text{g/L}$  or less.

Table 66: No observable effect concentrations, the average percent inhibition ( $\pm$ s.d.) at the NOEC, IC25 values ( $\pm$ 95% c.i.) and IC50 values ( $\pm$ 95% c.i.) for the effect of glyphosate upon <i>Myriophyllum sibiricum</i> endpoint parameters.				
GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	% I at NOEC	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	331.8	$5.3 \pm 7.6$	1 100.3 (654.1; 1 851.2) <sup>a</sup>	> 2 987 <sup>a,b</sup>
Increase in Shoot Length	331.8	$-1.7 \pm 5.7$	1 283.1 (760.4; 2 165.3) <sup>a</sup>	> 2 987 <sup>a,b</sup>
Root Length	331.8	$7.5 \pm 20.6$	598.5 (360.9; 992.7) <sup>a</sup>	844.0 (673.3; 1 058.0) <sup>a</sup>
Root Number	995.5	$-8.9 \pm 16.5$	2 324.6 <sup>c</sup>	2 797.6 <sup>c</sup>
Fresh Weight	110.6	$3.9 \pm 3.1$	593.5 (387.3; 909.5) <sup>a</sup>	1 474.1 (1 057.9; 2 054.0) <sup>a</sup>
Membrane Integrity	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Plant Area	110.6	$3.5 \pm 4.2$	627.7 (458.5; 859.4) <sup>a</sup>	2 090.2 (1 627.8; 2 683.9) <sup>a</sup>
Chlorophyll <i>a</i> (apical dry wt)	331.8	$9.4 \pm 15.5$	558.8 (217.2; 1438.6) <sup>a</sup>	2 157.4 (1 002.4; 4 643.2) <sup>a</sup>
Chlorophyll <i>b</i> (apical dry wt)	12.3	$3.4 \pm 13.2$	285.8 (63.5; 1 289.7) <sup>a</sup>	1 928.7 (717.3; 5 185.7) <sup>a</sup>
Carotenoid (apical dry wt)	331.8	$4.2 \pm 14.2$	761.6 (346.2; 1 676.2) <sup>a</sup>	2 629.0 (1 248.9; 5 534.3) <sup>a</sup>

a IC<sub>p</sub> values and 95% confidence intervals calculated with the cumulative normal distribution model.

b IC<sub>p</sub> and 95% confidence values calculated with the weibull method.

c The IC<sub>p</sub> values were obtained from second order polynomial curve fitting techniques. Confidence intervals could not be estimated.

Kruskal-Wallis statistical analysis of chlorophyll *b* showed that there was no significant difference between the control plants and certain treatments (4.1, 12.3 and 110.6  $\mu\text{g/L}$ ) ( $\alpha = 0.05$ ). Because the chlorophyll *b* content of control plants differed from the pigment content of apices treated with 36.9  $\mu\text{g/L}$  at  $\alpha = 0.05$ , the NOEC was determined to be 12.3  $\mu\text{g/L}$ . The LOEC concentration for chlorophyll *b* would be 331.9  $\mu\text{g/L}$  (Section 5.3). The lowest IC25 of 285.8  $\mu\text{g/L}$  was obtained for chlorophyll *b* (Table 66). After chlorophyll *b* content, fresh weight and plant area had the next lowest NOEC of 110.6  $\mu\text{g/L}$  (Table 66). The most severely affected endpoints correlate with glyphosate's mode of action of inhibiting aromatic amino acid synthesis (Rubin *et al.*, 1984; Amrhein *et al.*, 1980) and destroying photosynthetic pigments (Kitchen *et al.*, 1981a; 1981b; Trotter *et*

*al.*, 1990). Membrane integrity was not affected at any concentration of glyphosate, which correlates with the results obtained from the EEC experiments with glyphosate (Section 5.3 and Section 5.7.5.3.1) and the results of other researchers (Fletcher *et al.*, 1980). For root number, IC values could not be calculated using either the weibull or cumulative normal programs because percent inhibition jumped from an average of  $-8.9 \pm 16.5\%$  at 995.5  $\mu\text{g/L}$  (Table 66) to  $62.2 \pm 9.9\%$  at a glyphosate concentration of 2987  $\mu\text{g/L}$ . Using curve fitting techniques, IC values were estimated (Table 66). Slight stimulation in root length of *M. sibiricum* was also observed at glyphosate concentrations of 36.9  $\mu\text{g/L}$ . Root stimulation by low glyphosate concentrations has been observed in other studies (Bowmer *et al.*, 1986). For root length, IC<sub>50</sub> values of 844.0  $\mu\text{g/L}$  (c.i. = 673.3 - 1 058.0) were obtained. This was the lowest IC<sub>50</sub> value (Table 66) and corresponds to the strong inhibition of roots and rhizomes observed by other researchers (Claus and Behrens, 1976; Hoagland and Duke, 1982). IC<sub>50</sub> values could not be calculated for area under the growth curve and plant height. Even at the highest concentration tested, shoot height and area under the growth curve were not inhibited by 50% when compared to the control plants (Table 66). This corresponds to the results obtained during the EEC experiment (Table 30 and Table 63). For all the endpoint parameters, the IC<sub>25</sub> and IC<sub>50</sub> values had very wide confidence intervals. Repeating the dilution series of glyphosate with a narrower range of concentrations (e.g., 50 to 2987  $\mu\text{g/L}$ ) would possibly produce narrower confidence intervals. Perkins (1997) used a narrower concentration range with formulated and technical glyphosate (0.4 to 25.6 mg ae/L).

It is currently undetermined if there is a correlation between the glyphosate results obtained in this laboratory study and those observed in an aquatic field situation. Glyphosate displayed greater toxicity to quackgrass when tested in the greenhouse as compared to the field (1.94 times more sensitive in the greenhouse) (Fletcher *et al.*, 1990). Other possible endpoints that could be used in this axenic toxicity test, such as chlorophyll fluorescence, might not be sensitive to the effects of glyphosate. The chlorophyll fluorescence assay did not detect the effects of Roundup® (a.i. = glyphosate) upon *Tradescantia* leaves (Judy *et al.*, 1990).

The results obtained in this study may slightly underestimate the phytotoxicity of glyphosate to *M. sibiricum*. In Canada, a maximum of 5.76 kg a.i. glyphosate per

hectare may be used to control perennial weeds (Trotter *et al.*, 1990). This is slightly higher than the MLR of 4.48 kg/ha used in the current study. For glyphosate, there are no American standards or guidelines for water quality parameters (Nowell and Resek, 1994). A glyphosate level of 0.2 g/m<sup>3</sup> has been established in Australia as the maximum residue in potable water and in the United Kingdom as the level allowed in irrigation water (Bowmer *et al.*, 1986). Canada has set an interim water quality limit of 65 µg/L for the protection of freshwater aquatic life (Trotter *et al.*, 1990). Based upon results from the current study, the maximum level of glyphosate that should be allowed in freshwater systems to protect aquatic plants should be no more than 110.6 µg/L. This is less than the Australian and British levels but in agreement with the interim Canadian guideline. In freshwater enclosures, fifty percent dissipation from an initial concentration of 7.68 kg ae/ha occurred for Rodeo® within 7.5 days and for Roundup® within 13.2 days. During the 212 day monitoring period, glyphosate remained detectable in the sediments (MacKenzie, 1996). The World Health Organization (WHO, 1994a) summarised 50% dissipation ranges for glyphosate in aquatic situations to be from a few days to two weeks. Thus, aquatic macrophytes would be exposed to glyphosate concentrations in both the water and sediment for the fourteen day experimental period utilised in this study. After aerially spraying 2.0 kg/ha of Roundup®, a maximum stream water residue of 162 µg/L was detected (Feng *et al.*, 1990). Following an application of 5.6 kg/ha of Roundup® to two canals, 153 and 161 µg/L were detected 0.3 km downstream (Comes *et al.*, 1976). Water levels of 0.27 mg/L glyphosate were detected in a forest stream within an aerial application area (Newton *et al.*, 1984). These levels would cause a phytotoxic effect upon non-target aquatic macrophytes and the use of buffer zones may be appropriate.

#### 5.7.5.3.3 Aquatic Plant Toxicity Comparison

Table 67 is a summary of examples of glyphosate toxicity from the scientific literature. In general, higher concentrations of glyphosate were required for toxic effects to be exhibited by algae than the concentrations that affected axenic cultures of *Myriophyllum sibiricum*. After examining glyphosate toxicity to thirteen species of algae, it was determined that *Chlorella pyrenoidosa* was the least sensitive species, with an EC50 for growth of 590 mg/L. *Chlorococcum hypnosporum* Starr was the most sensitive species with an EC50 of 68 mg/L (Maule and Wright, 1984) (Table 67). Total

Table 67: Selected from the scientific literature, a summary of EC50 or IC50 values for aquatic plant species exposed to glyphosate. Test conditions differed for the different species but general trends in sensitivity can be observed. The submersed macrophytes are more sensitive to the effects of glyphosate followed closely by the floating macrophyte, *Lemna minor*, and the alga, *Anabaena variabilis*. Some algal species, such as *Chlorella pyrenoidosa*, are resistant to the effects of glyphosate.

Species	Endpoint	Formulation	Exposure Duration	EC50/IC50	Reference
<b>Algae</b>					
<i>Anabaena variabilis</i>	Growth	Technical	48 hours	2 mg/L	Hutber <i>et al.</i> (1979)
<i>Chlamydomonas reinhardtii</i>	Chlorophyll Production	Technical	4 days	5.3 mg/L	Fedtke (1992)
<i>Chlorococcum hypnosporum</i>	Growth	Technical	4 days	68 mg/L	Maule and Wright (1984)
<i>Chlorella pyrenoidosa</i>	Growth	Technical	4 days	590 mg/L	Maule and Wright (1984)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	Technical	96 hours	13.5 mg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	Technical	96 hours	7.8 mg/L	St. Laurent <i>et al.</i> (1992)
<b>Floating Macrophytes</b>					
<i>Lemna gibba</i>	Fresh Weight	Technical	7 days 10	9.98 mg ae/L	Perkins (1997)
<i>Lemna paucicostata</i>	Leaf Surface Area	Technical	days 14	3.9 mg/L	Retzlaff (1992)
<i>Lemna minor</i>	Fronde Number	Roundup®	days	2.0 mg/L	Hartman and Martin (1984)
<b>Submersed Macrophytes</b>					
<i>Myriophyllum spicatum</i>	Branch Number	Roundup®	5 days 14	1.6 mg/L	Bird (1993)
<i>Myriophyllum sibiricum</i>	Root Length	Technical	days 14	1.56 mg ae/L	Perkins (1997)
<i>Myriophyllum sibiricum</i>	Fresh Weight	Technical	days 14	3.3 mg ae/L	Perkins (1997)
<i>Myriophyllum sibiricum</i>	Plant Area	Technical	days 14	4.82 mg ae/L	Perkins (1997)
<i>Myriophyllum sibiricum</i>	Root Length	Technical	days 14	0.84 mg a.i./L	This Document
<i>Myriophyllum sibiricum</i>	Fresh Weight	Technical	days 14	1.47 mg a.i./L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	Technical	days	2.09 mg a.i./L	This Document

growth inhibition of *Anabaena variabilis* occurred at 10 mg/L and an EC50 was estimated at 2 mg/L glyphosate (Hutber *et al.*, 1979) (Table 67). Glyphosate was slightly to moderately toxic to *Selenastrum capricornutum*. EC50s of 13.5 mg/L (c.i. = 11.1 - 16.6 mg/L) and 7.8 mg/L (c.i. = 3.0 - 12.7 mg/L) were obtained for the flask and microplate bioassays, respectively (St. Laurent *et al.*, 1992) (Table 67). In experiments conducted by Bozeman *et al.* (1989), an EC50 of  $7.8 \pm 1.5$  mg/L was obtained for the effect of technical glyphosate upon the chlorophyll fluorescence in mobile *S. capricornutum* cells (Bozeman *et al.*, 1989). Based on cell growth of *S. capricornutum* in water and liquid growth medium, a 96 hour EC50 of 2.6 mg/L was obtained for the Roundup® formulation of glyphosate. Roundup® was less toxic in a soil elutate as indicated by a slight stimulatory effect in *S. capricornutum* growth was observed 10 days after treatment (Thomas *et al.*, 1990). There appears to be a typographical error in the abstract of the paper by Thomas *et al.* (1990) where the 96 hr EC50 of Roundup® in water was listed as 2 600 µg/mL and this differs by a factor of 1 000 from the number in the text, as cited above. Glyphosate, in the form of Roundup®, was one of the least toxic of nineteen chemicals tested on thirteen species of algae. For all species of algae, the median concentration that produced no detectable growth was 11 mg/L (Blanck *et al.*, 1984). In an estuarine system, edaphic algae were not affected fourteen days after application of Rodeo® applied at 4.7 L/ha (Simenstad, 1996). *Euglena gracilis* growth and chlorophyll content were inhibited by glyphosate at concentrations equal to or greater than  $1.2 \cdot 10^{-3}$  M (202.9 mg/L) (Richardson *et al.*, 1979). The growth rate of *Chlorella sorokiniana* was reduced by more than half by a glyphosate concentration of  $17.7 \cdot 10^{-6}$  M (2.99 mg/L) and halted by glyphosate concentrations equal to or greater than  $23.7 \cdot 10^{-6}$  M (4 mg/L) (Christy *et al.*, 1981). After 4 days of treatment, the chlorophyll production of *Chlamydomonas reinhardtii* was inhibited 50% by a technical glyphosate concentration of  $3.2 \cdot 10^{-5}$  M (5.3 mg/L) (Fedtke, 1992) (Table 67). Glyphosate at a concentration of  $7 \cdot 10^{-4}$  M (118.4 mg/L) caused 50% inhibition of the oxygen production in *Scenedesmus* cultures. The toxic effect of glyphosate increased with higher light intensities and higher temperatures (van Rensen, 1974). The interaction of light and temperature with glyphosate was not examined in the *Myriophyllum sibiricum* axenic toxicity test. The World Health Organization (WHO,

1994a) summarised the phytotoxic effects of glyphosate in water and soil to other algal species (WHO, 1994a).

Fifty percent inhibition of the total leaf surface area of *Lemna paucicostata* occurred at a technical glyphosate concentration of  $2.3 \cdot 10^{-5}$  M (3.9 mg/L) after ten days of incubation (Retzlaff, 1992) (Table 67). For *L. gibba*, the parameter that was most sensitive to technical glyphosate was fresh weight with an IC50 of 9.98 mg ae/L (c.i. = 4.13 - 29.03) (Perkins, 1997) (Table 67). Glyphosate increased the cellular leakage of *Lemna minor* plants at concentrations of  $10^{-1}$  mM (16.9 mg/L) after 12 and 24 hours of treatment and  $10^{-2}$  mM (1.69 mg/L) after 48, 72 and 96 hours of exposure. A slight yellowing of the fronds was noted after 96 hours (O'Brien and Prendeville, 1979). Cell deterioration in *Chlorella sorokiniana* cells was observed after glyphosate treatment (Christy *et al.*, 1981). Cell membrane permeability of *M. sibiricum* plants was not affected by glyphosate at the concentrations tested (Table 63, Table 64 and Table 66). An ED50 of 2.0 mg/L of glyphosate (Roundup®) was obtained for *Lemna minor* frond number (Table 67). When suspended sediment was added to the test system, the glyphosate toxicity decreased to 10.0 mg/L (Hartman and Martin, 1984). Compared to *L. minor* in aqueous solution, *M. sibiricum* root length and weight were more sensitive to glyphosate as no toxicity was observed at concentrations below 331.8 µg/L and 110.6 µg/L, respectively. The effect of suspended sediment on the *M. sibiricum* test system was not examined. The interaction of glyphosate with different iron concentrations and its effect upon *M. sibiricum* growth was examined in Section 5.4. *Myriophyllum sibiricum* was more sensitive to technical glyphosate than were any of the species of duckweed. In studies by Davis (1980) and Forney and Davis (1981), concentrations of glyphosate up to 320 µg/L had no significant effect on the growth or dry weight of any of the aquatic plant species tested (*Cabomba caroliniana*, *Egeria densa*, *Elodea canadensis*, *Myriophyllum spicatum*, *Potamogeton perfoliatus*, *Vallisneria americana* and *Lemna perusilla*) (Davis, 1980; Forney and Davis, 1981). In the current study, the NOEC for *M. sibiricum* plant height was 331.8 µg/L, which was slightly greater than the highest concentration tested by Forney and Davis (1981). Glyphosate at a concentration of 1 mg/L was translocated from the shoot into the root systems of *Potamogeton pectinatus* and *P. richardonii* (Marquis *et al.*, 1981). In another study, sprouting of *P. pectinatus* tubers was not affected by glyphosate (Roundup®) at concentrations up to 10 mg/L. At

1.0 mg/L, glyphosate stimulated tuber growth, as measured by increase in fresh weight, over a fourteen day period (Hartman and Martin, 1985). Technical grade glyphosate was tested in three toxicity testing systems using *Potamogeton pectinatus* as the test organism. Biomass production over the four week treatment period was not affected in the autotrophic system. In the heterotrophic system, there was a slight but insignificant stimulation in biomass at glyphosate concentrations of 0.001, 0.01, 0.1 and 1.0 mg/L. Ten milligrams of glyphosate per litre decreased biomass by  $35 \pm 7\%$  when compared to the control. In the microcosm test system, there was a decrease in *P. pectinatus* biomass production of  $54 \pm 6\%$  of the controls at the 10 mg/L glyphosate level (Fleming *et al.*, 1991).

The phytotoxic effect of glyphosate upon the production of new axillary buds, leaves, roots and branches of *Myriophyllum spicatum* was examined in tissue culture (Bird, 1993; Christopher and Bird, 1992). Five days post-treatment, glyphosate at a concentration of 1.6 mg/L caused a fifty percent reduction in the number of branches produced (Bird, 1993) (Table 67). Stimulation of bud production was observed at a glyphosate concentration of 2.5 mg/L. Inhibition of leaves occurred at glyphosate concentrations greater than or equal to 2.5 mg/L; inhibition of branches occurred at concentrations greater than or equal to 1.0 mg/L; and, total new structures were inhibited at concentrations equal to or greater than 5.0 mg/L. Root production did not differ from the control at any tested concentration (Christopher and Bird, 1992). The endpoints examined in the *M. sibiricum* axenic toxicity test were more sensitive to the toxic effects of glyphosate. The differences observed between the bioassay conducted by Bird (1993) and Christopher and Bird (1992) and the *M. sibiricum* toxicity test may also be a result of species differences between the two species of *Myriophyllum*.

As also seen in the current study, Perkins (1997) observed that root length was the most sensitive parameter for the effects of glyphosate upon *M. sibiricum*. An IC<sub>50</sub> of 1.56 mg ae/L (c.i. = 1.29 - 1.89) for the effect of technical glyphosate upon root length of *M. sibiricum* was obtained in the study by Perkins (1997). IC<sub>50</sub>s of 3.3 mg ae/L (c.i. = 1.34 - 8.12) and 4.82 mg ae/L (c.i. = 2.18 - 10.66) were obtained for fresh weight and plant area, respectively (Perkins, 1997) (Table 67). These values were very similar to those obtained in the current study.

#### 5.7.5.4 Conclusions

*Myriophyllum sibiricum* and most of the other aquatic plants that have been studied would be only slightly affected by the presence of the EEC (2.99 mg/L) of glyphosate in an aquatic environment. Concentrations about one-third of the EEC would inhibit root length by approximately 50%. Populations of *M. sibiricum* should be able to recover from glyphosate levels close to the EEC. If 1% of the EEC was to reach an aquatic ecosystem, chlorophyll *b* content would be the only endpoint parameter that might be slightly affected. Long term ecosystem level effects of a minor decrease in chlorophyll *b* content of *M. sibiricum* plants has not been determined.

Most of the scientific literature examined the effects of formulated glyphosate upon aquatic plants. Section 5.3 includes a review of the effects of formulated glyphosate upon aquatic plants, as detailed in the literature. Other literature examines the efficacy of glyphosate on aquatic weed problems (Kay, 1995; Lopez, 1993; Smith *et al.*, 1993; Trotter *et al.*, 1990). No EC50/IC50 values were found in the literature for emergent aquatic plants to allow a comparison with the *M. sibiricum* toxicity test. Based on EC50 and IC50 values, submersed macrophytes were the aquatic plant species that were most severely affected by glyphosate (Table 67). Using root length of *M. sibiricum* as the most sensitive endpoint, *M. sibiricum* was twice as sensitive to glyphosate as *Lemna minor* frond number and *Anabaena variabilis* growth. *M. sibiricum* was 9 times more sensitive to glyphosate than *Selenastrum capricornutum* population growth (Table 67). Comparing the plant area of *M. sibiricum* plants with the leaf surface area of *L. paucicostata*, *M. sibiricum* was almost twice as sensitive to the effects of glyphosate. The results from *M. sibiricum* toxicity tests should be included in aquatic risk assessments with glyphosate and similar herbicides.

#### 5.7.6 Hexazinone

##### 5.7.6.1 Introduction

In Canada, hexazinone (Figure 74) is registered for aerial application for site preparation in woodland management (Canadian Pulp and Paper Association and Forestry Canada, 1992; 1993b). In Ontario, hexazinone is primarily used on non-crop land for conifer reforestation (OMAF, 1994a). It has other registered markets in the United States, including use in alfalfa, sugar cane and pineapple. This herbicide is phytotoxic because it inhibits photosynthesis by binding to the Q<sub>a</sub> binding site on the D1

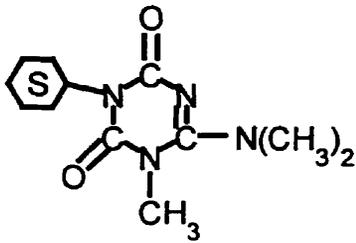


Figure 74: The chemical formula for the photosynthetic inhibitor, hexazinone.

protein of photosystem II. Thus, carbon dioxide fixation and production of ATP and NADPH<sub>2</sub> are halted. Other secondary reactions include the formation of triplet state chlorophyll, singlet oxygen, lipid peroxidation, loss of chlorophyll and carotenoids and leaky membranes.

Visual symptoms include chlorosis followed by necrosis (WSSA, 1994).

In Ontario during 1993, 1,833 kg a.i. hexazinone was sprayed by licensed applicators (Hunter and McGee, 1994). In a typical field soil, hexazinone has a 90 day half-life (WSSA, 1994). In a limnocorral study, an initial application of 16.75 µg/L formulated hexazinone (Velpar L) was not detectable 21 days after application and a higher dose of 167.5 µg/L was undetectable 42 days post-application. The calculated half-lives were 3.7 and 3.8 days for these two application rates (Solomon *et al.*, 1988). Average hexazinone concentrations of 442 ± 53 µg/L were detected in storm runoff three days after application of a granular formulation (Neary *et al.*, 1980). Hexazinone residues were detected in the lower Mississippi River at a maximum concentration of 71 ng/L (Pereira and Hostettler, 1993).

#### 5.7.6.2 Methods

For hexazinone, a MLR of 12 kg a.i. per hectare was used, which converted to an EEC of 8.0 mg/L. Technical hexazinone (98%) from Dupont was used in the EEC and dilution experiments. Hexazinone is extremely soluble in water (Table 27) so this compound was dissolved into sterile nanopure water prior to addition to the growth medium. The toxicity of hexazinone at the EEC was evaluated twice with initiation dates of February 21, 1994 and June 5, 1995. Stock plants used in the first experiment were cultured in M & S medium while plants used in the second EEC experiment were grown in modified Andrews' medium.

In a dilution series, the toxicity of hexazinone was also evaluated twice. The first dilution series (125, 250, 500, 1 000, 2 000, 4 000 and 8 000 µg/L) was initiated on August 8, 1994 and the second dilution series (0.0, 32.9, 98.8, 296.3, 888.9, 2 666.7 and 8 000 µg/L) was initiated on May 15, 1995. Stock plants were cultured in M & S

medium for the first dilution experiment and in modified Andrews' medium for the second dilution experiment.

### 5.7.6.3 Results and Discussion

#### 5.7.6.3.1 Expected Environmental Concentration

Visually, there did not appear to be much difference in the height of plants treated with the EEC of hexazinone and the control plants (Figure 75 and Figure 76). For area under the growth curve, there was no statistical difference between hexazinone treated plants and control plants (Figure 75, Table 68 and Table 69). Six days after experimental initiation, plants treated with the EEC of hexazinone were pale and spindly. This effect continued until termination of the experiment (Figure 76). Hexazinone treated plants had chlorotic leaves and stems, fewer nodes and few roots (Figure 76, Table 68 and Table 69). Because hexazinone interferes with photosynthesis, endpoints such as dissolved oxygen, fresh weight, root number and length, plant area (Table 68 and Table 69) and pigment content were also affected. Pigment content was not as severely affected when the results were presented in terms of the dry weight of the apex (Table 70). Membrane integrity was not affected. Total root length and root number were the two parameters most severely affected by hexazinone. The results from the

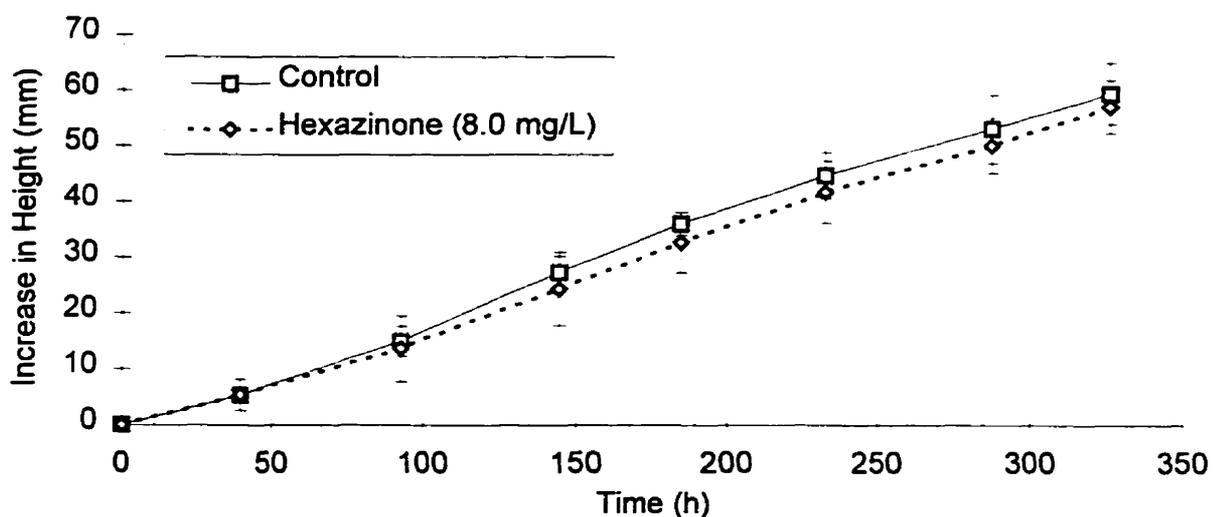


Figure 75: Fourteen day growth curves for 8.0 mg/L of hexazinone and control plants. Stock plants used for this experiment were cultured in M & S medium. Experimental plants were grown in modified Andrews' medium spiked with either sterile water or the technical pesticide dissolved in water.

two experiments were very similar even though the experiments were conducted more than fifteen months apart (Table 68 and Table 69). The pigment content (fresh weight) extracted from the control plants was slightly higher in the second EEC experiment than in the first experiment (Table 70) and this may have been due to the different media in which the stock plants were cultured.

The MLR of 12 kg a.i./ha (WSSA, 1989) used in this study might underestimate the effects of hexazinone upon non-target aquatic macrophytes because the recommended rate has been increased to 15 kg a.i./ha when applied to non-crop land including industrial sites, railroads, highway right-of-ways and storage areas (WSSA, 1994). There exists the possibility of hexazinone drift or runoff from these non-crop areas into aquatic systems including roadside ditches, ponds and streams where the non-target macrophytes may be affected more severely than predicted in this study.

Table 68: Percent inhibition caused by the EEC of hexazinone (8.0 mg/L) on <i>Myriophyllum sibiricum</i> growth and development. The plants were cultured in modified Andrews' medium with or without technical hexazinone. Hexazinone severely inhibited root growth.		
GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)	
	First Experiment	Second Experiment
Area Under the Growth Curve	12.7 $\pm$ 15.7	6.5 $\pm$ 7.7
Increase in Shoot Length	-2.5 $\pm$ 9.7	14.9 $\pm$ 5.4
Increase in Node Number	59.5 $\pm$ 9.9	67.2 $\pm$ 3.9
Nodes per Centimetre	61.0 $\pm$ 5.6	61.5 $\pm$ 4.9
Root Number	97.0 $\pm$ 6.7	94.0 $\pm$ 5.5
Root Length	99.7 $\pm$ 0.7	99.5 $\pm$ 0.7
D.O.	60.1 $\pm$ 11.5	78.6 $\pm$ 1.8
Fresh Weight	76.4 $\pm$ 2.5	71.8 $\pm$ 1.4
Membrane Integrity	19.7 $\pm$ 4.0	5.9 $\pm$ 4.6
Plant Area	89.8 $\pm$ 2.6	59.1 $\pm$ 3.9
Chlorophyll a (fresh)	14.8 $\pm$ 7.6	62.8 $\pm$ 1.9
Chlorophyll b (fresh)	12.6 $\pm$ 10.0	56.1 $\pm$ 4.4
Carotenoid (fresh)	17.1 $\pm$ 6.1	62.8 $\pm$ 15.4
Chlorophyll a (dry)	Not measured	9.7 $\pm$ 12.1
Chlorophyll b (dry)	Not measured	17.9 $\pm$ 7.6
Carotenoid (dry)	Not measured	3.1 $\pm$ 13.0



Table 69: The effect of hexazinone (8.0 mg/L) upon *Myriophyllum sibiricum* growth and development. The experimental plants were grown for 14 days in axenic modified Andrews' medium.

Treatment	Area under the growth curve	Increase in Plant Length (mm)	Increase in Node Number	Nodes/cm	Root Number	Total Root Length (mm)	D.O. (%)	Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
<b>First Expected Environmental Concentration Experiment</b>										
Control	9 807.9 ± 1 000.7 <sup>a</sup>	59.5 ± 5.6 <sup>a</sup>	12.3 ± 0.6 <sup>a</sup>	2.1 ± 0.2 <sup>a</sup>	6.7 ± 0.6 <sup>a</sup>	363.7 ± 98.2 <sup>a</sup>	48.7 ± 8.1 <sup>a</sup>	567.1 ± 37.8 <sup>a</sup>	8.6 ± 0.4 <sup>a</sup>	7.2 ± 0.6 <sup>a</sup>
Hexazinone	8 558.9 ± 1 535.8 <sup>a</sup>	61.0 ± 5.8 <sup>a</sup>	5.0 ± 1.2 <sup>b</sup>	0.8 ± 0.1 <sup>b</sup>	0.2 ± 0.4 <sup>b</sup>	1.2 ± 2.7 <sup>b</sup>	19.4 ± 5.6 <sup>b</sup>	133.8 ± 14.4 <sup>b</sup>	11.5 ± 2.3 <sup>a</sup>	0.7 ± 0.2 <sup>b</sup>
<b>Second Expected Environmental Concentration Experiment</b>										
Control	9 644.6 ± 772.0 <sup>a</sup>	60.8 ± 3.4 <sup>a</sup>	11.6 ± 1.3 <sup>a</sup>	1.9 ± 0.2 <sup>a</sup>	10.0 ± 1.9 <sup>a</sup>	532.9 ± 54.6 <sup>a</sup>	80.4 ± 7.2 <sup>a</sup>	843.3 ± 67.9 <sup>a</sup>	8.5 ± 0.6 <sup>a</sup>	14.1 ± 0.7 <sup>a</sup>
Hexazinone	8 897.2 ± 806.8 <sup>a</sup>	51.7 ± 3.3 <sup>b</sup>	3.8 ± 0.4 <sup>b</sup>	0.7 ± 0.1 <sup>b</sup>	0.6 ± 0.5 <sup>b</sup>	2.7 ± 3.7 <sup>b</sup>	17.2 ± 1.5 <sup>b</sup>	237.4 ± 11.7 <sup>b</sup>	10.0 ± 1.9 <sup>a</sup>	2.28 ± 0.6 <sup>b</sup>

a,b For each EEC experiment, any means in the same column with the same superscript were not significant different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test. For all parameters, n = 3 for the control of the first EEC experiment and n = 5 for the other treatments. The data presented are the untransformed mean ± s.d.

Table 70: The pigment content of *Myriophyllum sibiricum* apices after fourteen days of incubation in sterile modified Andrews' medium with or without the EEC of hexazinone (8.0 mg/L).

Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
First Expected Environmental Concentration Experiment						
Control	0.47 ± 0.01 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>	0.16 ± 0.003 <sup>a</sup>	Not Measured	Not Measured	Not Measured
Hexazinone	0.40 ± 0.04 <sup>b</sup>	0.16 ± 0.02 <sup>a</sup>	0.13 ± 0.01 <sup>b</sup>	Not Measured	Not Measured	Not Measured
Second Expected Environmental Concentration Experiment						
Control	0.64 ± 0.05 <sup>a</sup>	0.24 ± 0.02 <sup>a</sup>	0.22 ± 0.02 <sup>a</sup>	6.8 ± 0.7 <sup>a</sup>	2.5 ± 0.2 <sup>a</sup>	2.3 ± 0.2 <sup>a</sup>
Hexazinone	0.26 ± 0.02 <sup>b</sup>	0.09 ± 0.004 <sup>b</sup>	0.10 ± 0.01 <sup>b</sup>	6.1 ± 0.8 <sup>a</sup>	2.1 ± 0.2 <sup>b</sup>	2.2 ± 0.3 <sup>a</sup>

a,b Within each EEC experiment, any means in the same column with the same superscript were not significant different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test or the parametric t-test. For all parameters, n = 3 for the control of the first EEC experiment and n = 5 for the controls in the second EEC experiment and the hexazinone treatments. The data presented are the untransformed mean ± s.d.

#### 5.7.6.3.2 Dilution Series

Stimulation of *M. sibiricum* growth occurred at low concentrations of hexazinone (Figure 77 and Figure 78). When calculating the NOEC, stimulation in area under the growth curve occurred at 125 µg/L in the first dilution experiment and at 32.9, 98.8 and 296.3 µg/L in the second dilution experiment. At the other concentrations, plant height and area under the growth curve did not approach the 50% inhibition level and therefore IC50 values could not be calculated (Table 71 and Table 72). For the first dilution series experiment, SC20 values of 189.1 µg/L and 81.3 µg/L were obtained for area under the growth curve and shoot length, respectively. During the second experiment with a series of hexazinone concentrations, SC20 values of 546.0 µg/L and 874.2 µg/L were calculated for area under the growth curve and increase in shoot length, respectively. Low concentrations of herbicides often cause hormesis (Ries, 1976). The phytotoxic effects of hexazinone upon *M. sibiricum* might have been underestimated because the sucrose in the liquid growth medium might allow the plants to continue growing even if they were unable to photosynthesise. Even though stimulation in plant height was observed, there was not a corresponding increase in node number or fresh weight (Table 71 and Table 72). At low hexazinone concentrations, stimulation was also observed in pigment content based on fresh weight of the apex (first and second experiments) and on dry weight of the apex (Table 72 and Table 74). At higher concentrations, the reduction in pigment content did not reach the 50% inhibition level, so IC50s could not be interpolated from the data. High concentrations of hexazinone resulted in a decreased number of nodes per centimetre, root growth, total fresh weight, plant area and dissolved oxygen concentration. For most of the endpoint parameters, the NOEC was 125 µg/L for the first experiment and 98.8 µg/L for the second dilution experiment. In the second dilution experiment, percent inhibition of root length and root number went from stimulation (-5 and -10%, respectively) to inhibition (88 and 75%, respectively). This slope was too steep for either the weibull or cumulative normal distribution models to calculate an IC50 so the IC25 and IC50 values were estimated using curve fitting techniques.

If hexazinone concentrations in runoff water average  $442 \pm 53$  µg/L (Neary *et al.*, 1983), phytotoxic effects would be observed in *M. sibiricum* growth and development. In the United States, there are no standards or guidelines recommending limits for

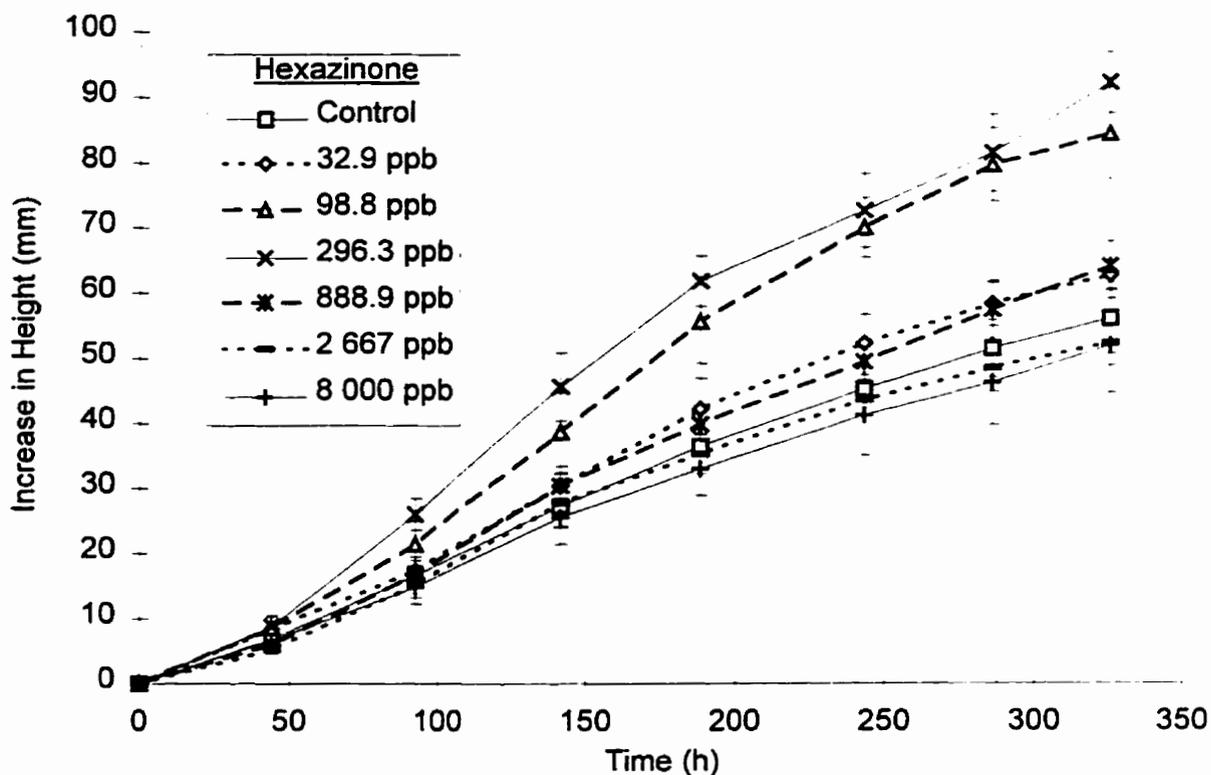


Figure 78: Fourteen day growth of *Myriophyllum sibiricum* apices exposed to a series of hexazinone concentrations in sterile modified Andrews' medium. Stimulation in plant height occurred at low concentrations.

quantities of hexazinone in water (Nowell and Resek, 1994). In order to protect aquatic plants, guidelines for the maximum residue in freshwater should be set between 32.9 and 98.8  $\mu\text{g/L}$ .

#### 5.7.6.3.3 Aquatic Plant Toxicity Comparison

Hexazinone was quite toxic to *Selenastrum capricornutum* tested in the flask assay (96 hour  $\text{EC}_{50}$  = 24.5  $\mu\text{g/L}$  (c.i. = 14.5 - 33.1  $\mu\text{g/L}$ ) and the microplate assay (96 hour  $\text{EC}_{50}$  = 27.7  $\mu\text{g/L}$  (c.i. = 22.7 - 32.5  $\mu\text{g/L}$ ) (Table 73) (St. Laurent *et al.*, 1992). Technical hexazinone was also toxic as indicated by chlorophyll *a* content of *S. capricornutum* but the toxicity decreased as the duration of the bioassay increased. After 3, 5 and 7 days of incubation, the  $\text{EC}_{50}$ s were 56  $\mu\text{g/L}$ , 85  $\mu\text{g/L}$  and 126  $\mu\text{g/L}$ , respectively (Table 73) (Abou-Waly *et al.*, 1991b). Formulations of hexazinone were slightly more toxic to *S. capricornutum* population growth than the technical compound. Growth of *S. capricornutum* in the presence of a soil eluate and two formulations of

hexazinone (the liquid formulation Velpar L<sup>®</sup> (25 % a.i.) and the granular formulation Velpar ULW<sup>®</sup> (75% a.i.)) was completely inhibited one hour, five days and ten days after treatment. For this algal species, the EC50 of these hexazinone formulations applied to water was determined to be 2.5 µg/L for Velpar L<sup>®</sup> and 1.2 µg/L for Velpar ULW<sup>®</sup> (Table 73) (Thomas *et al.*, 1990). There appears to be a typographical error in the abstract of the paper by Thomas *et al.* (1990) where the EC50s are reported to be 2.5 and 1.2 µg/mL for Velpar L<sup>®</sup> and Velpar ULW<sup>®</sup>, respectively. Based on the EC50 values, *S. capricornutum* appears to be more susceptible to hexazinone than *M. sibiricum*.

Technical hexazinone reduced the chlorophyll content of *Anabaena flos-aquae* but this species was not as sensitive as *S. capricornutum* (Abou-Waly *et al.*, 1991a; 1991b). The toxicity of hexazinone decreased over time, indicated by the 3 day EC50 was 2.014 mg/L, the 5 day EC50 was 2.375 mg/L and the 7 day EC50 was 2.752 mg/L (Table 73) (Abou-Waly *et al.*, 1991b).

Hexazinone, at a concentration of 2.867 mg/L, was extremely toxic (percent inhibition between 95 and 100%) to seven algal species (*Cyclotella meneghiana*, *Nitzschia* sp., *Scenedesmus quadricauda*, *Selenastrum capricornutum*, *Microcystis aeruginosa*, *Pseudoanabaena* sp., *Aphanizomenon flos-aquae*) and duckweed (*Lemna minor*). Using <sup>14</sup>C uptake, supplied as NaH<sup>14</sup>CO<sub>3</sub>, two other species of algae were not as greatly affected. *Oscillatoria* sp. was inhibited by 76% and *Anabaena inaequalis* was inhibited by 58% when compared to the controls (Peterson *et al.*, 1994). Hexazinone affected *M. sibiricum* growth to a greater extent than it affected *Oscillatoria* and *A. inaequalis*.

In a limnocorral study in a northern forest situation, there was a dose dependent effect of hexazinone upon dissolved oxygen concentration possibly caused by inhibition of algal photosynthesis (Solomon *et al.*, 1988). In a pond study with aquatic macrophytes, periphyton, invertebrates and fish, dissolved oxygen decreased from 8 mg/L to 0.2 mg/L by 6 days post-treatment (Anderson, 1981a). A decline in dissolved oxygen was also observed in the *Myriophyllum sibiricum* toxicity tests.

As determined by Anderson (1981a), 14 days after treatment with hexazinone at 1 mg/L, *Potamogeton nodosu* Poir. (American pondweed) and *P. pectinatus* were decomposing and by 28 days after treatment these plants were considered dead. Other

Table 71: The effect of hexazinone upon *Myriophyllum sibiricum* growth and development endpoint parameters presented as no observable effect concentrations. Plant height was stimulated at low concentrations and did not differ from the control at high concentrations.

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	
	First Dilution Series	Second Dilution Series
Area Under the Growth Curve	Stimulation	Stimulation
Increase in Shoot Length	Stimulation	Stimulation
Increase in Node Number	125	98.8
Nodes per Centimetre	125	All different from control
Root Length	125	98.8
Root Number	125	98.8
Dissolved Oxygen	125	All different from control
Fresh Weight	125	98.8
Membrane Integrity	No Effect	No Effect
Plant Area	125	98.8
Chlorophyll a (fresh)	< 125 <sup>a</sup>	32.9 <sup>b</sup>
Chlorophyll b (fresh)	< 125 <sup>a</sup>	98.8 <sup>b</sup>
Carotenoid (fresh)	< 125 <sup>a</sup>	98.8 <sup>b</sup>
Chlorophyll a (dry)	Not measured	32.9 <sup>c</sup>
Chlorophyll b (dry)	Not measured	98.8 <sup>c</sup>
Carotenoid (dry)	Not measured	32.9 <sup>c</sup>

a Stimulation of pigment content occurred at low concentrations (125 and 250  $\mu\text{g/L}$ ) and pigments were inhibited at concentrations greater than 500  $\mu\text{g/L}$ .

b In the second experiment, stimulation of pigment content occurred only at 296.3  $\mu\text{g/L}$ .

c Stimulation of pigment, based on the dry weight of the apex, occurred at 296.3 and 888.9  $\mu\text{g/L}$ .

aquatic plants studied by Anderson (1981a), including *Eleocharis acicularis* (L.) Roem. & Schult. (slender spikerush), *Typhus* sp. (cattail), *Chara* sp. and a filamentous alga (*Spirogyra* sp.), were controlled by 45 days post-spray. *Elodea canadensis* required 100 days of treatment before control was achieved. After hexazinone treatment, periphyton was also affected in terms of number of species and total numbers of algal cells. Water and hydrosol collected from ponds 15 months after treatment with 1 mg/L showed no phytotoxicity to *P. nodosu* and *P. pectinatus* germination or growth (Anderson, 1981a). The 1.0 mg/L hexazinone that controlled the aquatic plants in the

Table 72: The effect of a series of concentrations of hexazinone upon *Myriophyllum sibiricum* growth and development parameters, expressed as IC25 and IC50 values. Values in brackets are the 95% confidence intervals.

GROWTH PARAMETER	First Dilution Series		Second Dilution Series	
	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	2 110.2 <sup>a</sup>	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>
Increase in Shoot Length	1 565.8 <sup>a</sup>	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>
Increase in Node Number	< 125 <sup>c,d</sup>	277.4 (95.4, 807.0) <sup>c</sup>	220.3 (46.6, 1 042.0) <sup>d</sup>	978.4 (319.4, 2 997.1) <sup>d</sup>
Nodes per Centimetre	< 125 <sup>c,d</sup>	152.4 (49.9, 465.9) <sup>d</sup>	38.5 (10.5, 140.8) <sup>d</sup>	332.6 (111.9, 988.9) <sup>d</sup>
Root Length	< 125 <sup>c,d</sup>	< 125 <sup>c,d</sup>	125.1 <sup>a</sup>	289.6 <sup>a</sup>
Root Number	< 125 <sup>c,d</sup>	< 125 <sup>c,d</sup>	130.3 <sup>a</sup>	334.9 <sup>a</sup>
Dissolved Oxygen	< 125 <sup>c,d</sup>	931.2 (213.9; 4 053.3) <sup>c</sup>	< 32.9 <sup>c,d</sup>	128.2 (46.9; 350.4) <sup>c</sup>
Fresh Weight	< 125 <sup>d</sup>	< 125 <sup>d</sup>	125.8 (28.4, 557.4) <sup>d</sup>	538.6 (175.6; 1 651.9) <sup>d</sup>
Membrane Integrity	No Effect	No Effect	No Effect	No Effect
Plant Area	< 125 <sup>d</sup>	156.2 (96.4; 253.1) <sup>d</sup>	214.9 (134.5, 343.3) <sup>d</sup>	369.4 (243.2; 561.1) <sup>d</sup>
Chlorophyll a (fresh)	1 061.7 <sup>a</sup>	> 8 000 <sup>b</sup>	844.9 <sup>a</sup>	2 222.5 <sup>a</sup>
Chlorophyll b (fresh)	1 070.8 <sup>a</sup>	> 8 000 <sup>b</sup>	1 274.7 <sup>a</sup>	2 320.7 <sup>a</sup>
Carotenoid (fresh)	1 066.4 <sup>a</sup>	> 8 000 <sup>b</sup>	880.0 <sup>a</sup>	2 374.4 <sup>a</sup>
Chlorophyll a (dry)	Not measured	Not measured	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>
Chlorophyll b (dry)	Not measured	Not measured	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>
Carotenoid (dry)	Not measured	Not measured	> 8 000 <sup>b</sup>	> 8 000 <sup>b</sup>

a Stimulation occurred in these endpoint parameters so curve fitting techniques were used to calculate IC50 values. 95% confidence intervals could not be calculated.

b Stimulation was observed at low levels. At high concentrations, percent inhibition did not reach the level being examined.

c IC50 and 95% confidence values were calculated with the cumulative normal distribution model.

d IC50 values and 95% confidence intervals were calculated using the weibull model.

Table 73: A summary of IC50 and EC50 values for aquatic plant species exposed to hexazinone. The test conditions differed between the species but general trends in sensitivity may be observed. *Myriophyllum sibiricum* was 10 times less sensitive to the effects of hexazinone than was *Selenastrum capricornutum*.

Species	Endpoint	Formulation	Exposure Duration	IC50/EC50	Reference
<b>Algae</b>					
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	Technical	3 days	2.014 mg/L	Abou-Waly (1991b)
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	Technical	5 days	2.375 mg/L	Abou-Waly (1991b)
<i>Anabaena flos-aquae</i>	Chlorophyll a Content	Technical	7 days	2.752 mg/L	Abou-Waly (1991b)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	Technical	96 hours	24.5 µg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	Technical	96 hours	27.7 µg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Cell Count	Velpar ULW®	96 hours	1.2 µg/L	Thomas <i>et al.</i> (1990)
<i>Selenastrum capricornutum</i>	Cell Count	Velpar L®	96 hours	2.5 µg/L	Thomas <i>et al.</i> (1990)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	Technical	3 days	56 µg/L	Abou-Waly (1991b)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	Technical	5 days	85 µg/L	Abou-Waly (1991b)
<i>Selenastrum capricornutum</i>	Chlorophyll a Content	Technical	7 days	126 µg/L	Abou-Waly (1991b)
<b>Macrophytes</b>					
<i>Myriophyllum sibiricum</i>	Dissolved Oxygen	Technical	14 days	128.2 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Root Length	Technical	14 days	289.6 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Nodes per Centimetre	Technical	14 days	332.6 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	Technical	14 days	369.4 µg/L	This Document

study by Anderson (1981a) would have controlled the *M. sibiricum*, as determined in the current study.

The toxic effects of hexazinone to *Tradescantia* leaves were detected within 25 hours of exposure by measuring chlorophyll fluorescence. Ten and eighty percent of Velpar® ULW in solution with a soil eluate created over an eighty percent reduction in the size of the electron pool (Judy *et al.*, 1990). This detection of inhibitory effects was more rapid than that obtained by the *Myriophyllum sibiricum* toxicity test but the *M. sibiricum* test was more sensitive because root length was greatly inhibited (99%) when hexazinone was applied at the EEC.

The *Myriophyllum sibiricum* toxicity test was more sensitive to the effects of hexazinone than it was to the effects of atrazine (Section 5.7.1) even though both of these herbicides inhibit photosynthesis. Comparing the most sensitive parameter for hexazinone (dissolved oxygen) (Table 72 and Table 73) with the most sensitive parameter for atrazine (root length) (Table 45 and Table 47), hexazinone was 8 times more toxic to *M. sibiricum* than was atrazine. When the IC50 values for dissolved oxygen were compared, it was determined that *M. sibiricum* was 15.6 times more sensitive to the effects of hexazinone than to the effects of atrazine. This supported the hypothesis that this population of *M. sibiricum* might be resistant to atrazine or that there was a minor interaction between the technical atrazine and the solvent methanol. This helped clarify that the addition of sucrose to the medium was not prohibiting the detection of photosynthetic inhibitors. On occasion, sucrose has been included in *Lemna* experimental medium (Nasu and Kugimoto, 1981).

#### 5.7.6.3.4 Conclusions

It was determined that if 8.0 mg/L (expected environmental concentration) of hexazinone entered an aquatic system, *Myriophyllum sibiricum* would be affected. One-sixtieth of this concentration would cause a 50% reduction in oxygen production and one-thirtieth of this concentration would produce a 50% decrease in root length. One percent of the EEC would have no major effects upon the growth and development of *M. sibiricum*.

From the peer reviewed scientific literature, EC50/IC50 values could only be found for *Anabaena flos-aquae* and *Selenastrum capricornutum*. *S. capricornutum* was 5 times more sensitive to the effects of technical hexazinone but *A. flos-aquae* was 15

times less sensitive than was *M. sibiricum* in the axenic toxicity test (Table 73). Thus, *S. capricornutum* populations would also be affected by the EEC of hexazinone. More toxicological research with other aquatic plant species would be required before the value of the *M. sibiricum* toxicity test can be determined in the registration process of photosynthetic inhibiting herbicides.

## 5.7.7 Metolachlor

### 5.7.7.1 Introduction

The herbicide, metolachlor (Figure 79), is registered for used in corn, cotton (*Gossypium hirsutum* L.), peanuts (*Arachis hypogaea* L.), soybeans and other beans, potatoes, vegetables, sugar beets (*Beta vulgaris* L.), sorghum, fruit trees, nuts and

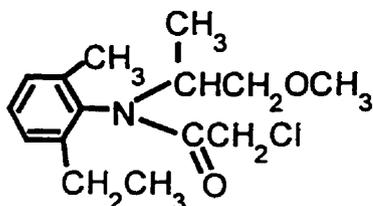


Figure 79: Metolachlor.

ornamentals and is applied as a soil surface spray or soil incorporated treatment (Wauchope *et al.*, 1992; OMAF, 1994a). Metolachlor is more effective in controlling some monocotyledonous plants than dicotyledonous weeds (Fuerst, 1987; Peter and Weber, 1985). It has been registered for use in Canada since 1977 (Kent *et al.*, 1991).

Metolachlor was the most widely used herbicide in Ontario during 1993 with a total of 1 327 315 kg a.i. being applied to field crops, fruits and vegetables (Hunter and McGee, 1994).

Metolachlor is considered to be relatively nonvolatile and is not degraded rapidly by sunlight (Kochany and Maguire, 1994; Lebaron *et al.*, 1988). In soils, biodegradation of metolachlor is moderately slow (Johnson and Fuhrmann, 1993) but microorganisms capable of degrading metolachlor have been identified. Numerous metolachlor metabolites have been identified under different environmental conditions. In field studies, the half-life of metolachlor ranged from seventeen days in clay loam to seventy days in sandy loam (Chesters *et al.*, 1989). In contrast, Liu *et al.* (1995) found no biodegradation or biotransformation of metolachlor in water from three lakes after 170 days (Liu *et al.*, 1995). The Dual<sup>®</sup> formulation of metolachlor is rated as a medium risk for surface runoff and leaching (Becker *et al.*, 1989). Of radioactive metolachlor applied to soil columns, 11.1% was detected in the leachate (Peter and Weber, 1985).

Residues of metolachlor have been detected in surface and subsurface runoff (Buttle,

1990; Chesters *et al.*, 1989; Gaynor *et al.*, 1995; Southwick *et al.*, 1990a; 1990b; Wietersen *et al.*, 1993) with the majority of the metolachlor in the runoff in the adsorbed phase (Buttle, 1990). The maximum total annual metolachlor loss from corn plots was 121.0 g/ha (Gaynor *et al.*, 1995) and the highest metolachlor concentration in runoff was 29.3 µg/L (Southwick *et al.*, 1990a). Due to accidental spillage, runoff and drift, metolachlor has been detected in drinking water wells (Frank *et al.*, 1987a; 1990a) and farm ponds. The metolachlor concentration in one farm pond was 190 µg/L (Frank *et al.*, 1990b). Metolachlor residues were detected in Great Lakes water samples to a level of 28 ng/L (Schottler and Eisenreich, 1994). Metolachlor was detected at twenty-six sites along the Mississippi River with 1918 ng/L as the maximum reported metolachlor residue (Pereira and Hostettler, 1993). In a study of drainage from Iowa farm land, metolachlor residues were detected 22 times with a mean concentration of 1.0 µg/L and a range from 0.10 - 5.0 µg/L (Wang *et al.*, 1995). For the protection of aquatic life, the interim Canadian water quality guideline has been set at 8 µg/L (Kent *et al.*, 1991). The toxicology and environmental fate of metolachlor are summarised by Chesters *et al.* (1989), Kent *et al.* (1991) and Lebaron *et al.* (1988).

The mode of metolachlor phytotoxic action is not well understood but herbicides in the chloroacetamide herbicide family are classified as growth inhibitors because they inhibit biosynthesis of fatty acids and lipids, isoprenoids, flavonoids (Fuerst, 1987; Lebaron *et al.*, 1988; WSSA, 1994), protein (Deal *et al.*, 1980; Fuerst, 1987; Lebaron *et al.*, 1988; WSSA, 1994), acetyl-coenzyme A, gibberellins and anthocyanins (Fuerst, 1987). It acts as a growth retardant in grasses. Chloroacetamide herbicides inhibit root and shoot growth (Lebaron *et al.*, 1988). Deal and Hess (1980) determined that in roots of terrestrial species both cell division and cell enlargement were affected (Deal and Hess, 1980).

#### 5.7.7.2 Methods

The water solubility of metolachlor is cited as ranging between 480 mg/L (WSSA, 1994) and 530 mg/L (Lebaron *et al.*, 1988; Wauchope *et al.*, 1992). Despite this, the technical metolachlor used in this study (98% as obtained from Chem Service<sup>16</sup>) was in non-polar, organic liquid form and did not dissolve readily into water or liquid growth medium. For most of the experiments, the metolachlor was dissolved in methanol before being added to the modified Andrews' medium. Metolachlor is very soluble in

methanol (Kent *et al.*, 1991). Deal and Hess (1980) and Deal *et al.* (1980) dissolved metolachlor in one percent dimethyl sulfoxide before treating oats (*Avena sativa* L.).

The recommended maximum label rate is 4.48 kg/ha and this converts to an expected environmental concentration in 15 cm of water of 2.99 mg/L. The EEC was tested twice. For the first EEC experiment, the stock plants were cultured in M & S medium, while in the second EEC experiment, the stock plants were cultured in modified Andrews' medium. For both EEC experiments, 250 mL of modified Andrews' medium were spiked with 1 mL of sterile nanopure water (water control), methanol (solvent control) or the metolachlor stock solution made in methanol. For detailed experimental and statistical methods, check Section 4.6.

All stock plants used in the dilution series experiments were cultured in modified Andrews' medium. The first metolachlor dilution series was conducted with metolachlor concentrations ranging between 4.1 µg/L and 2 987 µg/L plus a water control and a methanol control. Each treatment and the controls were replicated five times. The second metolachlor dilution series contained metolachlor concentrations of 12.3, 36.9, 110.6, 331.9, 995.6 and 2 987 µg/L and a water control. For the second dilution experiment, 8.08 µg metolachlor was dissolved in 1 L of sterile modified Andrews' medium. This metolachlor stock solution was serially diluted with sterile modified Andrews' medium to produce final concentrations of 12.3, 36.9, 110.6, 331.8, 995.6 and 2 987 µg/L. Undiluted modified Andrews' medium was used for the five control replicates. Refer to Section 4.6 for more detailed methods and statistical analysis.

### 5.7.7.3 Results and Discussion

#### 5.7.7.3.1 Expected Environmental Concentration

At the EEC of 2.99 mg/L, metolachlor inhibited the growth of *Myriophyllum sibiricum* plants in axenic culture. Shoot elongation was inhibited (Figure 81 and Figure 80) so there was a corresponding inhibition of area under the growth curve (Table 74). The raw data in Table 75 shows this trend for both EEC experiments conducted with metolachlor. For both experiments, the pigment content based on the fresh weight of the apices is misleading since it shows that there was a large stimulation (Table 80 and Table 81). For the second experiment, the dry weight of the apices was also measured and when the pigment content is expressed on this basis, there is no significant difference between the control and treated values (Table 76).

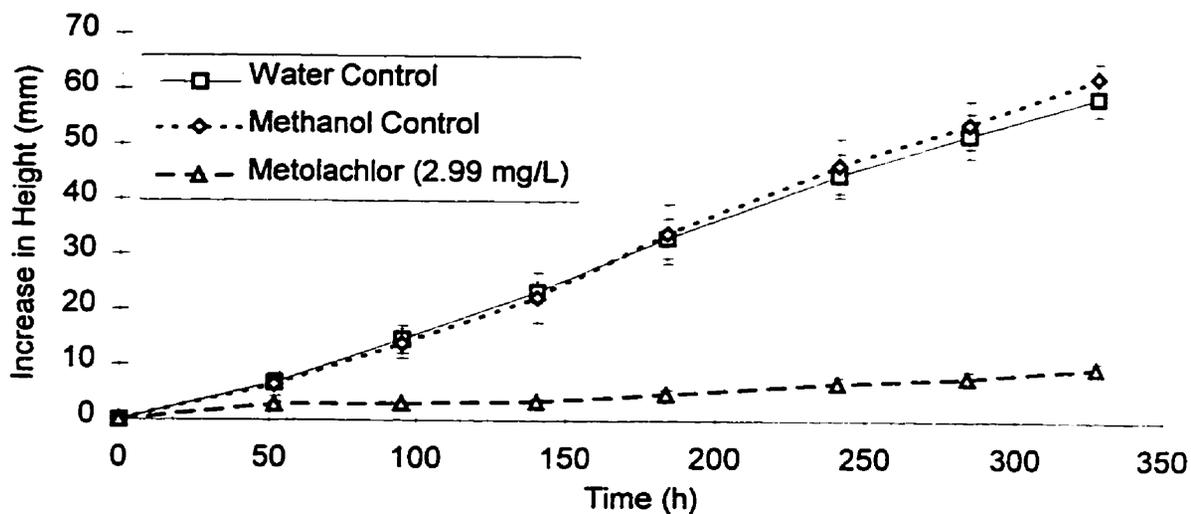


Figure 80: Metolachlor (2.99 mg/L) inhibited the plant height of *Myriophyllum sibiricum* plants, as seen in these fourteen day growth curves from the first metolachlor EEC experiment. There was no significant difference between the plant height or area under the growth curve for the water and methanol controls. All experimental plants were axenically cultured in modified Andrews' medium. Each data point is the mean  $\pm$  s.d. of five replicates.

Table 74: After fourteen days of growth in axenic culture with modified Andrews' medium plus the EEC of metolachlor (2.99 mg/L), *Myriophyllum sibiricum* growth and development were inhibited when compared with the control plants.

GROWTH PARAMETER	PERCENT INHIBITION (mean $\pm$ s.d.)	
	First Experiment	Second Experiment
Area Under the Growth Curve	83.8 $\pm$ 1.9 <sup>a</sup>	61.8 $\pm$ 8.1 <sup>a</sup>
Increase in Shoot Length	79.5 $\pm$ 1.4 <sup>a</sup>	63.5 $\pm$ 3.8 <sup>a</sup>
Root Number	91.8 $\pm$ 4.6 <sup>a</sup>	63.1 $\pm$ 14.3 <sup>a</sup>
Root Length	99.6 $\pm$ 0.6 <sup>a</sup>	98.4 $\pm$ 0.9 <sup>a</sup>
Fresh Weight	83.1 $\pm$ 2.7 <sup>a</sup>	73.7 $\pm$ 3.6 <sup>a</sup>
Membrane Integrity	14.6 $\pm$ 5.2 <sup>b</sup>	5.0 $\pm$ 1.4 <sup>b</sup>
Plant Area	83.3 $\pm$ 3.4 <sup>a</sup>	70.8 $\pm$ 6.4 <sup>a</sup>
Chlorophyll <i>a</i> (fresh)	-60.5 $\pm$ 14.4 <sup>a</sup>	-65.6 $\pm$ 14.2 <sup>b</sup>
Chlorophyll <i>b</i> (fresh)	-83.0 $\pm$ 17.9 <sup>a</sup>	-87.8 $\pm$ 13.4 <sup>b</sup>
Carotenoid (fresh)	-45.4 $\pm$ 15.0 <sup>a</sup>	-55.7 $\pm$ 13.0 <sup>b</sup>
Chlorophyll <i>a</i> (dry)	Not measured	8.1 $\pm$ 12.7 <sup>a</sup>
Chlorophyll <i>b</i> (dry)	Not measured	-6.9 $\pm$ 12.9 <sup>a</sup>
Carotenoid (dry)	Not measured	14.0 $\pm$ 11.8 <sup>a</sup>

a The percent inhibition was calculated based on the mean of all ten control plants.

b Only the mean of the five methanol control plants was utilised in the calculation of percent inhibition.

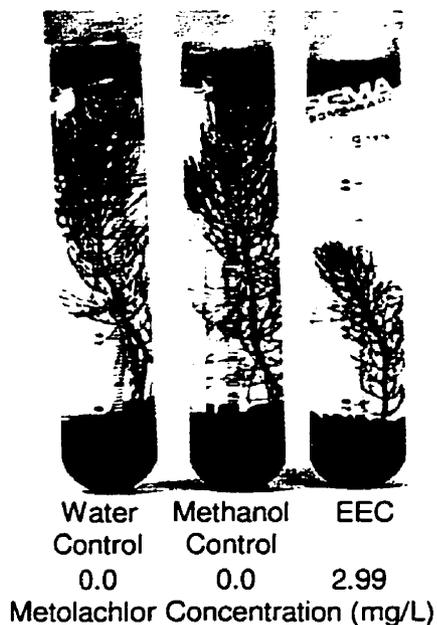


Figure 81: Influence of metolachlor (2.99 mg/L, right) on the growth of *M. sibiricum* as compared with the water (left) and methanol (middle) control plants. The plants were grown axenically for fourteen days in modified Andrews' medium.

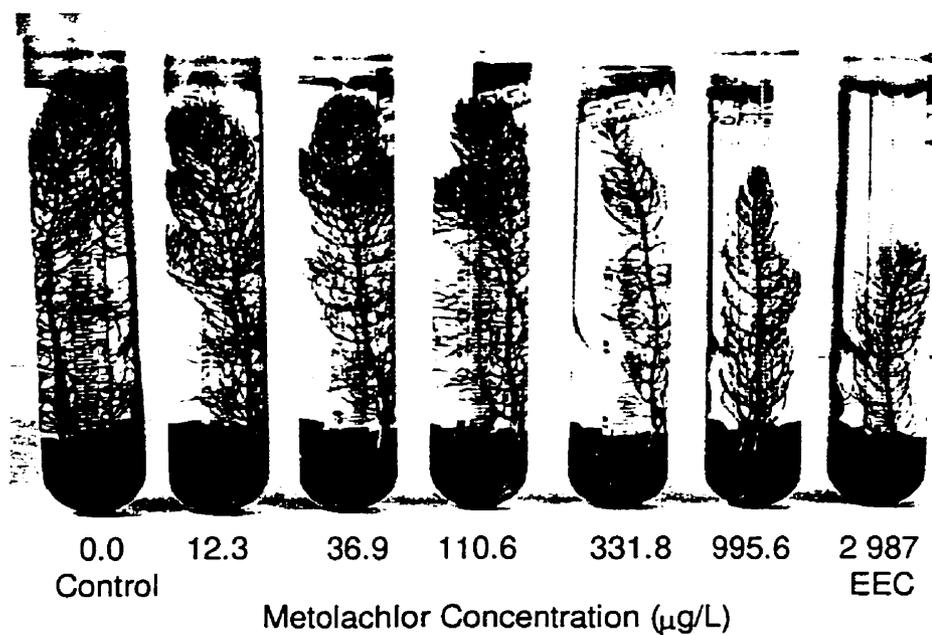


Figure 82: In the second dilution series, metolachlor visually affected *M. sibiricum* plants in axenic culture at a level of 331.9  $\mu\text{g/L}$ .

Table 75: The effect of metolachlor (2.99 mg/L) upon <i>Myriophyllum sibiricum</i> growth and development. The plants were grown for 14 days in axenic culture with modified Andrews' medium as determined in two EEC experiments.								
Treatment	Area under the growth curve	Increase in Plant Length (mm)	Root Number	Total Root Length (mm)	D.O. (%)	Fresh Weight (mg)	Membrane Permeability (%)	Plant Area (cm <sup>2</sup> )
First Expected Environmental Concentration Experiment								
Combined Controls	9 501.8 ± 931.2 <sup>a</sup>	65.9 ± 4.5 <sup>a</sup>	9.7 ± 1.5 <sup>a</sup>	442.2 ± 50.0 <sup>a</sup>	71.7 ± 6.1 <sup>a</sup>	544.9 ± 68.5 <sup>a</sup>	—	11.9 ± 1.7 <sup>a</sup>
Methanol Control	—	—	—	—	—	—	8.5 ± 0.6 <sup>a</sup>	—
Metolachlor	1 542.6 ± 181.8 <sup>b</sup>	13.5 ± 1.0 <sup>b</sup>	0.8 ± 0.4 <sup>b</sup>	1.7 ± 2.6 <sup>b</sup>	75.6 ± 2.3 <sup>a</sup>	92.3 ± 14.6 <sup>b</sup>	14.7 ± 2.7 <sup>b</sup>	2.0 ± 0.4 <sup>b</sup>
Second Expected Environmental Concentration Experiment								
Combined Controls	9 652.5 ± 1 019.2 <sup>a</sup>	59.7 ± 4.8 <sup>a</sup>	8.8 ± 1.3 <sup>a</sup>	382.2 ± 122.3 <sup>a</sup>	46.5 ± 5.7 <sup>a</sup>	730.9 ± 62.4 <sup>a</sup>	—	14.0 ± 1.5 <sup>a</sup>
Methanol Control	—	—	—	—	—	—	10.2 ± 1.0 <sup>a</sup>	—
Metolachlor	3 525.1 ± 685.4 <sup>b</sup>	21.8 ± 2.2 <sup>b</sup>	3.3 ± 1.3 <sup>b</sup>	6.0 ± 3.5 <sup>b</sup>	49.8 ± 3.6 <sup>a</sup>	192.0 ± 26.1 <sup>b</sup>	10.9 ± 0.7 <sup>a</sup>	4.1 ± 0.9 <sup>b</sup>

a,b For each EEC experiment, any means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test and the parametric t-test. For all parameters,  $n = 4$  for the metolachlor treatment of the second EEC experiment.  $n = 5$  for all the controls except the membrane integrity methanol control had  $n = 4$ . The control data were combined if there was no significant difference between the water and methanol controls. The data presented are the untransformed mean  $\pm$  s.d.

Table 76: As determined in two experiments, the effect of metolachlor (2.99 mg/L) upon the pigment content of <i>Myriophyllum sibiricum</i> grown for fourteen days in axenic culture with modified Andrews' medium.						
Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
First Expected Environmental Concentration Experiment						
Combined Controls	0.51 ± 0.04 <sup>a</sup>	0.17 ± 0.02 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>	Not Measured	Not Measured	Not Measured
Methanol Control	—	—	—	Not Measured	Not Measured	Not Measured
Metolachlor	0.82 ± 0.07 <sup>b</sup>	0.31 ± 0.03 <sup>b</sup>	0.25 ± 0.03 <sup>b</sup>	Not Measured	Not Measured	Not Measured
Second Expected Environmental Concentration Experiment						
Combined Controls	—	—	—	2.89 ± 0.22 <sup>a</sup>	1.03 ± 0.07 <sup>a</sup>	1.00 ± 0.08 <sup>a</sup>
Methanol Control	0.50 ± 0.02 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>	0.17 ± 0.01 <sup>a</sup>	—	—	—
Metolachlor	1.00 ± 0.09 <sup>b</sup>	0.41 ± 0.03 <sup>b</sup>	0.32 ± 0.03 <sup>b</sup>	2.66 ± 0.37 <sup>a</sup>	1.10 ± 0.13 <sup>a</sup>	0.86 ± 0.12 <sup>a</sup>

a,b For each EEC experiment, any means in the same column with the same superscript were not significantly different at  $\alpha = 0.05$  as tested by the nonparametric Mann-Whitney U-test or the parametric t-test. For all parameters,  $n = 4$  for the metolachlor treatment in the second EEC experiment and  $n = 5$  for each of the controls. If there was no significant difference between the methanol and water controls ( $\alpha = 0.05$ ), the data were combined for statistical analysis and presentation in this table. The data presented are the untransformed mean  $\pm$  s.d.

### 5.7.7.3.2 Dilution Series

Only the results from the second metolachlor dilution series are presented. In the first metolachlor dilution series, eighteen of the forty-five experimental plants were discarded due to contamination. It was decided not to include this data set because too many plants were lost. However, upon examination of the remaining data from the first dilution experiment, it was determined that the results between the two dilution series were similar.

Visually, metolachlor was toxic to *Myriophyllum sibiricum* at concentrations equal to or greater than 331.9  $\mu\text{g/L}$  (Figure 82). Most of the endpoint parameters did not differ from the controls ( $\alpha = 0.05$ ) at metolachlor concentrations of 110.6 or 331.9  $\mu\text{g/L}$  (Figure 83 and Table 77). The most sensitive parameter to the phytotoxic effects of metolachlor

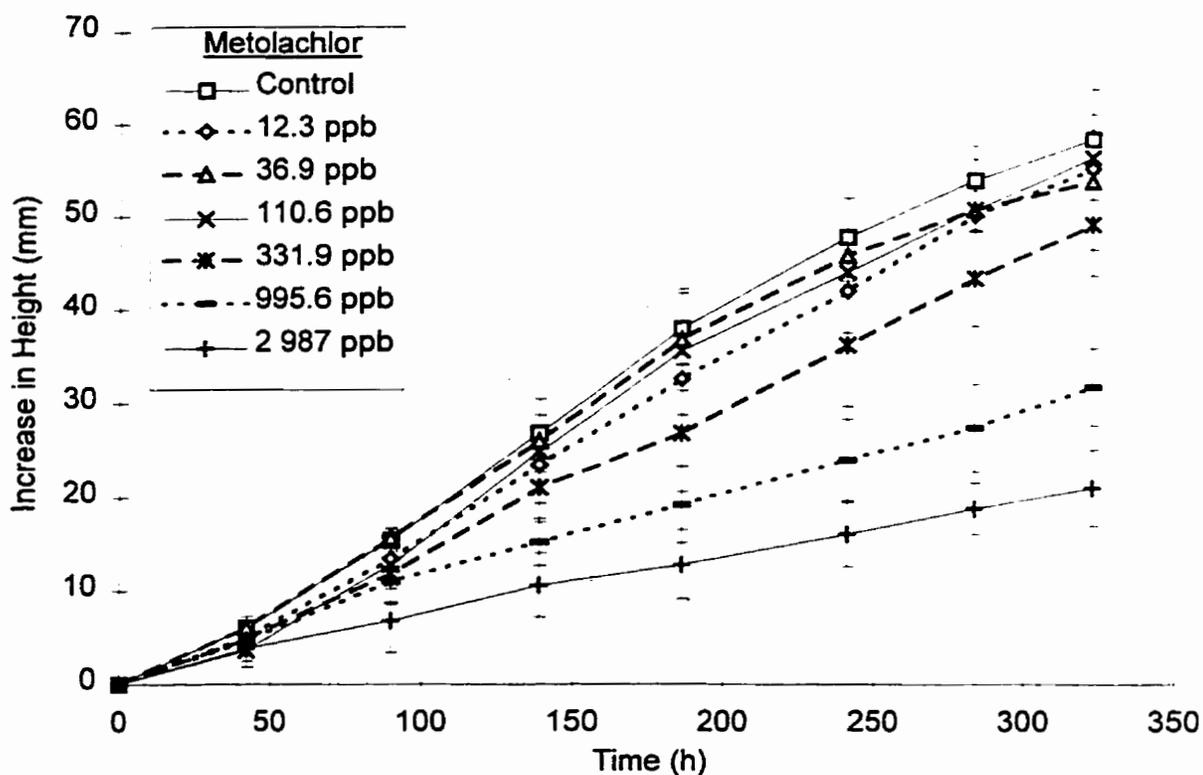


Figure 83: The effect of metolachlor at various concentrations (12.3  $\mu\text{g/L}$  to 2.99 mg/L) upon the growth of *Myriophyllum sibiricum*. The plants were cultured for fourteen days in modified Andrews' medium with the appropriate amount of metolachlor or sterile nanopure water. Data points are the mean of five replicates and the bars on the data points indicate the standard deviation.

**Table 77: The phytotoxic effects of metolachlor upon *Myriophyllum sibiricum* growth and development endpoint parameters, expressed as the no observable effect concentration, the average percent inhibition ( $\pm$  s.d.) at the NOEC, the IC25 and IC50 values (upper and lower 95% c.i.).**

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	% I at NOEC	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	110.6	8.1 $\pm$ 4.7	448.1 (277.2, 724.4) <sup>a</sup>	1 535.2 (1 116.6, 2 110.7) <sup>a</sup>
Increase in Shoot Length	331.9	10.5 $\pm$ 9.6	675.1 (435.7, 1 046.2) <sup>a</sup>	1896.0 (1348.5, 2665.8) <sup>a</sup>
Root Length	110.6	7.1 $\pm$ 15.1	375.7 (307.0, 459.7) <sup>a</sup>	670.1 (583.5, 769.5) <sup>a</sup>
Root Number	331.9	11.9 $\pm$ 13.6	522.9 (185.4, 1 475.4) <sup>a</sup>	1 684.8 (676.8, 4 194.0) <sup>a</sup>
Fresh Weight	36.9	-2.7 $\pm$ 11.6	196.6 (103.7, 372.6) <sup>a</sup>	606.7 (389.8, 944.2) <sup>a</sup>
Membrane Integrity	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Plant Area	110.6	6.2 $\pm$ 10.7	149.8 (42.1, 533.5) <sup>a</sup>	579.6 (241.6, 1 390.9) <sup>a</sup>
Chlorophyll <i>a</i> (fresh wt)	110.6	6.4 $\pm$ 7.2	Stimulation	Stimulation
Chlorophyll <i>b</i> (fresh wt)	110.6	7.0 $\pm$ 9.3	Stimulation	Stimulation
Carotenoid (fresh wt)	110.6	5.4 $\pm$ 7.2	Stimulation	Stimulation
Chlorophyll <i>a</i> (dry wt)	331.9	2.8 $\pm$ 17.8	> 2 987 <sup>a,b</sup>	> 2 987 <sup>a,b</sup>
Chlorophyll <i>b</i> (dry wt)	331.9	-2.6 $\pm$ 16.8	> 2 987 <sup>a,b</sup>	> 2 987 <sup>a,b</sup>
Carotenoid (dry wt)	331.9	4.2 $\pm$ 18.5	1 324.8 (449.5; 3 904.9) <sup>b</sup>	> 2 987 <sup>a,b</sup>

a IC<sub>p</sub> and 95% confidence values calculated with the cumulative normal distribution model.

b IC<sub>p</sub> values and 95% confidence intervals calculated with the weibull method.

was total fresh weight, which was not different from the average control plant weight at concentrations equal to or less than 36.9  $\mu\text{g/L}$  (Table 77). This corresponded with metolachlor acting as a plant growth inhibitor (Table 27). Pigment content, expressed on the basis of the fresh apical weight, differed from the control at concentrations greater than 110.6  $\mu\text{g/L}$ . This difference was caused by an apparent stimulation of pigment content. In the second dilution series, the amount of apparent stimulation at 2.99 mg/L was very similar to values obtained in the EEC experiments (Table 74).

Based upon the percent of control, stimulation of chlorophyll *a*, chlorophyll *b* and carotenoid content at 2.99 mg/L was  $-58.5\% \pm 15.7\%$ ;  $-64.4\% \pm 14.6\%$ ;  $-41.4\% \pm 13.6\%$ , respectively. The metolachlor concentrations that stimulated chlorophyll *a*, chlorophyll *b* and carotenoid content by 20 % were calculated using curve fitting techniques to be 217.4, 183.4 and 224.0 mg/L, respectively. When pigment concentrations were calculated based upon the dried apical weight, the pigment extracted from control plants did not differ significantly from plants treated with metolachlor concentrations less than 995.6 $\mu$ g/L ( $\alpha = 0.05$ ). The IC50 values for the pigment concentration were greater than 2.99 mg/L (Table 77).

In 1994, there were no American standards or guidelines for the protection of aquatic life from the effects of metolachlor (Nowell and Resek, 1994). In Canada, the water quality guideline has been set at 8  $\mu$ g of metolachlor per litre to protect aquatic life (Kent *et al.*, 1991). Based upon the results from this axenic toxicity test, this guideline should be sufficient to protect aquatic plants from the effects of metolachlor.

The MLR of 4.48 kg a.i. metolachlor per hectare (WSSA, 1989) used in the aquatic macrophyte toxicity tests has not changed for an initial herbicide application but for subsequent applications the amount could be as high as 6.7 kg a.i./ha in corn and peanuts and 8.7 kg a.i./ha in certain turfgrass species (WSSA, 1994). If metolachlor were applied at the higher rates in sequential applications, toxic effects upon non-target macrophytes would be more severe than demonstrated in this study.

In terrestrial plant species, chloroacetamide herbicides inhibit root and shoot growth (Lebaron *et al.*, 1988). Deal and Hess (1980) determined that within 6 hours, oat roots were inhibited by 47% when exposed to a  $1 \cdot 10^{-4}$  M (28.4 mg/L) metolachlor solution and within 24 hours the roots were inhibited by 80%. These authors concluded that both cell division and cell enlargement were affected (Deal and Hess, 1980). In the *Myriophyllum* toxicity test, both root and shoot growth were inhibited by metolachlor.

Membrane leakage has been reported in a susceptible species (onion - *Allium cepa* L.) after exposure to  $1 \cdot 10^{-4}$  M (28.4 mg/L) and  $1 \cdot 10^{-5}$  M (2.8 mg/L) metolachlor. Within 72 hours, leakage from the onion roots was significantly different from the controls at the  $1 \cdot 10^{-4}$  M concentration. By 144 hours after initiation of the experiment, leakage was 41 times the control value. Two tolerant species (corn and soybean) were not affected and an intermediate amount of cellular leakage occurred from the roots of

two moderately susceptible species (cotton and cucumber (*Cucumis sativus* L.)). These authors could not determine whether metolachlor was exerting a direct effect on the membranes or if the leakage was a secondary effect (Mellis *et al.*, 1982). Other authors have hypothesised that the chloroacetamides affect the synthesis of the components of the membrane lipids and thus the membrane leakage is a secondary effect (Lebaron *et al.*, 1988). Membrane leakage was not observed in the *M. sibiricum* toxicity test (Table 77).

#### 5.7.7.3.3 Aquatic Plant Toxicity Comparison

There seems to be a wide range of metolachlor toxicities to different aquatic plant species (Table 78 and Table 79). *Selenastrum capricornutum* was sensitive to metolachlor. Following the U.S. EPA flask protocol, this species exhibited a 96 hour EC50 of 55.5 µg/L (c.i. = 52.1 - 58.9 µg/L) (Table 78). In microplate studies, the 96 hour EC50 was 50.9 µg/L (95% c.i. = 44.8 - 56.8 µg/L) (Table 78) (St. Laurent *et al.*, 1992) and the 72 hour IC50 ranged from 37.2 to 55.8 µg/L (Table 78) (Day and Hodge, 1996). After four days of treatment, the chlorophyll production of *Chlamydomonas reinhardtii* was inhibited 50% by a technical metolachlor concentration of  $5.0 \cdot 10^{-6}$  M (1.4 mg/L) (Table 78) (Fedtke, 1992). In contrast, *Anabaena cylindrica* was not affected by up to 5 mg/L metolachlor after 72 hours of exposure (Table 78). *Lemna gibba* was of intermediate sensitivity with a seven day IC50 between 304 and 788 µg/L for decrease in frond number and 766 µg/L for change in dry weight (Table 78) (Day and Hodge, 1996). *Lemna minor* fronds were exposed to 50 µM (16.5 mg/L) of metolachlor but there was no change in the amount of photosensitive tetrapyrrole derivatives present in the cells (Chollet, 1992b). After two weeks of exposure, metolachlor at a concentration of 0.1 mg/L produced morphological changes in the free-floating freshwater fern, *Salvinia natans*. EC50 values of 0.075, 0.08, 0.05, 0.15 and 0.55 mg/L were obtained for the growth of leaves, the amount of chlorophyll *a* and chlorophyll *b*, wet weight and length of stems, respectively (Table 78) (Göncz and Sencic, 1994). Respiration rates were decreased by metolachlor concentrations of 274 µg/L in a periphyton community composed of diatoms, green algae, cyanobacteria and protozoans in simulated small agricultural streams (Day, 1993).

For metolachlor in water, the LOEC (lowest observable effect concentration) on seedling dry weight for two varieties of *Echinochloa crusgalli* was 0.25 mg/L and for

Table 78: Selected from the scientific literature, a summary of EC50/IC50 values for aquatic plant species exposed to metolachlor. Test conditions differed between the species but general trends in sensitivity may be observed. Based on EC50/IC50, *Selenastrum capricornutum* and *Salvinia natans* were the most sensitive aquatic plants to metolachlor. *Myriophyllum sibiricum* and *Lemna gibba* were approximately 10 times more resistant to metolachlor.

Species	Endpoint	Exposure Duration	EC50 or IC50	Reference
<b>Algae</b>				
<i>Anabaena cylindrica</i>	Growth	72 hours	> 5 000 µg/L	Day and Hodge (1996)
<i>Chlamydomonas reinhardtii</i>	Chlorophyll	4 days	1 400 µg/L	Fedtke (1992)
<i>Selenastrum capricornutum</i>	Growth	72 hours	37.2 - 56.8 µg/L	Day and Hodge (1996)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	96 hours	55.5 µg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	50.9 µg/L	St. Laurent <i>et al.</i> (1992)
<b>Floating Macrophytes</b>				
<i>Lemna gibba</i>	Frond Number	7 days	304 - 788 µg/L	Day and Hodge (1996)
<i>Lemna gibba</i>	Dry Weight	7 days	766 µg/L	Day and Hodge (1996)
<i>Salvinia natans</i>	Chlorophyll <i>b</i>	4 weeks	50 µg/L	Gönca and Sencic (1994)
<i>Salvinia natans</i>	Stem Length	4 weeks	550 µg/L	Gönca and Sencic (1994)
<b>Macrophytes</b>				
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	579.6 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Fresh Weight	14 days	606.7 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Root Length	14 days	670.1 µg/L	This Document

Table 79: Lowest observable effect concentration for macrophytes. Based on LOEC, *Myriophyllum sibiricum* was over two thousand times as sensitive to metolachlor as compared with the other two macrophytes.

Species	Endpoint	Exposure Duration	LOEC	Reference
<b>Macrophytes</b>				
<i>Echinochloa crusgalli</i>	Seedling Dry Weight	2 weeks	250 µg/L	Walsh <i>et al.</i> (1991b)
<i>Spartina alterniflora</i>	Seedling Dry Weight	4 weeks	500 µg/L	Walsh <i>et al.</i> (1991b)
<i>Myriophyllum sibiricum</i>	Fresh Weight	14 days	110.6 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	331.9 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Root Length	14 days	331.9 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Pigment Content	14 days	331.9 µg/L	This Document

*Spartina alterniflora* was 0.5 mg/L (Table 79). Seedling survival of one variety of *E. crusgalli* was affected by metolachlor with a LOEC of 0.5 mg/kg in natural and synthetic sediments. The LOEC for *S. alterniflora* seedling survival was 2.5 mg/kg in synthetic sediment and 7.5 mg/kg in natural sediment. Metolachlor did not affect seed germination in either of these species (Walsh *et al.*, 1991b). The lowest LOEC for *Myriophyllum sibiricum* tested in this study was 110.6 µg/L for fresh plant weight. This was followed by LOEC of 331.9 µg/L for root length, plant area and pigment concentration based on fresh weight (Table 79).

#### 5.7.7.3.4 Conclusions

*Myriophyllum sibiricum* plants would be affected by 2.99 mg/L (EEC) of metolachlor entering an aquatic system. One-fifth of this concentration would produce a 50% reduction in plant area, fresh weight and root length of this submersed macrophyte species. There should not be any detrimental effects to *M. sibiricum* plants if 1% of the EEC for metolachlor entered non-target aquatic areas.

Thus, next to the algal species *Selenastrum capricornutum*, *Myriophyllum sibiricum* was one of the more sensitive aquatic plant species to metolachlor. Based on EC50/IC50 values, *M. sibiricum* was 15 times less sensitive to metolachlor than *S. capricornutum* after 72 hours of exposure (Table 78). The toxicity of metolachlor to the floating macrophytes was comparable to its toxicity to *M. sibiricum* (Table 78). Compared to *M. sibiricum*, *Chlamydomonas reinhardtii* and *Anabaena cylindrica* were 2 and 8 times more resistant to metolachlor, respectively (Table 78). Based on LOEC, *M. sibiricum* was over 2 000 times more sensitive to metolachlor than the two other macrophytes for which LOEC were available (Table 79). However, the difference in toxicity among plant species re-emphasises the importance of utilising a battery of test species to determine environmental impact.

### 5.7.8 Triclopyr

#### 5.7.8.1 Introduction

Triclopyr, as shown in Figure 84, is an auxin-type selective herbicide used to control woody plants and annual broadleaf weeds in non-crop areas and in rice. In Canadian

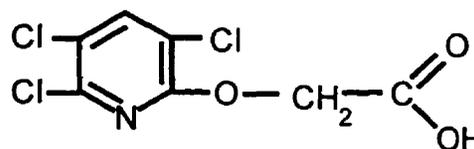


Figure 84: The molecular structure of triclopyr, a growth regulating herbicide.

forestry, it is commonly used for conifer release (WSSA, 1989; 1994) and the formulation Release<sup>®</sup> (triclopyr butoxyethyl ester) has been registered for arial application. Triclopyr is readily absorbed by both plant leaves and roots, translocates through the symplast and accumulates in meristematic tissue (OMAF, 1994a; WSSA, 1989; 1994). The precise mode of action of triclopyr is currently unknown. This herbicide interferes with DNA, RNA and protein biosynthesis. The symptoms of triclopyr toxicity include epinasty in stems and petioles, swelling of the stem (particularly at the nodes) and leaf curling. This is followed by growth inhibition, necrosis and chlorosis at the apices (WSSA, 1994).

In Ontario, a total of 1,235 kg a.i. triclopyr was sprayed by licensed applicators in 1993 (Hunter and McGee, 1994). When conditions are favourable for microbial degradation, the soil half-life is approximately 30 days (OMAF, 1994a). In northern Ontario sand and clay soils, the Garlon<sup>®</sup> 4E butoxyethanol ester of triclopyr dissipated to fifty percent within two weeks. Runoff water contained triclopyr residues between 0.01 and 0.96 µg/L (Stephenson *et al.*, 1990). Triclopyr is degraded rapidly by photodecomposition in aquatic environments (Johnson *et al.*, 1995; McCall and Gavit, 1986). At the water surface, average summer half-lives are 2.1 hours for triclopyr acid and 12.5 hours for triclopyr ester. Triclopyr ester would migrate into the sediments where it would be transformed to the acid (McCall and Gavit, 1986). When triclopyr was applied at a rate of 0.43 kg a.i./ha to rice paddies, fifty percent of the triclopyr dissipated from the water within four days (Johnson *et al.*, 1995). Solomon *et al.* (1988) applied Garlon<sup>®</sup> 4E to limnocorrals within a northern watershed and observed that the two treatments of 12 µg/L and 120 µg/L dissipated to below 5% within 15 days after application and by day 42 triclopyr was no longer detectable. They calculated half-lives of 4.3 days for the higher concentration and 3.8 days for the lower concentration (Solomon *et al.*, 1988). In a limnocorral study in an agricultural pond in southern Ontario, Garlon<sup>®</sup> 3A and Garlon<sup>®</sup> 4 were applied at 12.96 kg ae/ha. There was no difference between the dissipation rates of either formulation from the water column. Fifty percent of the Garlon<sup>®</sup> 3A dissipated within 18.7 to 21.2 days and Garlon<sup>®</sup> 4 dissipated to fifty percent of the applied level within 16.4 to 19.4 days. Seven days after application, triclopyr residues in the sediments reached a maximum. Triclopyr was no longer detectable in either water or sediment by the eighty-ninth day after treatment (MacKenzie, 1996). In another study, Garlon<sup>®</sup> 4 was applied directly to a boreal forest

stream in northern Ontario at a rate of 3.84 kg/ha. During the first 12 to 14 hours after application, average ester concentration in the stream water ranged from 0.05 to 0.11 mg/L. Levels declined to below detection levels within 72 hours after application. Triclopyr acid residues were detected in the water and the maximum level was 0.14 mg/L 6 hours after treatment. Triclopyr residues were detected in the aquatic plants, *Carex retrorsa* Schwein and *Sagittarius latifolia* Willd. (broad-leaved arrowhead), with fifty percent dissipation rates of 7.0 and 0.4 days, respectively (Thompson *et al.*, 1991).

#### 5.7.8.2 Methods

Technical triclopyr acid (99.6%) was obtained from Dow Elanco and was tested at the MLR of 10 kg/ha (WSSA, 1989). This converted to an EEC of 6.67 mg a.i./L. In Section 5.3, a comparison was made between the phytotoxicity of technical triclopyr and two formulations at the EEC. In addition to that study, the EEC of triclopyr was tested for toxic effects to *Myriophyllum sibiricum* starting on February 20, 1995. The triclopyr dilution series was initiated on the same day and consisted of treatments with 0.0, 9.1, 27.4, 82.3, 246.9, 740.7, 2 222.2 and 6 666.7 µg a.i./L of technical triclopyr. Because it is water soluble (Table 27), the technical triclopyr was dissolved in sterile nanopure water before addition to the sterile growth medium. All stock and experimental plants were grown in modified Andrews' medium. The experimental and statistical methods followed those outlined in Section 4.6.

#### 5.7.8.3 Results and Discussion

##### 5.7.8.3.1 Expected Environmental Concentration

Plants treated with the EEC of triclopyr demonstrated the classic symptoms of auxin toxicity including epidermal rupture, leaf bending and stem twisting (Figure 86). The symptoms were not quite as severe as those observed in the 2,4-D (another auxin mimic) treated plants (Figure 56). The toxic symptoms were definitely evident within the first week after treatment. Triclopyr reduced plant height (Figure 86) and thus area under the growth curve was also inhibited (Figure 85, Table 80 and Table 82). For all the endpoints measured, the control plants were significantly different from the treated plants (Table 82 and Table 83).

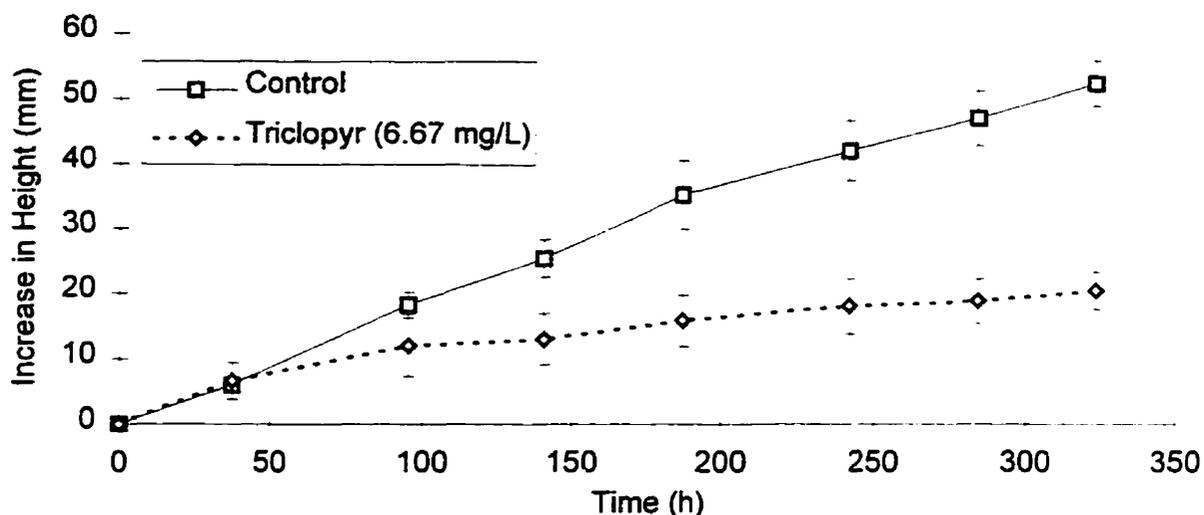


Figure 85: Plant height of untreated control plants and plants treated with 6.67 mg/L of technical triclopyr was measured every second day during the 14 day exposure period. For each treatment, the average of five plants ( $\pm$  s.d.) was plotted versus time.

Table 80: Percent inhibition for some of the endpoint parameters measured on five *Myriophyllum sibiricum* plants exposed to the EEC (6.67 mg/L) of technical triclopyr.

Treatment	Area under the Growth Curve	Plant Length	Root Number	Total Root Length	Total Fresh Weight	Membrane Permeability	Plant Area (cm <sup>2</sup> )
Triclopyr	52.3 $\pm$ 12.3	52.5 $\pm$ 5.6	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	49.1 $\pm$ 2.9	29.0 $\pm$ 6.0	50.5 $\pm$ 6.6

Table 81: Pigment content of apices was expressed as percent inhibition for *Myriophyllum sibiricum* plants exposed to the EEC of triclopyr. Pigment content is presented on both a fresh and dry weight basis. Values are the average  $\pm$  s.d. of 5 plants.

Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
Triclopyr	93.2 $\pm$ 1.5	92.7 $\pm$ 2.6	89.5 $\pm$ 2.3	94.4 $\pm$ 1.4	94.2 $\pm$ 2.2	91.6 $\pm$ 2.1



Table 82: Untransformed means  $\pm$  s.d. of numerous endpoint parameters measured on *Myriophyllum sibiricum* plants that were cultured for fourteen days in the presence or absence of 6.67 mg/L triclopyr.

Treatment	Area under the growth curve	Shoot Length (mm)	Root Number	Total Root Length (mm)	Total Fresh Weight (mg)	Membrane Permeability (%)	Plant area (cm <sup>2</sup> )
Control	9 172.2 $\pm$ 974.1 <sup>a</sup>	53.8 $\pm$ 2.7 <sup>a</sup>	7.4 $\pm$ 0.5 <sup>a</sup>	473.9 $\pm$ 96.4 <sup>a</sup>	825.6 $\pm$ 67.1 <sup>a</sup>	8.7 $\pm$ 1.1 <sup>a</sup>	12.1 $\pm$ 1.0 <sup>a</sup>
Triclopyr	4 377.8 $\pm$ 1 129.9 <sup>b</sup>	25.6 $\pm$ 3.0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>b</sup>	420.2 $\pm$ 24.1 <sup>b</sup>	23.1 $\pm$ 3.0 <sup>b</sup>	6.0 $\pm$ 0.8 <sup>b</sup>

a,b Any two means in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  as determined with either the nonparametric Mann-Whitney U-test or the parametric t-test. Values are means  $\pm$  s.d. of five replicate treatments.

Table 83: Pigment content detected in apices from *Myriophyllum sibiricum* plants grown for fourteen days in axenic culture. Plants were either untreated or treated with the EEC of technical triclopyr.

Treatment	Chlorophyll a Content (mg/g fresh weight)	Chlorophyll b Content (mg/g fresh weight)	Carotenoid Content (mg/g fresh weight)	Chlorophyll a Content (mg/g dry weight)	Chlorophyll b Content (mg/g dry weight)	Carotenoid Content (mg/g dry weight)
Control	0.56 $\pm$ 0.01 <sup>a</sup>	0.20 $\pm$ 0.004 <sup>a</sup>	0.20 $\pm$ 0.004 <sup>a</sup>	6.7 $\pm$ 0.9 <sup>a</sup>	2.39 $\pm$ 0.27 <sup>a</sup>	2.36 $\pm$ 0.24 <sup>a</sup>
Triclopyr	0.04 $\pm$ 0.008 <sup>b</sup>	0.01 $\pm$ 0.005 <sup>b</sup>	0.02 $\pm$ 0.004 <sup>b</sup>	0.37 $\pm$ 0.09 <sup>b</sup>	0.14 $\pm$ 0.05 <sup>b</sup>	0.20 $\pm$ 0.05 <sup>b</sup>

a,b Any means in the same column followed by the same superscript were not significantly different at  $\alpha = 0.05$  as determined with the nonparametric Mann-Whitney U-test. The values presented are the untransformed means  $\pm$  s.d. of five replicates.

#### 5.7.8.3.2 Dilution Series

Epinastic effects were visible at all the triclopyr concentrations tested, down to 9.1 µg/L (Figure 87). Shoot growth and area under the growth curve were inhibited at triclopyr concentrations greater than 9.1 µg/L (Figure 88 and Table 84). For the IC25 and IC50 values, these endpoint parameters had wide confidence intervals. At low concentrations (9.1 to 246.9 µg/L), triclopyr stimulated root growth, whereas at high triclopyr concentrations (2.2 and 6.7 mg/L) root growth was completely inhibited. Using either weibull or cumulative normal programs, IC25 and IC50 values could not be calculated for root number or total root length. Root number went from a control average of  $8.4 \pm 0.9$  to a maximum of  $32.6 \pm 4.6$  at 82.3 µg/L triclopyr and then down to  $0.0 \pm 0.0$  at 2.2 and 6.7 mg/L. Stimulation of root length had the same pattern, increasing from a control average length of  $458.7 \pm 76.7$  mm to a treatment maximum of  $1\ 141.34 \pm 98.0$  mm at 82.3 µg/L triclopyr and then totally inhibited at the two highest concentrations tested (2.2 and 6.7 mg/L) (Table 84). Curve fitting techniques were used to calculate the IC25, IC50 (Table 84) and SC20 values. SC20 values were calculated to be 675.2 and 281.9 µg/L for root number and length, respectively. Stimulation of plant growth has been equated with a beneficial effect. In the case of triclopyr stimulating root number and length, it was not apparently beneficial for a 60 cm plant segment to produce approximately 40 roots with a total root length of 130 cm. Fresh weight was stimulated at triclopyr concentrations of 82.3 and 246.9 µg/L. Chlorophyll *a*, chlorophyll *b* and carotenoid content were the most sensitive parameters to the phytotoxic effects of triclopyr. The IC25 and IC50 values had small confidence intervals. The NOEC for pigment content, expressed on a dry weight basis, was less than the lowest concentration tested (9.1 µg/L) (Table 84). The effect of technical triclopyr, at concentrations as low as 0.0432 µg ae/L, was tested on *Myriophyllum sibiricum* growth and development by Perkins (1997). In the study by Perkins (1997), root number and length were stimulated at low triclopyr concentrations. Technical triclopyr could be tested in the *Myriophyllum sibiricum* axenic toxicity test at concentrations less than 82.3 µg/L to ascertain the NOEC for root number, root length, and pigment content on a dry weight basis.

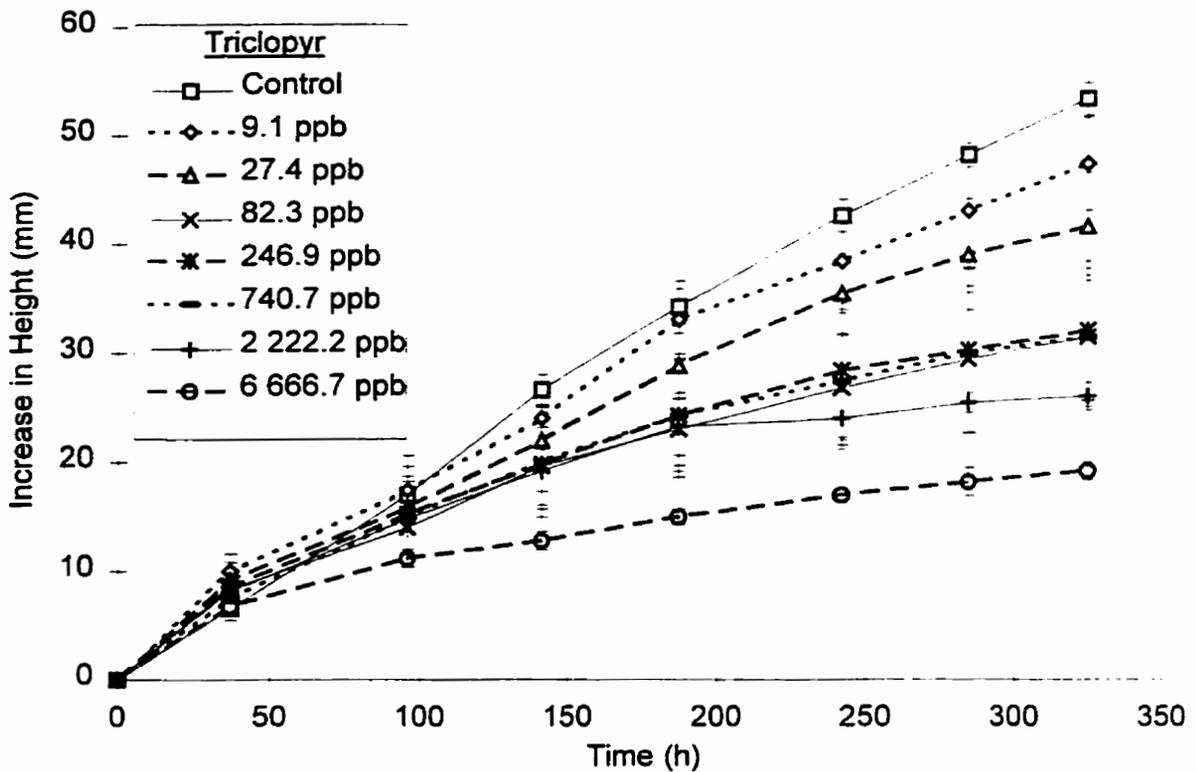


Figure 88: Fourteen day growth curves for *Myriophyllum sibiricum* in response to various concentration of triclopyr. For both area under the growth curve and total increase in plant height, only the plants treated with 27.1  $\mu\text{g/L}$  or higher were significantly different from the control plants, as determined with a one-way ANOVA ( $\alpha = 0.05$ ).

#### 5.7.8.3.3 Aquatic Plant Toxicity Comparison

The phytotoxic effects of triclopyr on aquatic plants are summarised in Table 85 and Table 86. Within 25 hours of exposure, the *Tradescantia* chlorophyll fluorescence assay did not successfully detect the effects of triclopyr (as Garlon® 4) at concentrations up to 3 mg/L (Judy *et al.*, 1990). Triclopyr is currently being investigated as a possible control mechanism for *Lythrum salicaria* (purple loosestrife). In a field assessment of the effects of triclopyr amine on *L. salicaria* and non-target wetland species (grasses and sedges), it was determined that 12 kg/ha prevented the regrowth of mature *L. salicaria* plants during the following growing season. In plots treated with 4 and 8 kg/ha, *L. salicaria* densities were higher in the second growing season. There was no apparent affect of any treatment on the stem density of grass species. Sedge

Table 84: The effect of triclopyr upon *M. sibiricum* growth and development endpoint parameters, expressed as the no observable effect concentration, the average percent inhibition ( $\pm$  s.d.) at the NOEC, IC25 and IC50 values (upper and lower 95% c.i.).

GROWTH PARAMETER	NOEC ( $\mu\text{g/L}$ )	% I at NOEC	IC25 ( $\mu\text{g/L}$ )	IC50 ( $\mu\text{g/L}$ )
Area Under the Growth Curve	9.1	6.9 $\pm$ 6.1	149.6 (19.0, 1 185.2) <sup>b</sup>	6 460.0 (1 394.6, 2 9923.1) <sup>b</sup>
Increase in Shoot Length	9.1	5.8 $\pm$ 10.8	1463.9 (795.7, 2 693.9) <sup>b</sup>	4 574.9 (2 810.8, 7 446.2) <sup>b</sup>
Root Length	Stimulated	Stimulated	450.1 <sup>d</sup>	560.0 <sup>d</sup>
Root Number	Stimulation	Stimulation	897.2 <sup>d</sup>	1 051.0 <sup>d</sup>
Fresh Weight <sup>a</sup>	Fluctuated	Fluctuated	2 660.0 (1 248.8, 5 668.0) <sup>b</sup>	6 386.1 (3 221.8, 12 658.2) <sup>b</sup>
Membrane Integrity	9.1	-14.4 $\pm$ 7.3	692.7 (20.1, 24 094.2) <sup>b</sup>	> 6 666.7 <sup>b,c</sup>
Plant Area	9.1	9.1 $\pm$ 8.4	24.8 (6.9, 89.6) <sup>b</sup>	> 6 666.7 <sup>b,c</sup>
Chlorophyll <i>a</i> (fresh wt)	27.4	-4.0 $\pm$ 8.0	53.1 (36.2, 77.8) <sup>b</sup>	92.6 (68.9, 124.6) <sup>b</sup>
Chlorophyll <i>b</i> (fresh wt)	27.4	-5.9 $\pm$ 11.4	53.4 (37.2, 76.5) <sup>b</sup>	78.8 (64.6, 96.2) <sup>b</sup>
Carotenoid (fresh wt)	27.4	-0.2 $\pm$ 6.9	45.8 (24.0, 87.1) <sup>b</sup>	94.0 (58.8, 150.3) <sup>b</sup>
Chlorophyll <i>a</i> (dry wt)	< 9.1	Not Calculable	20.6 (11.9, 35.7) <sup>b</sup>	55.0 (39.2, 77.1) <sup>b</sup>
Chlorophyll <i>b</i> (dry wt)	< 9.1	Not Calculable	23.3 (13.7; 39.6) <sup>b</sup>	52.0 (38.0, 71.3) <sup>b</sup>
Carotenoid (dry wt)	< 9.1	Not Calculable	16.7 (8.0; 35.0) <sup>b</sup>	52.2 (32.9; 82.8) <sup>b</sup>

- a The total plant fresh fluctuated from an average control weight of 831.7  $\pm$  69.5 mg up to the maximum average of 1013.9  $\pm$  95.0 mg at a triclopyr concentration of 246.9  $\mu\text{g/L}$  and then decreased to an average of 429.3  $\pm$  71.3 mg at the EEC. The NOEC could not be calculated.
- b IC<sub>p</sub> and 95% confidence values were calculated with the cumulative normal distribution model.
- c IC<sub>p</sub> values and 95% confidence intervals were calculated with the weibull method.
- d IC<sub>p</sub> values were calculated using a third order polynomial curve fitting technique.

densities were lower during the first season in the 12 kg/ha plots but these plants recovered (Gabor *et al.*, 1995). Another field study was conducted to examine the potential impact of Garlon<sup>®</sup> 3A on non-target aquatic organisms if this herbicide was used to control *L. salicaria*. At a treatment rate of 5 L/ha, there was no significant change in the average number of *Lemna gibba* fronds after 48 hours (Gardner and

Grue, 1996). Treatment rates of 3.41 and 4.54 kg/ha triclopyr, as Garlon® 3A, reduced *L. salicaria* cover in a field study. In treated plots, the percent cover for non-target dicots was significantly decreased compared to untreated controls (Nelson *et al.*, 1996). All the treatment rates tested by Gabor *et al.* (1995), Gardner and Grue (1996) and Nelson *et al.* (1996) will have a detrimental effect upon *M. sibiricum* populations if these rates were applied to aquatic systems.

For six species of algae, a triclopyr concentration of 2.56 mg/L had no significant effect upon <sup>14</sup>C uptake when it was supplied as NaH<sup>14</sup>CO<sub>3</sub>. This level of herbicide significantly stimulated <sup>14</sup>C uptake in *Selenastrum capricornutum* by 24% and *Aphanizomenon flos-aquae* by 34% but inhibited <sup>14</sup>C uptake in *Pseudoanabaena* sp. by 13% and frond production in *Lemna minor* by 23% (Peterson *et al.*, 1994). This stimulation by triclopyr in two species resembles the triclopyr stimulation observed in *Myriophyllum sibiricum* at low levels.

In combination with a soil eluate, triclopyr (as Garlon® 4) was slightly inhibitory to *S. capricornutum* cell growth. After 10 days of incubation, the Garlon® 4 inhibited cell division in this algal species by 15.9% when compared to the control. As applied in liquid growth medium, Garlon® 4 produced a 96 hour EC50 of 5 mg/L upon *S. capricornutum* cell growth (Thomas *et al.*, 1990). There appears to be a typographical error in the abstract of the article by Thomas *et al.* (1990) because the EC50 is 1 000 times less toxic than the values in the text of the document.

Unpublished data conducted by Dow Chemical Co. for the toxic effect of Garlon® 3A upon *Selenastrum capricornutum*, *Navicula pelliculosa* and *Anabaena flos-aquae* were cited in Cowgill *et al.* (1989b). For *S. capricornutum* total cell count, the EC50 of 16.7 mg/L was given. Total cell counts for *N. pelliculosa* and *A. flos-aquae* were given as 24 mg/L (c.i. = 16 - 32 mg/L) and 6 mg/L (c.i. = 2.4 - 16.6 mg/L), respectively (Table 85) (Cowgill *et al.*, 1989b). *Skeletonema costatum* total cell count was reduced to the 50% level by a Garlon® 3A concentration of 11.1 mg/L (c.i. = 4.3 - 28.5mg/L) (Table 85). The NOELs for total cell count and total cell volume of *S. costatum* were 1 mg/L and 13 mg/L, respectively (Table 86) (Cowgill *et al.*, 1989a). As determined in a microplate bioassay, triclopyr inhibited *Chlorella vulgaris* and *C. pyrenoidosa* cell counts by 50% at concentrations of 11 mg/L and 80 mg/L, respectively (Baarschers *et al.*, 1988). *M. sibiricum*, as tested in this axenic toxicity test, was more sensitive to triclopyr than any of the algal species.

The EC50 for *Lemna gibba* plant number to triclopyr triethylamine salt was determined to be 9 mg/L with a range of 2.1 to 38.4 mg/L, while the EC50 for frond number was determined to be 9.1 mg/L (95% c.i. = 2.9 - 28.4 mg/L) (Cowgill and Milazzo, 1987). The phytotoxic effect of Garlon® 3A was examined upon four clones of *Lemna minor* and *Lemna gibba* over seven and fourteen day test periods. Concentrations ranged from 100 to 2.8 mg/L in a 60% dilution series. The endpoints measured included dry weight, plant number, frond number and % chlorophyll *a* and % chlorophyll *b*, as measured with a high pressure liquid chromatograph. For fourteen day exposure, the lowest NOEL was 1 mg/L for frond number of one *L. minor* clone, followed by a NOEL of 4.7 mg/L for plant number for another *L. minor* clone. All other NOELs were 7.8 mg/L or greater (Table 86). *L. gibba*, based on a fourteen day dry weight EC50 of 26 mg/L (c.i. = 12 - 61 mg/L), was more sensitive to triclopyr than the clones of *L. minor*. The EC50 values for other endpoints (Table 85), as reported by Cowgill *et al.* (1989b), were even higher and demonstrate that these floating aquatic plant species were less affected by triclopyr (Cowgill *et al.*, 1989b) than was *M. sibiricum*.

Four aquatic plant species (*Myriophyllum spicatum*, *Elodea canadensis*, *Potamogeton pectinatus* and *Vallisneria americana*) were exposed to triclopyr (Garlon® 3A) at concentrations of 1 mg ae/L for 12 hours and 2.5 mg ae/L for 24 hours. *E. canadensis* and *V. americana* were unaffected by either treatment concentration after five weeks. A significant reduction in *P. pectinatus* dry weight was observed at the highest treatment concentration. Twenty days after herbicide treatment, *P. pectinatus* plants began to lose turgor followed by tissue necrosis and foliage chlorosis. Treated *M. spicatum* exhibited curvature in apical and axillary shoots and epidermal rupture by three days post-treatment. Fourteen days after treatment, no living stems or leaves remained. Measurements of peroxidase activity indicated that there was a significant increase in the amount of peroxidase in *M. spicatum* tissue (Sprecher and Stewart, 1995). *M. sibiricum*, tested in this axenic toxicity test, experienced similar symptoms to *M. spicatum* as noted by Sprecher and Stewart (1995). The chlorophyll analysis conducted in the axenic toxicity test appeared to be more sensitive than the endpoints used by Sprecher and Stewart (1995) but a comparison was difficult because peroxidase content was not measured in the *M. sibiricum* toxicity test and there were

Table 85: Selected from the scientific literature, a summary IC50/EC50 values for aquatic plant species exposed to triclopyr. Test conditions differed between the species but general trends in sensitivity can be observed. *Myriophyllum sibiricum* was 100 times more sensitive to the effects of triclopyr than the most sensitive algal species (*Selenastrum capricornutum* and *Anabaena flos-aquae*). The floating macrophytes were approximately 200 times less sensitive to triclopyr than *M. sibiricum*. *Chlorella pyrenoidosa*, was tolerant to high levels of triclopyr.

Species	Endpoint	Formulation	Exposure Duration	IC50/EC50	Reference
<b>Algae</b>					
<i>Anabaena flos-aquae</i>	Cell Counts	Garlon® 3A	Time not given	6 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Chlorella pyrenoidosa</i>	Cell Counts	Technical	10 - 14 days	80 mg/L	Baarschers <i>et al.</i> (1988)
<i>Chlorella vulgaris</i>	Cell Counts	Technical	10 - 14 days	11 mg/L	Baarschers <i>et al.</i> (1988)
<i>Navicula pelliculosa</i>	Cell Counts	Garlon® 3A	Time not given	24 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Selenastrum capricornutum</i>	Cell Counts	Garlon® 3A	Time not given	16.7 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Selenastrum capricornutum</i>	Cell Growth	Garlon® 4	96 hours	5 mg/L	Thomas <i>et al.</i> (1990)
<i>Skeletonema costatum</i>	Cell Counts	Garlon® 3A	5 days	11.1 mg/L	Cowgill <i>et al.</i> (1989a)
<b>Floating Macrophytes</b>					
<i>Lemna gibba</i>	Dry Weight	Garlon® 3A	7 days	26 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Lemna gibba</i>	FronD Number	Garlon® 3A	14 days	30 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Lemna gibba</i>	Plant Number	Technical	7 days	11.62 mg ae/L	Perkins (1997)
<i>Lemna gibba</i>	FronD Number	Technical	7 days	11.73 mg ae/L	Perkins (1997)
<i>Lemna minor</i>	Dry Weight	Garlon® 3A	7 days	48 mg/L	Cowgill <i>et al.</i> (1989b)
<i>Lemna minor</i>	FronD Number	Garlon® 3A	14 days	35 mg/L	Cowgill <i>et al.</i> (1989b)
<b>Submersed Macrophytes</b>					
<i>Myriophyllum sibiricum</i>	Chorophyll <i>b</i> (fresh)	Technical	14 days	40 µg ae/L	Perkins (1997)
<i>Myriophyllum sibiricum</i>	Carotenoid (fresh)	Technical	14 days	80 µg ae/L	Perkins (1997)
<i>Myriophyllum sibiricum</i>	Chorophyll <i>b</i> (dry)	Technical	14 days	52.0 µg a.i./L	This Document
<i>Myriophyllum sibiricum</i>	Carotenoid (dry)	Technical	14 days	52.2 µg a.i./L	This Document

Table 86: No observable effect concentration for macrophytes. Based on NOEC, *Myriophyllum sibiricum* was 110 times as sensitive to triclopyr as compared without the other two macrophytes.

Species	Endpoint	Exposure Duration	NOEC	Reference
<b>Algae</b>				
<i>Skeletonema costatum</i>	Total Cell Count	5 days	1 mg/L	Cowgill et al. (1989a)
<i>Skeletonema costatum</i>	Total Cell Volume	5 days	13 mg/L	Cowgill et al. (1989a)
<b>Macrophytes</b>				
<i>Lemna gibba</i>	Fron and Plant Number	14 days	7.8 mg/L	Cowgill et al. (1989b)
<i>Lemna minor</i>	Fron Number	14 days	1 mg/L	Cowgill et al. (1989b)
<i>Lemna minor</i>	Plant Number	14 days	4.7 mg/L	Cowgill et al. (1989b)
<i>Myriophyllum sibiricum</i>	Membrane Integrity	14 days	9.1 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Plant Area	14 days	9.1 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Increase in Shoot Length	14 days	9.1 µg/L	This Document
<i>Myriophyllum sibiricum</i>	Pigment Content	14 days	27.4 µg/L	This Document

only two triclopyr concentrations used by Sprecher and Stewart (1995) so NOEC and EC50 values could not be calculated.

In comparative studies between *Lemna gibba* G3 and *Myriophyllum sibiricum*, the *L. gibba* was less sensitive to technical triclopyr (Perkins, 1997). With technical triclopyr, the most sensitive parameters for *L. gibba* were plant number with an IC50 of 11.62 mg ae/L (c.i. = 9.18 - 14.98) and frond number with an IC50 of 11.73 mg ae/L (c.i. = 7.05 - 17.98) (Table 85). The most sensitive parameters for *M. sibiricum* were chlorophyll *b* with an IC50 of 0.04 mg ae/L (c.i. = 0.0 to 2.72) and carotenoid content with an IC50 of 0.08 mg ae/L (c.i. = 0.0 - 2.43) (Table 85) (Perkins, 1997). In both the study conducted by Perkins (1997) and the one discussed in this report, pigment content was the most sensitive parameter to the phytotoxic effects of triclopyr.

#### 5.7.8.3.4 Conclusions

*Myriophyllum sibiricum* in aquatic systems would be severely affected by 6.67 mg/L of triclopyr. Concentrations less than 100 times this expected environmental concentration would severely inhibit pigment content of this species. At this point, it is not known how a decrease in pigment content would affect aquatic macrophyte populations. Longer term studies would be required to answer this question. Even, one-tenth of the EEC would inhibit root production by 50%, which would inhibit individual plant survival.

Based on both EC50/IC50 and NOEC values (Table 85 and Table 86), *Myriophyllum sibiricum* proved to be the most sensitive aquatic plant species that has been tested to date with the most sensitive parameters being chlorophyll *b* and carotenoid content (Table 86). For the toxic effects of triclopyr as determined with EC50 and IC50 values, *M. sibiricum* was 96 times more sensitive than *Selenastrum capricornutum* and 220 times more sensitive than *Lemna gibba* (Table 85). *M. sibiricum* was 110 times more sensitive to triclopyr than *Skeletonema costatum* and *L. minor* as determined by NOECs (Table 86). This emphasised the need to use multiple plant species for aquatic risk assessment and that the *M. sibiricum* toxicity test might be a valuable addition to the pesticide registration process.

#### 5.7.9 Summary of Pesticides Effects

*Myriophyllum sibiricum* in axenic culture was sensitive to most of the herbicides tested. The results of the pesticide testing are summarised in Table 87 and Table 88.

Table 87: Summary of the effect of eight pesticides upon numerous *Myriophyllum sibiricum* growth and development parameters as determined with the axenic culturing system. *M. sibiricum* was least sensitive to the effects of atrazine and most sensitive to the effects of 2,4-D, diquat and triclopyr. For diquat, the results of three experiments conducted with a series of concentrations were included.

HERBICIDE	IC50 Values (µg/L)							
	Shoot Length	Root Length	Fresh Weight	Plant Area	Membrane Integrity	Chlorophyll a (Fresh weight)	Chlorophyll b (Fresh weight)	Carotenoid (Fresh weight)
Atrazine	Stimulation	1 130.0	> 2 987	2 679.4	No Effect	Stimulation	Stimulation	Stimulation
2,4-D	> 1 467	13.1	> 1 467	957.4	801.3	313.7	253.7	308.5
Diquat	346.2; 403.8; 1 610.9	< 46.7; 79.7; 105.7	< 46.7; 78.2; 184.0	< 46.7; 56.2; 127.7	> 2 987; > 2 987; > 2 987	< 46.7; 71.5; 201.3	< 46.7; 36.9; 206.8	< 46.7; 139.2 249.6
Fluridone	> 3 000	650.3	784.2	262.3	No Effect	50.4	51.2	44.6
Glyphosate	> 2 998	844.0	1474.1	2 090.2	No Effect	2 090.2 (Dry)	1 928.7 (Dry)	2 629.0 (Dry)
Hexazinone	> 8 000	289.6	538.6	369.4	No Effect	2 222.5	2 320.7	2374.4
Metolachlor	1 896.0	670.1	606.7	579.6	No Effect	Stimulation	Stimulation	Stimulation
Triclopyr	4 574.9	560.0	6 386.1	> 6 666.7	> 6 666.7	92.6	78.8	94.0

Table 88: For the eight herbicides tested with the *Myriophyllum sibiricum* toxicity test, the IC50 values for the most sensitive *M. sibiricum* endpoint are presented along with IC50/EC50 values for other sensitive aquatic plant species. The data presented for the other aquatic plants are selected from the scientific literature and even though test conditions differed between the species, general trends in sensitivity may be observed.

Herbicide	Species	Endpoint	Exposure Duration	IC50/EC50	Reference
Atrazine	<i>Myriophyllum sibiricum</i>	Nodes per Centimetre	14 days	437.7 µg/L	This Document Larsen <i>et al.</i> (1986) Kemp <i>et al.</i> (1985)
	<i>Chlamydomonas reinhardtii</i>	<sup>14</sup> C Uptake	24 hours	19 µg/L	
	<i>Potamogeton perfoliatus</i>	Stem Dry Weight	4 weeks	30 µg/L	
2,4-D	<i>Myriophyllum sibiricum</i>	Root Length	14 days	0.01 mg/L	This Document St. Laurent <i>et al.</i> (1992) Göncü and Sencic (1994)
	<i>Selenastrum capricornutum</i>	Population Growth	96 hours	24.2 mg/L	
	<i>Salvinia natans</i>	Chlorophyll	4 weeks	0.3 mg/L	
Diquat	<i>Myriophyllum sibiricum</i>	Chlorophyll <i>b</i> (dry)	14 days	36.9 µg/L	This Document St. Laurent <i>et al.</i> (1992)
	<i>Selenastrum capricornutum</i>	Population Growth	96 hours	4.9 µg/L	
Fluridone	<i>Myriophyllum sibiricum</i>	Carotenoid (fresh)	14 days	44.6 µg/L	This Document Fedtke (1992)
	<i>Chlamydomonas reinhardtii</i>	Chlorophyll Production	4 days	2.1 mg/L	
Glyphosate	<i>Myriophyllum sibiricum</i>	Root Length	14 days	0.84 mg/L	This Document Hutber <i>et al.</i> (1979) Hartman and Martin (1984)
	<i>Anabaena variabilis</i>	Growth	48 hours	2 mg/L	
	<i>Lemna minor</i>	Fronnd Number	14 days	2.0 mg/L	
Hexazinone	<i>Myriophyllum sibiricum</i>	Dissolved Oxygen	14 days	128.2 µg/L	This Document St. Laurent <i>et al.</i> (1992)
	<i>Selenastrum capricornutum</i>	Population Growth	96 hours	24.5 µg/L	
Metolachlor	<i>Myriophyllum sibiricum</i>	Plant Area	14 days	579.6 µg/L	This Document Day and Hodge (1996)
	<i>Selenastrum capricornutum</i>	Growth	72 hours	37.2 µg/L	
Triclopyr	<i>Myriophyllum sibiricum</i>	Chlorophyll <i>b</i> (dry)	14 days	52.0 µg/L	This Document Thomas <i>et al.</i> (1990) Perkins (1997)
	<i>Selenastrum capricornutum</i>	Cell Growth	96 hours	5 mg/L	
	<i>Lemna gibba</i>	Plant Number	7 days	11.62 mg/L	

As determined with this toxicity test, the range of herbicide toxicity was 600 fold depending on the herbicide and the endpoint examined. For fluridone, the amount of herbicide required to inhibit pigment content (chlorophyll *a*, chlorophyll *b* and carotenoid) was almost 70 times less than the amount of herbicide required to adversely affect morphological parameters, such as area under the growth curve and plant height. For 2,4-D, root length was almost 25 times more sensitive than pigment content and more than 110 times more sensitive than shoot length.

This toxicity test was also capable of detecting hormesis. The shoots of plants grown with atrazine, hexazinone and fluridone demonstrated increased height over those of the controls. Pigment content of the apices (fresh weight) of plants treated with atrazine and metolachlor were also stimulated above control levels. Root number and length of *M. sibiricum* plants exposed to low concentrations of triclopyr were greatly stimulated above normal levels.

Some of the endpoint parameters used in this toxicity test were just as sensitive ( $\mu\text{g/L}$  range) or were more sensitive to herbicide effects than those observed in other aquatic plant tests. Root length is extremely sensitive towards the effects of 2,4-D ( $\text{IC}_{50} = 13.1 \mu\text{g/L}$ ) and diquat ( $\text{IC}_{50} \leq 105.7 \mu\text{g/L}$ ). For 2,4-D, diquat, fluridone and triclopyr, the amount of herbicide required to affect pigment content by 50% was extremely small ( $\text{IC}_{50} = 36.9 - 313.7 \mu\text{g/L}$ ).

The herbicide toxicity varies for the different endpoints. If the toxicity of the herbicides is ranked based on total root length, the order of decreasing herbicide toxicity is: 2,4-D > Diquat > Hexazinone > Triclopyr > Fluridone > Metolachlor > Glyphosate > Atrazine. If the herbicide toxicity is ranked by the effect upon chlorophyll *a*, chlorophyll *b* and carotenoid, then the decreasing order is: Fluridone > Triclopyr > Diquat > 2,4-D > Glyphosate > Hexazinone > Metolachlor = Atrazine.

Based on  $\text{EC}_{50}$  and  $\text{IC}_{50}$  values, the *Myriophyllum sibiricum* toxicity test was the most sensitive aquatic plant test for detecting the phytotoxic effects of 2,4-D, fluridone, glyphosate and triclopyr (Table 88). Compared to the other aquatic plant species, *M. sibiricum* was 30 times more sensitive to 2,4-D, 47 times more sensitive to fluridone, twice as sensitive to glyphosate and 96 times more sensitive to triclopyr. For diquat, *Selenastrum capricornutum* was slightly more sensitive than *M. sibiricum*. Sensitive algal species were 23 times more sensitive to 2,4-D, 5 times more sensitive to hexazinone and

15 times more sensitive to metolachlor than *M. sibiricum* in the axenic toxicity test (Table 88). Based on the success of the *M. sibiricum* toxicity test at detecting the phytotoxic effects of auxin mimicking herbicides, carotenoid biosynthesis inhibitors and inhibitors of aromatic amino acid synthesis, this toxicity test would be a beneficial addition to the pesticide registration process, especially for compounds with these or similar modes of action.

## 5.8 REFERENCE TOXICANTS

### 5.8.1 Introduction

The use of standardised reference toxicants in aquatic toxicity tests maximises comparability, replicability and reliability (Environment Canada, 1990; Rand and Petrocelli, 1985a; Yearley *et al.*, 1995). A reference substance is defined under GLP to be any chemical substance or mixture, any analytical standard, or material other than a test substance, nutrient or water, which is administered to or used in analysing the test system in the course of a study for the purpose of establishing a basis for comparison with the test substance for known biological or chemical measurements (U.S. EPA, 1983b). These substances provide fitness checks on the health of the organism by allowing comparisons with previous studies (St. Laurent *et al.*, 1992). Environment Canada (1990) recommends that reference toxicity tests be conducted at least once per month. Under quality assurance/quality control programs, biological testing laboratories will be required to conduct reference toxicant tests (Environment Canada, 1990). Numerous reference toxicants have been examined by different authors.

KCl has been used as a reference toxicant in studies with algae and macrophytes (Nelson and Fairchild, 1994). Day and Hodge (1996) used  $ZnSO_4$  as a reference toxicant with *Selenastrum capricornutum* and *Anabaena cylindrica*.  $ZnCl_2$  and phenol have been used in diatom studies (Patrick *et al.*, 1968). St. Laurent *et al.* (1992) used four reference toxicants including phenol,  $Cr^{6+}$  (as  $K_2Cr_2O_7$ ),  $Cu^{2+}$  (as  $CuSO_4$ ) and  $Zn^{2+}$  (as  $ZnCl_2$ ) in a comparative study between the *Selenastrum capricornutum* microplate and flask tests. Environment Canada (1990) listed phenol, hexavalent chromium, copper and zinc as suitable reference toxicants for testing during the 96 hour *S. capricornutum* growth inhibition test. The effect of six metal reference toxicants ( $Ni^{2+}$  as  $NiCl_2 \cdot 6H_2O$ ,  $Co^{2+}$  as  $CoSO_4$ ,  $Cd^{2+}$  as  $CdCl_2$ ,  $Zn^{2+}$  as  $ZnCl_2$ ,  $Cu^{2+}$  as  $CuSO_4$ , and  $Cr^{2+}$  as  $CrCl_2$ ) upon *S. capricornutum* cell counts were examined in a microplate study (Blaise *et al.*, 1986).

Thellen *et al.* (1989) used  $\text{Cd}^{2+}$  as  $\text{CdCl}_2$  and phenol in a multiple laboratory study with the *Selenastrum capricornutum* microplate bioassay. Zinc chloride, at a concentration of 153.8  $\mu\text{g/L}$ , was used as a positive control in studies examining the effect of herbicide contaminated soil on *S. capricornutum* (Thomas *et al.*, 1990). Wang (1987) recommended the chromate ion at 10 mg/L for use as a positive control during toxicity testing with millet (*Panicum miliaceum* L.) and duckweed (*Lemna minor*).

For these investigations, it was decided that an inorganic reference toxicant would be compared with an organic reference toxicant. Zinc (as  $\text{ZnCl}_2$ ) was selected as a possible inorganic reference toxicant because numerous studies have used this chemical and there is sufficient information for a comparison of toxicities. Phenol was chosen as the organic reference toxicant for use in the *Myriophyllum* toxicity test because this was the only organic reference toxicant for which toxicity data to other non-target aquatic plant species were found.

## 5.8.2 Inorganic Reference Toxicant (Zinc Chloride)

### 5.8.2.1 Introduction

Zinc was ranked the most suitable reference toxicant by Environment Canada (1990) because of its solubility, stability, long shelf life, availability in pure form, established toxicity database, analytical methods available, minor water quality effects and detection of abnormal organisms.

Zinc has been shown to accumulate in aquatic plants, such as *Lemna perpusilla*, at exposure levels of 0.1 mg/L. The plants accumulated higher concentrations than was detected in the water (Clark *et al.*, 1981). In *Elodea nuttallii* (Planch.) St. John, zinc uptake occurred faster in excised roots than in excised leaves (Marquenie-van der Werff and Ernst, 1979).

### 5.8.2.2 Methods

The effect of analytical grade zinc chloride ( $\text{ZnCl}_2$ ) upon the growth and development of *Myriophyllum sibiricum* was tested three times during the course of the current research. The first  $\text{ZnCl}_2$  experiment (range-finding test) was conducted between October 5 and 19, 1992, using concentrations of 0.0, 0.136, 0.68, 1.36, 6.8, 13.6, 68.1 and 136.3 mg/L. Based on the results from this first experiment, the  $\text{ZnCl}_2$  reference toxicant experiment was repeated twice using concentrations of 0.0, 4.26, 8.52, 17.04,

34.07, 68.15, 136.30 and 272.59 mg/L. All concentrations were replicated five times. These experiments were initiated on February 2, 1994 and September 2, 1995, respectively. The first two experiments were started using stock plants that had been cultured in half strength M & S medium while the stock plants utilised in the last reference toxicant experiment were cultured in full strength modified Andrews' medium. All three experiments were conducted in full strength modified Andrews' medium. The first experiment was conducted without a substrate. From the range-finding test, one experimental plant was lost due to fungal contamination and one plant was discarded because the apex was damaged during the transfer. One experimental plant was lost from the first dilution series experiment due to a broken test tube and no plants were lost from the second dilution series experiment. The remainder of the methods used for the reference toxicant experiments followed those outlined in Chapter 4.6.

At the end of the 14 day test periods, the experimental endpoints were determined. The data were tested for normality. Normal data or data that could be transformed to meet the requirements for normality were analysed using a one-way ANOVA followed by multiple comparisons. Data that could not be normalised were analysed using the nonparametric Kruskal-Wallis test followed by multiple comparison testing. The NOECs were determined from these analyses. IC50 values were determined using either the cumulative normal or weibull methods for continuous response data (Andersen *et al.* submitted).

#### 5.8.2.3 Results and Discussion

The ZnCl<sub>2</sub> was toxic to *M. sibiricum* at low levels. The growth curves for the second ZnCl<sub>2</sub> dilution experiment are displayed as representative curves (Figure 89). Figure 90 photographically depicts the *Myriophyllum* plants as exposed to this dilution series. The highest concentration of zinc (136.3 mg/L) inhibited growth completely and the plants in this treatment were necrotic. Plant height showed a good dose response with the selected concentrations. NOEC (Table 89) and IC50 values (Table 90) show the correlation between the three experiments. Based upon the highest NOEC of 4.26 mg/L, area under the growth curve, total root length and fresh weight were the most consistently sensitive endpoints (Table 89). Of the various endpoints measured on *M. sibiricum*, root length was determined to be the most sensitive to the toxic effects of zinc. The lowest IC50 value of 8.2 mg/L was obtained for root length. Root number, fresh

weight and plant area were also severely affected by the zinc and had low IC50 values. For most of the endpoint parameters, narrow confidence limits surrounded the IC50 and there was good correlation between the three experiments, even though they were conducted over a three year period (Table 90).

The EDTA in the modified Andrews' medium used to culture the *Myriophyllum sibiricum* may complex the zinc in solution. This was not examined but research with other aquatic plant species, metals and complexing agents in the growth medium has determined that free metal levels affecting growth are relatively constant even if the total metal concentration varied with the amount of complexing agent (Borgmann, 1983). At pH 7 and below, most of the zinc is free (Borgmann, 1983) and as long as the available EDTA is in excess of the metal concentration, complexation is minimal (Huebert and Shay, 1992a; 1992b). With the marine alga *Ditylum brightwellii* (West) Grunow, the

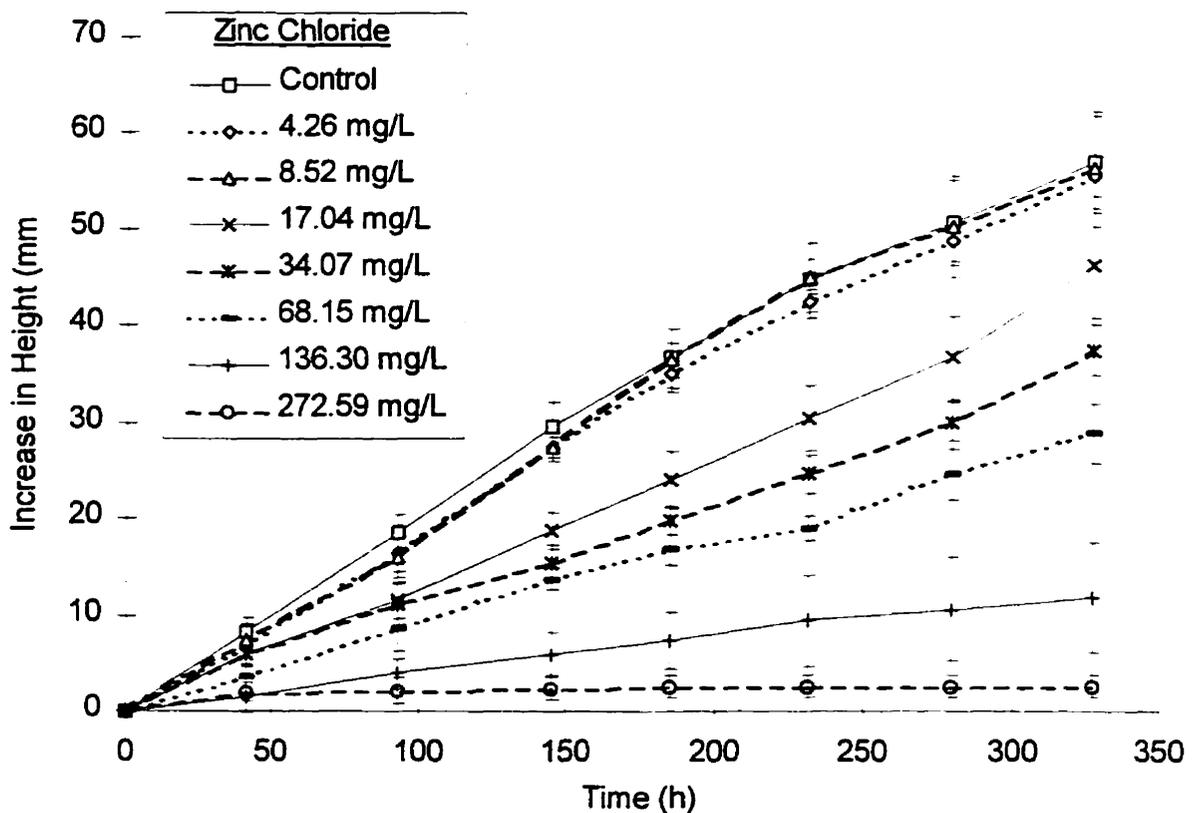


Figure 89: Fourteen day growth curves depicting the effect of different concentrations of ZnCl<sub>2</sub> on the shoot height of *Myriophyllum sibiricum* apices, as collected from the last ZnCl<sub>2</sub> experiment conducted. The experimental medium was modified Andrews' medium.

toxicity of zinc (as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) decreased as the EDTA concentration increased (Canterford and Canterford, 1980). Similar results were determined in studies examining the interaction of zinc and EDTA on the coastal diatom *Thalassiosira weissflogii* Grun. (Anderson and Morel, 1978). The 96 hour toxicity of zinc (as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) to *Selenastrum capricornutum* decreased from 4.4  $\mu\text{g/L}$  in medium without EDTA to 27  $\mu\text{g/L}$  in growth medium with 300  $\mu\text{g/L}$  EDTA. Over seven days, zinc toxicity to *S. capricornutum* decreased from 4.1  $\mu\text{g/L}$  in medium without EDTA to 32  $\mu\text{g/L}$  in medium supplemented with EDTA (Chiaudani and Vighi, 1978). In the *M. sibiricum* toxicity test, with a pH of 5.8, most of the zinc should be free to interact with the plants.

#### 5.8.2.4 Aquatic Plant Toxicity Comparison

Zinc applied as  $\text{ZnCl}_2$  inhibited *Selenastrum capricornutum* oxygen evolution by 50% at a concentration of 178  $\mu\text{g/L}$  (95% c.i. = 165 - 191  $\mu\text{g/L}$ ), while 21 day biomass was inhibited at a concentration of 50.9  $\mu\text{g/L}$  (c.i. = 48 - 53.7  $\mu\text{g/L}$ ) (Table 91) (Turbak *et al.*, 1986). As determined by a 72 hour microplate bioassay,  $\text{ZnSO}_4$  was toxic to *Selenastrum capricornutum* with an IC50 range of 34.49 - 39.00  $\mu\text{g/L}$  (Table 91) (Day and Hodge, 1996). In microplate studies,  $\text{ZnCl}_2$  produced 96 hour cell count EC50s at 52.6  $\mu\text{g/L}$  (95% c.i. = 31.9 - 72.7  $\mu\text{g/L}$ ) (St. Laurent *et al.*, 1992) and 44.7  $\mu\text{g/L}$  (Table 91) (Blaise *et al.*, 1986). In a *S. capricornutum* flask study, the  $\text{ZnCl}_2$  produced similar 96 hour EC50s of 65.4  $\mu\text{g/L}$  (95% c.i. = 56.7 - 73.7  $\mu\text{g/L}$ ) (Table 91) (St. Laurent *et al.*, 1992). In a 72 hour microplate bioassay, *Anabaena cylindrica* was affected at  $\text{ZnSO}_4$  concentrations between 33.51 - 40.31  $\mu\text{g/L}$  (Table 91) (Day and Hodge, 1996). A concentration of 4.3 mg/L  $\text{ZnCl}_2$  caused a 50% reduction in the number of *Nitzschia linearis* (a diatom) cells produced after 120 hours of incubation (Table 91) (Patrick *et al.*, 1968). The effect of zinc chloride upon the growth rate of five species of marine algae (*Pelvetia canaliculata* (L.) Dec. et Thur., *Fucus spiralis* L., *F. vesiculosus* L., *F. serratus* L. and *Ascophyllum nodosum* (L.) Le Jolis) was determined over a 10 day exposure period. For all of these species, the growth rate did not differ from the control at zinc concentrations up to 0.1 mg/L. After 2 days of exposure, a concentration of 1.4 mg/L caused significant reductions in the growth rate of *P. canaliculate*, *F. spiralis*, *F. serratus* and *Ascophyllum nodosum*. For *F. vesiculosus*, a zinc concentration of 7 mg/L caused a reduction in the growth rate. Sixty to eighty percent reduction in growth rate of all species was observed at 14 mg/L (Strömngren, 1979). Segments from the growing region

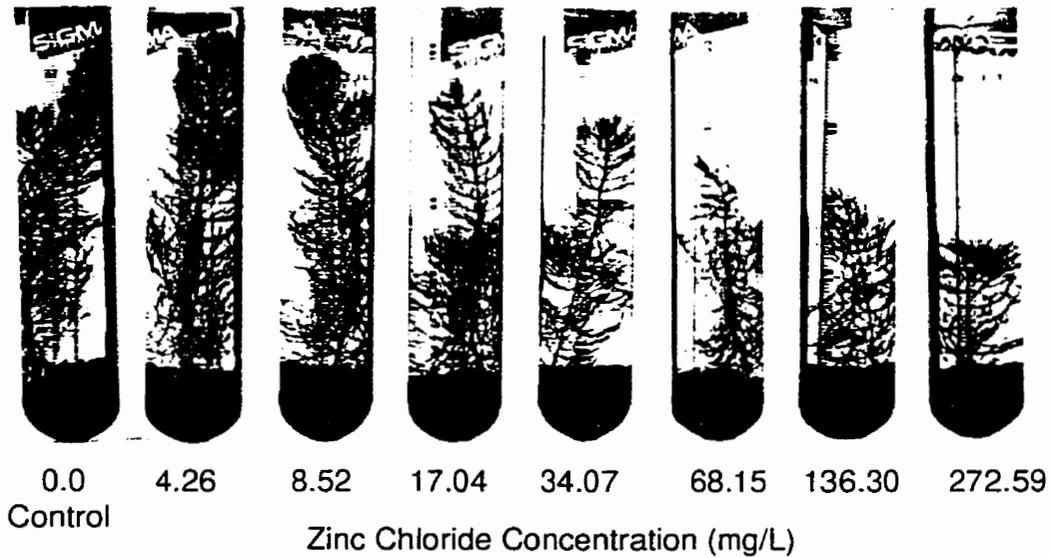


Figure 90: *Myriophyllum sibiricum* apices exposed to a concentration gradient of  $ZnCl_2$ , an inorganic reference toxicant.

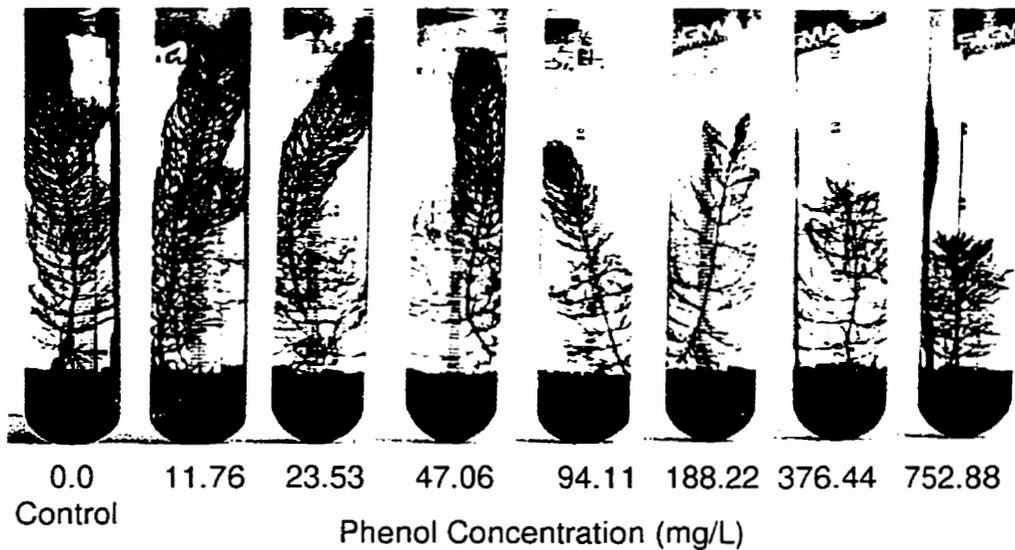


Figure 91: The visual effects of phenol on the growth of axenic *Myriophyllum sibiricum* apices after fourteen days of incubation. The plants were cultured in modified Andrews' medium.

Table 89: The effect of zinc chloride upon numerous *Myriophyllum sibiricum* growth and development parameters, expressed as NOEC.

GROWTH PARAMETER	NOEC (mg/L)		
	Range-finding Test	First Dilution Series	Second Dilution Series
Area Under the Growth Curve	13.6 <sup>a</sup>	4.26 <sup>b</sup>	8.52 <sup>b</sup>
Increase in Shoot Length	13.6 <sup>b</sup>	4.26 <sup>b</sup>	8.52 <sup>b</sup>
Root Number	6.8 <sup>b</sup>	4.26 <sup>b</sup>	17.04 <sup>a</sup>
Root Length	6.8 <sup>b</sup>	4.26 <sup>b</sup>	4.26 <sup>b</sup>
D.O.	Not measured	4.26 <sup>a</sup>	34.07 <sup>a</sup>
Fresh Weight	13.6 <sup>a</sup>	All differ from control <sup>b</sup>	8.52 <sup>b</sup>
Plant Area	13.6 <sup>a</sup>	17.04 <sup>b</sup>	8.52 <sup>a</sup>
Membrane Integrity	13.6 <sup>b</sup>	17.04 <sup>b</sup>	68.15 <sup>b</sup>
Chlorophyll <i>a</i> (fresh wt)	13.6 <sup>b</sup>	17.04 <sup>b</sup>	8.52 <sup>a</sup>
Chlorophyll <i>b</i> (fresh wt)	13.6 <sup>a</sup>	34.07 <sup>b</sup>	8.52 <sup>a</sup>
Carotenoid (fresh wt)	13.6 <sup>b</sup>	34.07 <sup>b</sup>	34.07 <sup>a</sup>
Chlorophyll <i>a</i> (dry wt)	Not measured	Not measured	8.52 <sup>b</sup>
Chlorophyll <i>b</i> (dry wt)	Not measured	Not measured	8.52 <sup>a</sup>
Carotenoid (dry wt)	Not measured	Not measured	8.52 <sup>a</sup>

a Data were normal or could be normalised. NOEC analysis was conducted using one-way ANOVA followed by Tukey-Kramer multiple comparison testing.

b Data were not normal so NOEC were determined with the nonparametric Kruskal-Wallis test followed by multiple comparison testing (Conover, 1980).

of the marine alga, *Laminaria digitata* (Huds.) Lamour., demonstrated growth inhibition starting at zinc concentrations of 100 µg/L after 42 days of incubation. Growth of these segments was completely inhibited at a zinc concentration of 515 µg/L (Bryan, 1969). As summarised by Stratton (1987b), Zn<sup>2+</sup> affected algal growth at EC50s ranging from more than 200 mg/L for *Chlorella pyrenoidosa* to 20 µg/L for *Ditylum brightwellii* (Stratton, 1987b). The *Myriophyllum sibiricum* tested in the current study was intermediate in sensitivity when compared to the algal sensitivity.

Table 90: IC50 values and 95% confidence levels for the effect of zinc chloride upon numerous *Myriophyllum sibiricum* growth and development endpoint parameters.

GROWTH PARAMETER	IC50 (mg/L)		
	Range-finding Test	First Dilution Series	Second Dilution Series
Area Under the Growth Curve	36.2 (26.1; 50.2) <sup>a</sup>	23.1 (18.7; 28.6) <sup>b</sup>	48.0 (34.5; 66.7) <sup>b</sup>
Increase in Shoot Length	29.5 (20.5; 42.4) <sup>a</sup>	27.8 (20.9; 37.1) <sup>b</sup>	66.8 (58.3; 76.5) <sup>b</sup>
Root Number	13.3 (5.7; 30.9)	14.7 (12.9; 16.7) <sup>b</sup>	38.6 (19.7; 75.9) <sup>b</sup>
Root Length	8.2 (2.3; 28.6) <sup>a</sup>	11.2 (10.1; 12.4) <sup>b</sup>	17.8 (13.3; 23.9) <sup>b</sup>
D.O.	Not measured	No IC50	No IC50
Fresh Weight	16.7 (5.0; 56.3) <sup>a</sup>	13.2 (8.2; 21.0) <sup>b</sup>	32.4 (23.2; 45.3) <sup>b</sup>
Membrane Integrity	66.9 (49.8; 89.8) <sup>a</sup>	65.1 (32.4; 133.3) <sup>b</sup>	> 272.59 <sup>a,b</sup>
Plant Area	28.5 (12.8; 63.5) <sup>a</sup>	16.9 (9.0; 31.7) <sup>b</sup>	38.3 (26.4; 55.5) <sup>b</sup>
Chlorophyll <i>a</i> (fresh wt)	78.4 (61.7; 99.6) <sup>a</sup>	90.0 (71.0; 114.1) <sup>b</sup>	100.5 (69.5; 145.3) <sup>b</sup>
Chlorophyll <i>b</i> (fresh wt)	126.2 (91.8; 173.5) <sup>a</sup>	143.4 (107.5; 191.2) <sup>b</sup>	134.3 (80.2; 225.0) <sup>b</sup>
Carotenoid (fresh wt)	91.4 (73.5; 113.7) <sup>a</sup>	229.0 (170.9; 306.6) <sup>b</sup>	232.0 (148.0; 363.6) <sup>b</sup>
Chlorophyll <i>a</i> (dry wt)	Not measured	Not measured	47.0 (28.5, 77.7) <sup>b</sup>
Chlorophyll <i>b</i> (dry wt)	Not measured	Not measured	55.7 (27.0, 114.9) <sup>b</sup>
Carotenoid (dry wt)	Not measured	Not measured	80.9 (36.8, 177.8) <sup>b</sup>

a IC50 and confidence limits estimated using the cumulative normal distribution.

b IC50 and 95% confidence intervals estimated using the weibull model.

Table 91: A summary of EC50/IC50 values for aquatic plant species exposed to zinc. Test conditions differed between the species but general trends in sensitivity may be observed. Based on the EC50 or IC50 values, *Selenastrum capricornutum* was the most sensitive aquatic plant species to the phytotoxic effects of zinc. In the axenic toxicity test, *Myriophyllum sibiricum* was over three hundred times less sensitive to zinc.

Species	Endpoint	Exposure Duration	EC50 or IC50	Reference
<b>Algae</b>				
<i>Anabaena cylindrica</i>	Growth	72 hours	33.51 - 40.31 µg/L	Day and Hodge (1996)
<i>Nitzschia linearis</i>	Cell Production	120 hours	4.3 mg/L	Patrick et al. (1968)
<i>Selenastrum capricornutum</i>	Oxygen Evolution	24 hours	178 µg/L	Turbak et al. (1986)
<i>Selenastrum capricornutum</i>	Biomass	21 days	50.9 µg/L	Turbak et al. (1986)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	72 hours	34.49 - 39.00 µg/L	Day and Hodge (1996)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	96 hours	65.4 µg/L	St. Laurent et al. (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	52.6 µg/L	St. Laurent et al. (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	44.7 µg/L	Blaise et al. (1986)
<b>Floating Macrophytes</b>				
<i>Lemna minor</i>	Plant Damage	14 days	67.7 mg/L	Brown and Rattigan (1979)
<i>Lemna trisulca</i>	FronD Multiplication Rate	2 weeks	0.92 mg/L	Huebert and Shay (1992c)
<i>Lemna trisulca</i>	Final Yield	2 weeks	0.33 mg/L	Huebert and Shay (1992c)
<b>Macrophytes</b>				
<i>Elodea canadensis</i>	Oxygen Evolution	24 hours	8.1 mg/L	Brown and Rattigan (1979)
<i>Elodea canadensis</i>	Plant Damage	28 days	22.5 mg/L	Brown and Rattigan (1979)
<i>Myriophyllum sibiricum</i>	Root Length	14 days	11.2 mg/L	This Document
<i>Myriophyllum sibiricum</i>	Fresh Weight	14 days	13.2 mg/L	This Document

When compared to control plants, leaf length of *Zostera marina* L. (eelgrass) was inhibited by a Zn concentration of 50  $\mu\text{M}$  (3.3 mg/L). Lower Zn concentrations did not significantly affect leaf growth when compared to control plants (Lyngby and Brix, 1984). In segments of *Elodea canadensis*, oxygen evolution measured after 24 hours of exposure to zinc (as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) was reduced to the IC50 level by concentration of 8.1 mg/L (Table 91) and to the IC90 level by a concentration of 23.1 mg/L. In whole plant experiments with *E. canadensis*, 22.5 mg/L produced 50% damage (Table 91) (Brown and Rattigan, 1979). Injury of leaf cells of *E. nuttallii* occurred at zinc (as sulphate) concentrations greater than 150  $\mu\text{M}$  (24 mg/L) (Marquenie-van der Werff and Ernst, 1979). *Lemna minor* was less severely affected because 50% damage was observed at a zinc concentration of 67.7 mg/L (Table 91) (Brown and Rattigan, 1979). Zinc, as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , inhibited frond multiplication of *L. paucicostata* at 1 mg/L in Bonner-Devirian's medium but inhibition was observed at zinc concentrations of 10 mg/L or higher in Hoagland type M-medium (Nasu and Kugimoto, 1981). Zinc at 6.12  $\mu\text{M}$  (0.4 mg/L) decreased the frond multiplication rate of *L. trisulca* by 28% and at 24.5  $\mu\text{M}$  (1.6 mg/L) growth was almost completely inhibited (Huebert and Shay, 1992a; 1992b). The final yield (mg dry weight) of *L. trisulca* was decreased by 72% at 6.12  $\mu\text{M}$  (0.4 mg/L) (Huebert and Shay, 1992a). In a medium replacement system, the effect of zinc was determined upon *L. trisulca*. For the two week frond multiplication rate, an EC50 of 14  $\mu\text{M}$  (0.92 mg/L) was determined and for final yield the EC50 was 5  $\mu\text{M}$  (0.33 mg/L) (Table 91) (Huebert and Shay, 1992c).

The toxicity of zinc to *Myriophyllum sibiricum*, as tested in this study, fits into the toxicity range of other macrophyte species and root length was the most sensitive endpoint. To the phytotoxic effects of zinc, *M. sibiricum* was approximately 300 times less sensitive than *Selenastrum capricornutum* and over 30 times less sensitive than *Lemna trisulca* (Table 91). *M. sibiricum* was 6 times more sensitive to zinc than *L. minor* (Table 91). This range in toxicity supported the suggestion that the *M. sibiricum* toxicity test might be as useful as the *Lemna* bioassay in toxicity assessments.

### 5.8.3 Organic Reference Toxicant (Phenol)

#### 5.8.3.1 Introduction

Phenol has been found in the environment from natural and anthropogenic sources. Once in the environment, it enters the water phase, is highly mobile in soil and

leaches into the ground water (Howard, 1989; WHO, 1994b). In Lake Huron, it has been detected at concentrations between 3 and 24 µg/L in the water and at 13 mg/L in the sediment (Howard, 1989). The World Health Organization (WHO, 1994b) identified phenol as a compound for which plant toxicity data were limited. As a reference toxicant, phenol was ranked moderately high by Environment Canada (1990). The main drawback to using phenol is that it is not very stable in solution (Environment Canada, 1990). Human health concerns can also be a consideration when using phenol because it is highly toxic (MSDS, 1991) so appropriate protective clothing should be employed when handling concentrated solutions.

#### 5.8.3.2 Methods

The phytotoxic effect of phenol upon *M. sibiricum* was evaluated three times during the course of this research project. The range-finding test was conducted between February 11 and 25, 1994, using concentrations between 2.95 and 188.2 mg/L. Using the results from this experiment, the concentration range was altered to between 11.76 and 752.8 mg/L and was repeated twice. These experiments were initiated on May 30, 1994 and January 30, 1995. The experimental plants were cultured in full strength modified Andrews' medium for all three phenol experiments. For the first two experiments, the stock plants were cultured in half strength M & S medium before experimentation. For the last experiment, the stock plants were cultured in the full strength modified Andrews' medium due to the problems with the M & S medium (Chapter 4.5.2). No experimental plants were lost from the range-finding test or the first dose response experiment but unfortunately five experimental plants were discarded from the second dose response experiment due to both fungal and bacterial contamination. Refer to the procedures outlined in Chapter 4.6 for the remainder of the methods utilised to initiate and terminate these reference toxicant experiments.

The experimental endpoints were tested for normality. Normal data or data that could be transformed were analysed using a one-way ANOVA followed by Tukey-Kramer multiple comparisons. Data that were not normal were analysed using the nonparametric Kruskal-Wallis test followed by multiple comparison testing. NOECs were determined from these analyses. IC50 values were determined using either the cumulative normal or weibull methods for continuous response data (Andersen *et al.*, submitted).

### 5.8.3.3 Results and Discussion

Visually, as the concentration of phenol increased, shoot height was reduced (Figure 91). This visual effect was confirmed by the growth curve data (Figure 92). In aquatic situations, phenol degrades completely to carbon dioxide (WHO, 1994b) and thus may become less toxic to aquatic plants over time. No decrease in toxicity over time was noticed in the *Myriophyllum sibiricum* growth curves.

The NOEC and IC50 ( $\pm 95\%$  c.i.) data for the endpoint parameters are summarised in Table 92 and Table 93, respectively. The highest NOEC was 11.76 mg/L for two endpoint parameters (root number and total root length) (Table 92). The lowest IC50 values were for root number, root length and fresh weight. These parameters demonstrated a good correlation between the three experiments and the 95% confidence intervals were narrow. Pigment content, based on mg/g fresh weight of the apex, was variable between experiments with large confidence intervals. This variability in pigment content was caused by stimulation of the pigments at low phenol concentrations (Table 93). For the range-finding experiment, SC20 values (Section 5.6) were determined to be 35.9, 64.1 and 48.1 mg/L for chlorophyll *a*, chlorophyll *b* and carotenoid content, respectively. SC20 values could not be calculated for pigment content (fresh weight) from the two dilution experiments. Using pigment content based on dry weight data from the second dilution experiment, SC20 values were 45.2, 29.4 and 109.6 mg/L for chlorophyll *a*, chlorophyll *b* and carotenoid content, respectively. For all the toxicants tested in the axenic *M. sibiricum* toxicity test, the highest level of membrane damage was observed with phenol. During the second experiment with phenol, the highest level of percent membrane damage was observed at 752.88 mg/L with the highest value being 80.4% and an average of  $72.5 \pm 7.1\%$ . This level of membrane damage did not occur at the other concentrations tested so the IC50 values were not extremely low.

The toxicity of phenol was slightly more variable than that of the  $ZnCl_2$ , possibly due to the moderate volatility of this compound at room temperature (WHO, 1994b). In multiple laboratory testing with *Selenastrum capricornutum* microplates, problems with the volatility of phenol were encountered (Thellen *et al.*; 1989). If this compound were to be used in reference toxicant testing, it would be important to ensure that all experimental test chambers were securely closed with laboratory sealant film and that care was taken when dispensing the compound and mixing the stock solutions. Phenol

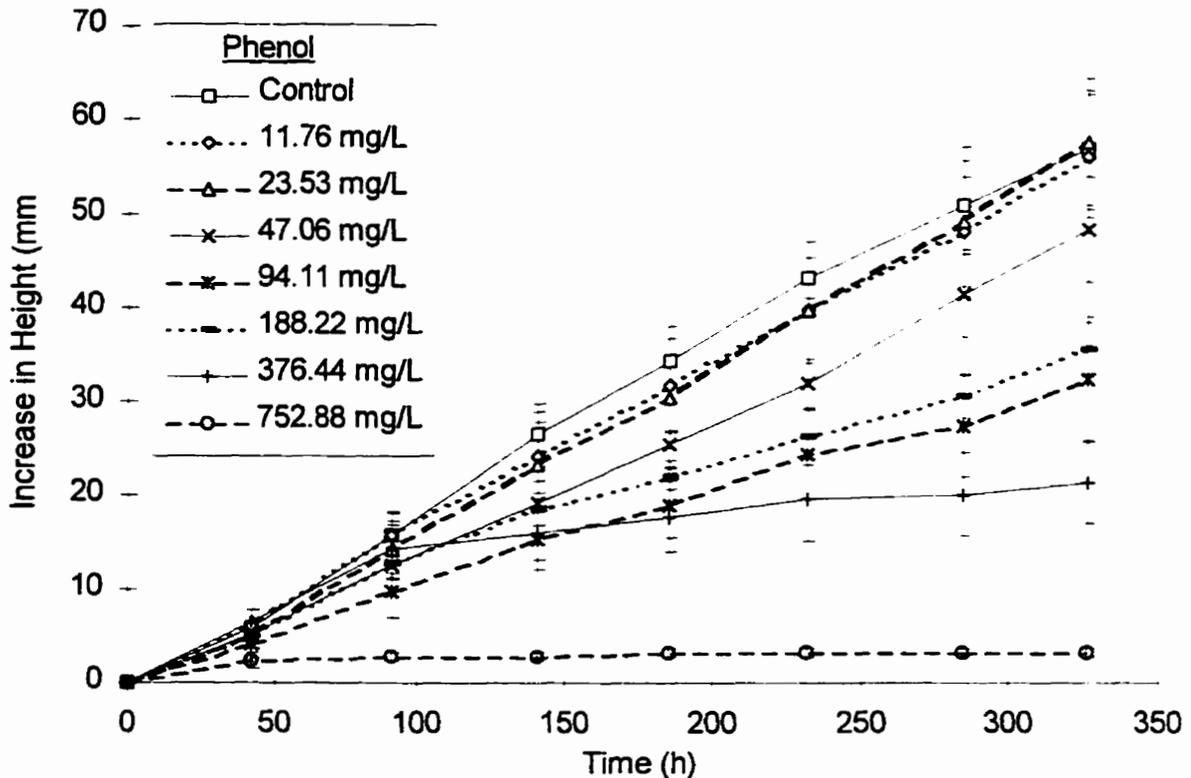


Figure 92: The effect of phenol (0.0 to 752.88 mg/L) on *Myriophyllum sibiricum* shoot height over 14 days. For area under the growth curve, the control does not differ from 11.76 and 23.53 mg/L phenol. The data presented in this graph was from the last phenol dilution series.

has been shown to be absorbed and stored in the cuticle of various plants (Shafer and Schönherr, 1985) and to be metabolised into immobile compounds bound in the roots (McFarlane *et al.*, 1987). In the current study, no attempt was made to determine if *Myriophyllum sibiricum* has the ability to store or metabolise phenol.

#### 5.8.3.4 Aquatic Plant Toxicity Comparison

Buikema *et al.* (1979) compiled the research results of studies on the effect of phenol on numerous algal species. Toxicity was apparent at concentrations ranging from less than 8 mg/L for total inhibition to 100 mg/L at the onset of toxicity (Kostyaev, 1973 as cited in Buikema *et al.*, 1979). The inhibition of cell multiplication in *Scenedesmus quadricauda* began at a phenol concentration of 7.5 mg/L (Bringmann and Kühn, 1980). When examining the effect of phenol upon total cell count of *Skeletonema costatum*, it was determined that the NOEC was 13 mg/L and the EC50

Table 92: No observable effect concentrations for the effect of phenol upon numerous <i>Myriophyllum sibiricum</i> growth and development parameters.			
GROWTH PARAMETER	NOEC (mg/L)		
	Range-finding Test	First Dilution Series	Second Dilution Series
Area Under the Growth Curve	47.06 <sup>a</sup>	All different from control <sup>a</sup>	23.53 <sup>a</sup>
Increase in Shoot Length	47.06 <sup>b</sup>	23.53 <sup>a</sup>	47.06 <sup>a</sup>
Root Number	11.76 <sup>b</sup>	11.76 <sup>b</sup>	23.53 <sup>b</sup>
Root Length	11.76 <sup>b</sup>	11.76 <sup>b</sup>	11.76 <sup>b</sup>
D.O.	No treatment difference <sup>a</sup>	No treatment difference <sup>a,d</sup>	No treatment difference <sup>a,e</sup>
Fresh Weight	11.76 <sup>b</sup>	11.76 <sup>b</sup>	47.06 <sup>b</sup>
Membrane Integrity	No treatment difference <sup>b,c</sup>	47.06 <sup>b</sup>	94.11 <sup>b</sup>
Plant Area	47.06 <sup>b</sup>	11.76 <sup>b</sup>	No treatment difference <sup>a</sup>
Chlorophyll <i>a</i> (fresh wt)	11.76 <sup>b</sup>	23.53 <sup>b</sup>	47.06 <sup>b</sup>
Chlorophyll <i>b</i> (fresh wt)	2.95 <sup>b</sup>	94.11 <sup>a</sup>	188.22 <sup>a</sup>
Carotenoid (fresh wt)	11.76 <sup>a</sup>	23.53 <sup>b</sup>	47.06 <sup>b</sup>
Chlorophyll <i>a</i> (dry wt)	Not measured	Not measured	11.76 <sup>b</sup>
Chlorophyll <i>b</i> (dry wt)	Not measured	Not measured	188.22 <sup>a</sup>
Carotenoid (dry wt)	Not measured	Not measured	< 11.76 <sup>b</sup>

a Data were normal or could be normalised. One-way ANOVA followed by Tukey-Kramer multiple comparison tests were conducted to determine the NOEC.

b Data were not normal so the NOEC were determined using the nonparametric test Kruskal-Wallis test followed by multiple comparisons.

c No difference between the control and the treatments except for 2.95 and 23.53 mg/L.

d No difference between the control and all the treatments except for 23.53 mg/L.

e No difference between the control and all the treatments except for 47.06 mg/L.

was 49.6 mg/L (c.i. = 24.7 - 74.6 mg/L) (Table 94) (Cowgill *et al.*, 1989a). Phenol at a concentration of 258 mg/L caused a 50% reduction in the number of *Nitzschia linearis* cells produced after 120 hours (Table 94) (Patrick *et al.*, 1968). Phenol produced an EC50 of 61.1 mg/L (95% c.i. = 37.1 - 84.5 mg/L) in *Selenastrum capricornutum* flask assays and an EC50 of 63.1 mg/L (c.i. = 18.8 - 104.4 mg/L) in microplate bioassays with the same algal species (Table 94) (St. Laurent *et al.*, 1992). Multiple laboratory testing

Table 93: The effect of phenol upon numerous *Myriophyllum sibiricum* growth and development parameters, expressed as an IC50 and 95% confidence limits.

GROWTH PARAMETER	IC50 (mg/L)		
	Range-finding Test	First Dilution Series	Second Dilution Series
Area Under the Growth Curve	163.2 (124.9; 213.4) <sup>a</sup>	111.4 (62.1; 200.0) <sup>b</sup>	239.8 (115.3; 498.6) <sup>b</sup>
Increase in Shoot Length	127.8 (101.1; 161.5) <sup>b</sup>	109.9 (90.2; 133.9) <sup>b</sup>	228.3 (145.4; 358.4) <sup>b</sup>
Root Number	45.8 (32.4; 64.7) <sup>b</sup>	57.0 (50.5; 64.2) <sup>b</sup>	55.0 (47.9; 63.1) <sup>b</sup>
Root Length	40.0 (34.9; 46.0) <sup>b</sup>	32.6 (30.4; 35.1) <sup>b</sup>	37.7 (32.5; 43.8) <sup>b</sup>
D.O.	No treatment difference	No treatment difference	No treatment difference
Fresh Weight	68.7 (51.8; 91.1) <sup>b</sup>	48.2 (32.5; 71.4) <sup>b</sup>	75.9 (45.0; 127.9) <sup>b</sup>
Membrane Integrity	249.2 (231.2; 268.7) <sup>a</sup>	335.8 (247.0; 456.6) <sup>b</sup>	732.4 (686.4; 781.4) <sup>b</sup>
Plant Area	81.5 (66.4; 100.1) <sup>a</sup>	52.3 (29.5; 92.6) <sup>b</sup>	No treatment difference
Chlorophyll <i>a</i> (fresh wt)	Not calculable <sup>c</sup>	293.4 (143.6; 599.2) <sup>a,e</sup>	344.6 (154.2; 770.3) <sup>b,d</sup>
Chlorophyll <i>b</i> (fresh wt)	Not calculable <sup>d</sup>	425.4 (163.2; 1 109.1) <sup>b,e</sup>	456.4 (177.8; 1 171.7) <sup>b,d</sup>
Carotenoid (fresh wt)	Not calculable <sup>c</sup>	400.1 (286.9; 558.1) <sup>b,e</sup>	453.4 (260.5; 789.0) <sup>b,d</sup>
Chlorophyll <i>a</i> (dry wt)	Not measured	Not measured	366.8 (194.8, 690.9) <sup>b,f</sup>
Chlorophyll <i>b</i> (dry wt)	Not measured	Not measured	560.1 (260.0, 1 206.4) <sup>a,e</sup>
Carotenoid (dry wt)	Not measured	Not measured	525.7 (254.1, 1 087.6) <sup>a,g</sup>

a IC50 values and 95% confidence limits calculated with the cumulative normal distribution.

b IC50 and confidence limits calculated using the weibull model.

c Stimulation occurred at 23.53, 47.06 and 94.11 mg/L, so IC50 values could not be determined.

d Statistically significant stimulation occurred at 94.11 mg/L.

e Statistically significant stimulation occurred at 47.06 mg/L.

f Statistically significant stimulation occurred at 23.53 and 47.06 mg/L.

g Statistically significant stimulation occurred at 11.76, 23.53, 47.06 and 94.11 mg/L.

Table 94: Selected from the scientific literature, a summary of EC50/IC50 values for aquatic plant species exposed to phenol. Test conditions differed between the species but general trends in sensitivity may be observed. Based on the EC50/IC50, *Myriophyllum sibiricum* was the most sensitive aquatic plant to the effects of phenol.

Species	Endpoint	Exposure Duration	EC50 or IC50	Reference
<b>Algae</b>				
<i>Nitzschia linearis</i>	Cell Production	120 hours	258 mg/L	Patrick <i>et al.</i> (1968)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	69.7 mg/L	Thellen <i>et al.</i> (1989)
<i>Selenastrum capricornutum</i>	Population Growth (flask assay)	96 hours	61.1 mg/L	St. Laurent <i>et al.</i> (1992)
<i>Selenastrum capricornutum</i>	Population Growth (microplate)	96 hours	63.1 mg/L	St. Laurent <i>et al.</i> (1992)
<i>Skeletonema costatum</i>	Cell Count	5 days	49.6 mg/L	Cowgill <i>et al.</i> (1989a)
<b>Floating Macrophytes</b>				
<i>Lemna gibba</i>	Vegetative Reproduction	7 days	50.82 mg/L	Barber <i>et al.</i> (1995), Sharma <i>et al.</i> (1997)
<i>Lemna minor</i>	Dry Weight	7 days	157 mg/L	Cowgill <i>et al.</i> (1991)
<i>Lemna minor</i>	Plant Number	7 days	312 mg/L	Cowgill <i>et al.</i> (1991)
<i>Lemna minor</i>	Growth	7 days	480 mg/L	Simon and Blackman (1953)
<i>Lemna minor</i>	Death	48 hours exposure: 24 hours in nutrient	1 505.76 mg/L	Blackman <i>et al.</i> (1955)
<b>Macrophytes</b>				
<i>Myriophyllum sibiricum</i>	Root Length	14 days	32.6 mg/L	This Document
<i>Myriophyllum sibiricum</i>	Fresh Weight	14 days	48.2 mg/L	This Document
<i>Panicum miliaceum</i>	Seedling Root Elongation	120 hours	120 - 170 mg/L	Wang (1986b)

was conducted using phenol with *S. capricornutum* microplate bioassays. The overall average 96 hour EC50 was 69.7 mg/L (Table 94) with a coefficient of variation of 34.9% (Thellen *et al.*, 1989). In 72 hour tests with *Chlorella pyrenoidosa*, 100 mg/L phenol reduced chlorophyll content by 30%, 500 mg/L inhibited the formation of new chlorophyll and phenol concentrations greater than 750 mg/L resulted in the destruction of chlorophyll (Huang and Gloyna, 1968). For millet root elongation inhibition, 120 h EC50 values of 120 to 170 mg/L have been obtained (Wang, 1986b). In the studies reported here, *Myriophyllum* was affected by phenol at lower concentrations than *Nitzschia* or millet but at concentrations comparable to those that affected *Selenastrum*.

Over a 7 day growth period, vegetative reproduction of *Lemna gibba* was also affected by phenol; the EC10 was  $0.08 \pm 0.0092$  mM ( $7.53 \pm 0.87$  mg/L) and the EC50 was  $0.54 \pm 0.037$  mM ( $50.82 \pm 3.48$  mg/L) (Table 94) (Barber *et al.*, 1995; Sharma *et al.*, 1997). A phenol concentration of  $5.1 \cdot 10^{-3}$  M (480 mg/L) caused a 50% reduction in growth of *Lemna minor* (Table 94) (Simon and Blackman, 1953). In a study by Blackman *et al.* (1955), 48 hours of exposure to a phenol concentration of  $1.6 \cdot 10^{-2}$  M (1 505.76 mg/L) followed by 24 hours in untreated nutrient medium induced death in 50% of the *L. minor* population (Table 94) (Blackman *et al.*, 1955). The effect of phenol upon plant number, frond number and dry weight of *L. gibba* and four clones of *L. minor* was examined. The seven day EC50 values ranged from a low of 157 mg/L (c.i. = 0 - 395 mg/L) for the dry weight of one *L. minor* clone to a high of 312 mg/L (c.i. = 151 - 472 mg/L) for the plant number of another *L. minor* clone (Table 94). The lowest NOEL for the effect of phenol upon duckweed was 5 mg/L for all three endpoint parameters of one clone of *L. minor* (Cowgill *et al.*, 1991).

In static tests with algae, the concentration of phenol that affected the growth of more than 3% of the algal population was determined to be 6 mg/L for *Microcystis aeruginosa* (Bringman and Kühn, 1978, as cited in WHO, 1994b) and 8 mg/L for *Scenedesmus quadricauda* (Bringman and Kuhn, 1980). Within fifteen minutes, one-sixteenth of a saturated solution of phenol halted cytoplasm movement in cells of *Nitella* sp. (Stom and Beym, 1976). It was not possible to compare these algal studies with the phenol data obtained for the *Myriophyllum* because of the differences in testing procedures.

Stimulation of photosynthesis by phenol has been observed in *Chlorella* sp. at concentrations between 10 - 40 mg/L (Lukima, 1970) and in *Oscillatoria splendida* Grew. at concentrations between 1 - 100 mg/L (Goncharova, 1975). *Myriophyllum sibiricum*, as cultured in this toxicity test, did not demonstrate stimulation in growth at low phenol concentrations, possibly because the system was axenic, however stimulation in pigment content (fresh weight) was observed (Table 93). Kostyaev (1973, as cited in Buikema *et al.*, 1979), hypothesised that bacteria decomposed the phenol and that the algae were able to utilise the degradation products. Another possibility was that a metabolite might have been responsible for the stimulation. This differs from the theory suggested by Lukima (1975, as cited in Buikema *et al.*, 1979), in which the algae were able to metabolise the phenol. In either case, the *M. sibiricum* system did not show stimulation.

Inhibition of photosynthesis and respiration was observed in *Oscillatoria splendida* at phenol concentrations greater than 100 mg/L (Goncharova, 1975). The amount of oxygen produced by *M. sibiricum* was not affected by phenol. Chlorophyll *a* was more sensitive to phenolics than was chlorophyll *b* (Kostyaev, 1973, as cited in Buikema *et al.*, 1979). The sensitivity difference between chlorophylls *a* and *b* to phenol was not noticed in the *Myriophyllum sibiricum* toxicity test.

Based on a comparison with phenol phytotoxicity data in the literature, *Myriophyllum sibiricum*, as tested in the axenic toxicity test, was the most sensitive aquatic plant. Root length was the most sensitive endpoint parameter in the *M. sibiricum* toxicity test. Based on the phenol EC50 and IC50 values, *M. sibiricum* was 1.5 times more sensitive than *Selenastrum capricornutum* and *Lemna gibba*, 4.8 times more sensitive than *L. minor* and almost 8 times more sensitive than *Nitzschia linearis* (Table 94). This reinforced the idea that multiple aquatic plant species are required for reliable aquatic risk assessment and pesticide registration.

## 5.9 VALIDATION OF HERBICIDE CONCENTRATION IN MEDIUM

### 5.9.1 Introduction

There are many ways to analytically determine pesticide concentration in a solution. The traditional method of residue extraction is to partition the pesticide into a suitable solvent and analyse it by gas liquid chromatographic methods (GLC) (Johnson *et al.*, 1995; MacKenzie, 1996; Solomon *et al.*, 1988; Stephenson *et al.*, 1990) or high

pressure liquid chromatography (HPLC). Triclopyr was analysed with traditional GC methods.

Under Good Laboratory Practice (DiGiulio and Malloy, 1985; U.S. EPA, 1983a; 1983b), the concentration, solubility and stability of the test substance in the nutrient medium should be determined under the toxicity test conditions. Conder *et al.* (1995) found that components in the algal nutrient medium interfered with determination of pesticide concentration. Most analytical methods are validated in water, and therefore it is important to verify the pesticide concentration in the matrix used in the toxicity test. Triclopyr-spiked nutrient medium samples were analysed. Due to time constraints, the other pesticides examined in the toxicity testing system were not analysed.

### 5.9.2 Methods

Medium samples were collected from the triclopyr dilution and MLR series both at the beginning and end of the experiment. A sample of the stock solutions was also saved. During the take-down procedures, medium from all replicate treatments was collected. Medium was placed into glass scintillation vials and frozen at  $-20^{\circ}\text{C}$  until chemical analysis was conducted. Samples were removed from the freezer and warmed up to room temperature before they were subsampled for analysis.

Triclopyr was extracted from the liquid medium following the extraction procedure developed by MacKenzie (1996). This method has been validated for water samples collected from limnocorrals. Samples were acidified to pH 1 - 2 using concentrated (37 N)  $\text{H}_2\text{SO}_4$ . Five or ten mL of triclopyr-spiked medium was loaded onto conditioned J.T. Baker<sup>®</sup> Bakerbond spe\* Octadecyl ( $\text{C}_{18}$ ) Extraction Columns. Under a vacuum of  $3.45 \cdot 10^4$  -  $6.90 \cdot 10^4$  Pa, methanol (2 x 3 mL) was used to elute the triclopyr from the column. The triclopyr acid samples were methylated using 0.5 mL  $\text{BF}_3$  as the catalyst and  $90^{\circ}\text{C}$  heat in a water bath for 30 min. Once the samples had cooled to room temperature, a liquid-liquid partition was performed by adding the sample to 25 mL of water in a 60 mL separatory funnel. Three partitions with petroleum ether (3 x 5 mL) were conducted. The petroleum ether fraction was dried through anhydrous sodium sulphate and collected in 25 mL screw-cap test tubes containing 1 mL of isooctane. The petroleum ether was evaporated to near-dryness with a stream of nitrogen, leaving the triclopyr methyl ester in the isooctane. The sample was transferred to a volumetric flask and brought up to the original volume (5 or 10 mL) with isooctane. All glassware was

rinsed with the appropriate solvent to reduce the loss of the compound. The triclopyr methyl ester was ready for direct injection into the gas chromatograph or if necessary, dilution to less than 100 µg/L. Samples could be stored at -2 °C until further analysis.

Samples were analysed on a Hewlett-Packard® (HP) 5890A gas chromatograph equipped with an electron capture detector and an autosampler (HP 7673 GC/SFC injector). The GC parameters were as follows: HP PAS-5 column, 25 m long x 0.32 mm I.D. x 0.52 mm film thickness; injection port, 250 °C; column, 100 °C for 1 min, 20 °C/min to 145 °C, hold 1 min, 15 °C/min to 225 °C, hold 6 min; detector, 300 °C; carrier gas, ultrapure helium at 1 mL/min; makeup gas, argon-methane (95%-5%); linearity range, 2.5 - 100 pg injected. Sample volumes of 1 µL were injected by a split-less injection technique (MacKenzie, 1996). Residue concentrations were quantified by comparison of sample peak height to mean peak height of standards. Output from the electron-capture detector was integrated on a Hewlett-Packard® 7673 Controller. Duplicate or triplicate samples were analysed. A standard curve using triclopyr methyl ester concentrations of 5, 10, 25, 50 and 100 µg/L was developed for every set of standards and samples injected on the GC.

Triclopyr recovery was determined by spiking autoclaved modified Andrews' medium with triclopyr acid, extracting the triclopyr methyl ester and quantifying the residue. Sample concentrations were corrected for recovery. The nominal concentrations were extracted twice, while the medium collected at the end of the 14 day experiment was extracted once and all five replicates of each concentration were analysed.

### 5.9.3 Results and Discussion

Appendix 8.7 contains a sample standard curve (Appendix 8.7.1, Figure 95), sample recovery data (Appendix 8.7.2, Table 105) and selected GC curves (Appendix 8.7.3). Recovery efficiency of triclopyr from spiked modified Andrews' medium was  $73.3\% \pm 1.15$  (mean  $\pm$  s.e.). The limit of detection (LOD) = 2 µg/L and the limit of quantification (LOQ) = 5 µg/L. The results from the initial triclopyr concentrations are in Table 95. The glass scintillation vial containing the concentrated triclopyr stock solution broke in the -20 °C freezer and the triclopyr had precipitated out of solution, which thus may have contributed to the concentration of triclopyr stock solution actually detected being higher than the actual value. The minor amount of triclopyr detected in the first

extraction of the Triclopyr Control #1 ( $4.23 \pm 0.64$ ) was most likely due to dirty glassware or cross-contamination of the sample because triclopyr was not detected in the second extraction of this sample or in Control # 9 from the MLR experiment. The triclopyr detected during the first extraction of Triclopyr #6 is low because the sample boiled over during the methylation step. Once corrected for recovery, the other nominal concentrations were all slightly greater than the expected concentration.

Table 95: Nominal concentrations ( $\mu\text{g/L}$ ) of triclopyr acid spike modified Andrews' medium as used in the <i>Myriophyllum</i> toxicity tests (corrected for recovery).			
	Expected Concentration	First Extraction (mean $\pm$ s.e.)	Second Extraction (mean $\pm$ s.e.)
Triclopyr Stock Solution	342 000	372 594.17 $\pm$ 663.00	466 049.18 $\pm$ 6 893.85
<b>DILUTION EXPERIMENT</b>			
Control-Triclopyr #1	0	4.23 $\pm$ 0.64	n.d.
Triclopyr #2	9.2	14.54 $\pm$ 0.13	19.68 $\pm$ 0.18
Triclopyr #3	27.6	32.70 $\pm$ 2.24	35.49 $\pm$ 0.50
Triclopyr #4	82.8	95.01 $\pm$ 1.15	104.56 $\pm$ 0.76
Triclopyr #5	248	311.76 $\pm$ 11.09	324.70 $\pm$ 2.87
Triclopyr #6	745	496.01 $\pm$ 34.56	936.31 $\pm$ 2.48
Triclopyr #7	2 235	2 665.05 $\pm$ 27.44	3 459.28 $\pm$ 143.25
Triclopyr #8 (MLR)	6 706	7 982.50 $\pm$ 62.60	8 529.37 $\pm$ 200.41
<b>MLR EXPERIMENT</b>			
Control-Triclopyr #9	0	n.d.	n.d.
Triclopyr #10 (MLR)	6 706	8 556.61 $\pm$ 24.51	9 530.70 $\pm$ 198.01

n.d. = not detectable

The average values (mean  $\pm$  s.e.) for the medium collected at the end of the 14 day experiment are given in Table 96. There was a slight decrease in medium concentration over the 14 day toxicity testing period. According to Solomon *et al.* (1988), triclopyr butoxyethanol ester added to limnocorrals in a northern forest watershed dissipated rapidly and was below 5% of the applied levels within 15 days. Within 42 days, triclopyr could no longer be detected in the limnocorrals. In a limnocorral study in a south western Ontario pond, MacKenzie (1996) determined that the Garlon<sup>®</sup> 3A formulation of triclopyr dissipated to the 50% level after 18.7 to 21.2 days. Ninety-five percent dissipation occurred after 28.5 to 46.3 days. The dissipation

times (DT) for the Garlon® 4 formulation of triclopyr ranged from 16.4 to 19.4 days for the DT50 (time to 50% dissipation) and from 33.6 to 43.4 days for the DT95 (time to 95% dissipation) (MacKenzie, 1996). In contrast, after the 14 day period in this study, the triclopyr acid remained at 53 - 105% of the expected concentration or 43 -58% of the detected nominal triclopyr concentration. These results indicate that in the toxicity testing system microbial breakdown of the pesticide is insignificant. It is also significant that no metabolite peaks were apparent. The portion of the triclopyr that had dissipated was most likely absorbed into the plant or adsorbed onto the plant, Turface®, glass test tube, glass measuring rod or the Tygon® tubing. Although, photolysis might be another minor breakdown pathway, it would also be insignificant because there was a negligible amount of UV light in the light supplied to the test plants. In order to determine the specific fate of herbicides in the axenic toxicity testing system, several additional studies would need to be conducted. Analysis of the plant tissue would be necessary to determine the concentration of triclopyr that was bound to the plant. In order to determine what percentage of the pesticide was adhering to the toxicity testing equipment and materials, test tubes would have to be set-up without the macrophyte tissue and placed under the growth cabinet conditions for the 14 day toxicity testing period. The lack of pesticide degradation is an important component of the toxicity testing system because this system is capable of detecting the direct interaction of the pesticide with the plant. There are no other interfering factors to breakdown the pesticide and change the pesticide concentration that is interacting with the plant.

Table 96: Triclopyr acid concentrations ( $\mu\text{g/L}$ ) in the modified Andrews' medium used in the <i>Myriophyllum</i> toxicity tests (corrected for recovery).		
	Expected Concentration	Detected Concentration (mean $\pm$ s.e.)
<b>DILUTION SERIES</b>		
DZ1 - DZ5	0	between n.d. and 5
DZ6 - DZ10	9.2	9.72 $\pm$ 1.43
DZ11 - DZ15	27.6	14.81 $\pm$ 2.60
DZ16 - DZ20	82.8	47.14 $\pm$ 3.55
DZ21 - DZ25	248	183.24 $\pm$ 4.31
DZ26 - DZ30	745	515.18 $\pm$ 29.01
DZ31 - DZ35	2 235	1 358.44 $\pm$ 101.85
DZ36 - DZ40	6 706	4 505.80 $\pm$ 349.58
<b>MLR SERIES</b>		
DZ41 - DZ45	0	n.d.
DZ46 - DZ50	6 706	4 595.93 $\pm$ 286.75

n.d. = not detectable

## 6. GENERAL DISCUSSION

### 6.1 COMPARISON OF THE *MYRIOPHYLLUM SIBIRICUM* ASSAY WITH THE ALGAL AND DUCKWEED ASSAYS

Laboratory derived toxicological data cannot be directly interpreted to predict environmental impact. This is due to a variety of external factors. Water chemistry is one of the primary differences. In the laboratory, water hardness, pH and temperature are rigorously controlled while in the field these parameters fluctuate. Suspended solids and organic matter that can interfere with the water quality are kept to a minimum in the laboratory (Chapman, 1983; Lee and Jones, 1983). Pesticides may undergo precipitation, complexation, abiotic/biotic sorption, acid/base alteration, gas transfer, photolysis, hydrolysis and volatilisation in field studies. Most of these processes can be minimised or controlled in enclosed laboratory assays.

There is large toxicological response variation between different species. If a variety of species are used in toxicity testing, a better range of effects can be determined (Lewis, 1990; 1995). Extrapolation from one species to another or to a natural plant community is not justified but if several plant species from a number of taxonomic divisions are used in the laboratory tests, the realism of the testing is increased (Chapman, 1983; Lewis, 1990). Most laboratory tests employ the use of continuous exposure to ensure constant levels of pesticides. In contrast, in the natural environment exposure is erratic and difficult to define because pesticide concentration increases dramatically after heavy rainfalls (Edwards, 1977) and where mobile aquatic organisms (e.g. *Chlamydomonas*) can avoid the toxic concentrations (Chapman, 1983).

A number of factors intrinsic to the study organism affect the comparison of laboratory and field toxicity. Life stage and size of the organism can affect the results (Chapman, 1983; Steeles and Thursby, 1983). Prior exposure to a pesticide can occur in the field situation allowing the population to acclimate or to be selected for resistance. Most laboratory organisms have never been stressed by previous exposure to any toxic chemical and are kept in a disease free state. Laboratory handling may affect the organisms (Chapman, 1983). The enriched nutrient conditions in the laboratory are rarely present in the natural ecosystem. Different laboratories may obtain variable results depending on the pesticide being tested (Chapman, 1983; Lewis, 1990).

Laboratory tests are important components in understanding the effects of pesticides upon non-target aquatic plants. They are useful so long as clear goals are kept in mind. Laboratory tests are useful in determining the relative toxicity of pesticides and estimating safe pesticide levels (Chapman, 1983). Only the test species in a single species laboratory test can be expected to be protected in a natural system. The ecosystem is so complex that laboratory results from one species cannot be expected to represent or predict the toxic concentrations to other organisms in the field (Cairns, 1988). For every chemical under examination, several types of species from different trophic levels should be tested (Martin, 1973). Multispecies tests are a viable alternative (Cairns, 1986). Laboratory tests are not reliable in predicting the actual effects of the pesticides upon release into the aquatic environment but give an indication of expected effects (Chapman, 1983).

The duckweed assay is not always applicable in determining the effects of heavy metals upon aquatic macrophytes because this genus seems to be particularly resistant to most metals (Mangi *et al.*, 1978). For other compounds, duckweed can be more sensitive than commonly tested algal species (Peterson *et al.*, 1994). Numerous studies have demonstrated that there is extreme variability between algal species and their sensitivity to toxicants (Blanck *et al.*, 1984; Peterson *et al.*, 1994; Slooff *et al.*, 1983; Wangberg and Blanck, 1988), that there is a range in toxicity thresholds for different microorganisms (Bringmann and Kühn, 1980) and that different clones and species of duckweed can vary in their toxicity (Cowgill *et al.*, 1991). The duckweed bioassay has the advantage that it can test the effects of both sprayed and dissolved herbicides (Lockhart *et al.*, 1989) and thereby can examine the effects of two routes of pesticide entry into aquatic systems. Thus, a test battery approach to toxicant testing has been recommended.

## 6.2 COMPARISON OF THE *MYRIOPHYLLUM SIBIRICUM* ASSAY WITH THE OTHER MACROPHYTE PROTOCOLS

The macrophyte toxicity test that has been developed for use with domestic rice and other native emergent plant species has several disadvantages that are not observed in the *M. sibiricum* toxicity test. In the emergent macrophyte toxicity test, there tends to be high variation between the replicates with the toxicants and species tested to date. In native species, this variation might be a result of the experiment being

started with field collected tubers or rhizomes that may be of variable size, different genetic stock and different nutritional histories. Since this test has been conducted in the greenhouse, there also can be high variation between results obtained at different times during the growing season. Plants grown in September and October produced a much lower dry weight than plants grown in July (Powell *et al.*, 1996). This is in contrast to the axenic *M. sibiricum* toxicity test described in this text. There is minor variation between replicates because they are genetically uniform and the plant material at the start of the experiment is the same height. Because the growing conditions are controlled, consistent results can be obtained at different times of the year.

For domestic rice, Powell *et al.* (1996) recommended that chlorophyll *a* was the most sensitive endpoint but they only compared chlorophyll content to visual assessment of the plants, dry weight and residue content. For the *Myriophyllum sibiricum* toxicity test described here, numerous physiologically different endpoints can be measured. The most sensitive endpoint depends upon the mode of phytotoxic action and root number and pigment content tend to be extremely sensitive.

The sediment toxicity test with *Vallisneria americana* (Biernacki *et al.*, 1997) also has several possible disadvantages. As an aquarium culture, there might be problems with algal, bacterial and aphid infestations. This toxicity test was conducted under a natural photoperiod and this might produce variable results during the year. Sediment from different field sites might be variable in nutrients and texture, which might influence the results. One similarity between the sediment bioassay with *V. americana* and the axenic *Myriophyllum sibiricum* toxicity test was the use of clones.

### 6.3 NEW TECHNOLOGIES AND BENEFITS

This document describes a new submersed aquatic macrophyte toxicity test that is capable of detecting the effects of low concentrations of pesticides and other phytotoxic compounds. The conditions for this toxicity test have been explored and the protocol contains a well defined liquid growth medium, environmental growth conditions and parameters for pesticide use. The recommended toxicity test endpoints were explored thoroughly. Determination of area under the growth curve was an endpoint that previously had not been used for macrophyte testing. The use of new regression-based statistical analyses of the toxicity data strengthened the results. Analysis of toxicant data with hormesis added beneficial information to the study. The toxicity test

was repeatable and reliable, and *Myriophyllum sibiricum* is ecologically relevant so this toxicity test meets the three requirements for ecotoxicological tests as prescribed by Calow (1992). As determined in this axenic toxicity test, *Myriophyllum sibiricum* was the most sensitive aquatic plant species to 2,4-D, fluridone, glyphosate, triclopyr and phenol based on values from the current literature. It is important to note that these herbicides represent four very different modes of phytotoxic action.

#### 6.4 FUTURE RESEARCH INTO ROOTED AQUATIC PLANT TOXICITY TESTS

Other endpoints could be examined. Peroxidase activity has been shown to be ubiquitous in the plant kingdom. High levels of peroxidase activity has been found in pollution (pulp and paper mill effluent) tolerant aquatic plant species while low peroxidase levels are evident in pollution intolerant species (Roy *et al.*, 1992). A research group at Clemson University has induced peroxidase activity in *Hydrilla* (Byl and Klaine, 1991; Byl *et al.*, 1994). Peroxidase activity has also been examined as an aquatic plant response to herbicide stress (Sprecher and Stewart, 1995; Sprecher *et al.*, 1993). The measurement of peroxidase activity in *Potamogeton pectinatus* was less successful at detecting low levels of metal induced stress than was the measurement of stress proteins (Siesko *et al.*, 1997). The induction of enzyme activity may be a more sensitive indicator of environmental stress or exposure than the morphological and physiological endpoints examined in this study. However, the relationship between measurable effects on the enzyme and growth is still unclear.

Chlorophyll fluorescence has also been examined as an endpoint. This method has successfully detected the effect of photosynthetic inhibitors within 5 to 25 hours of exposure (Judy *et al.*, 1990). It has also been used to determine the phytotoxic effects of chemicals to algae (Bozeman *et al.*, 1989; Caux *et al.*, 1992) and herbicides to terrestrial plants (MacDonald *et al.*, 1993). This method has been used to examine photoinduced toxicity of polycyclic aromatic hydrocarbons to *Lemna gibba* (Huang *et al.*, 1997). It would be interesting to determine if chlorophyll fluorescence could be used as an endpoint with the *Myriophyllum sibiricum* toxicity test.

Residue content of the plants could be determined. This has been beneficial in determining how the plant metabolises or stores the toxicant. Emergent macrophyte (*Oryza sativa*) residue content was determined by Powell *et al.* (1996). Other methods of studying the absorption and metabolism of phytotoxic compounds involved the use of

radiolabelled compounds and the examination of their fate over time. With aquatic plants, radioactive counting using a liquid scintillation detector (Barber *et al.*, 1995; Wang *et al.*, 1994a) or analysis on HPLC (Barber *et al.*, 1995) have been used. Pesticide fate or metabolism has been studied in the following aquatic plant species: *Eichhornia crassipes* (Wang *et al.*, 1994a), *Lemna gibba* G3 (Barber *et al.*, 1995) and *Myriophyllum sibiricum* (Turner, 1996). As was seen in the study by Turner (1996), this axenic *Myriophyllum* toxicity test was easily adapted to examine herbicide metabolism. It would be interesting to examine the metabolism of other phytotoxic compounds using this toxicity test.

The interaction between pesticides and solvents in the axenic *Myriophyllum sibiricum* toxicity test should be examined. It has been determined that solvents and pesticides interact synergistically, antagonistically or additively in other toxicity tests (Adams *et al.*, 1986; Stratton, 1985; Stratton *et al.*, 1980; 1982; Stratton and Corke, 1980; 1981b). It is possible that a methanol-atrazine interaction occurred (Section 5.7.1).

The effect of pesticide degradation products upon the growth and development of *Myriophyllum sibiricum* could also be examined. Other authors have found that the degradation products can be of equal toxicity, more toxic or less toxic than the parent compound (Baarschers *et al.*, 1988; Day, 1991; Jones and Winchell, 1984; Stratton, 1981; 1984). The effects of pesticide formulations on *Myriophyllum sibiricum* could also be examined. On marine unicellular algae, herbicide formulation affected the toxicity. The formulation could be of greater or reduced toxicity when compared with the parent compound (Walsh, 1972). Occasionally, the surfactant or adjuvants added to the herbicide can be differentially toxic. Another avenue of research might include adapting this toxicity test to determine the effects of growth hormones upon aquatic plant species by utilising methods similar to those of Kane and Gilman (1991). The effect of mixtures of chemicals would be another avenue of future research.

Standardisation and round-robin laboratory testing is an important component of the acceptance and wide-spread use of any newly developed biological test (Wang and Freemark, 1995). For example, the results produced using the standardised aquatic microcosm test were compared between four laboratories. Once the results were shown to be consistent, the test has more validity (Taub, 1993). The *Selenastrum capricornutum* microplate bioassay was validated by testing two chemicals in three

laboratories and two researchers per lab (Thellen *et al.*, 1989). For the axenic aquatic macrophyte toxicity test discussed in this thesis, round-robin testing is needed. Plants have been transferred to Analytical Bio-Chemistry Laboratories, Inc. in Columbia, MO where the initial stages of plant culturing are underway. The goal is also to include this toxicity test among those to be examined by the Aquatic Macrophyte Working Group that is currently being formed. At the University of Guelph, two students have successfully utilised the toxicity test without modification (Faber, 1997; Perkins, 1997) and one student has used the toxicity test with modification (McCann, 1997). The technology has been transferred to technical staff at the University of Guelph so experimental research with the macrophyte toxicity test can continue. The evaluation of intralaboratory and interlaboratory precision would further validate the use of this toxicity test.

Field validation of laboratory results is often recommended but field studies do not necessarily validate the laboratory. Studies under both conditions provide necessary information with the laboratory studies often providing results under "the worse-case scenario" (Cairns, 1983; Chapman, 1995a; 1995b).

## 6.5 SIGNIFICANCE OF THE RESULTS

In order to determine aquatic plant sensitivity to new pesticides, a species battery including the *Selenastrum capricornutum*, *Lemna gibba* and *Myriophyllum sibiricum* toxicity tests should be conducted. Compared to these other aquatic plants, *Myriophyllum sibiricum*, as tested in the axenic toxicity test, was highly sensitive to the phytotoxic effects of the expected environmental concentration of 2,4-D, fluridone, glyphosate, triclopyr and phenol. Even 1% of the EEC of 2,4-D, diquat, fluridone and triclopyr could cause significant inhibitory effects on *M. sibiricum* plants. Thus, buffer zones should be employed to prevent oversprays or spray drift onto non-target aquatic areas.

In conclusion, this static, axenic toxicity test is a fast, simple and accurate method that can determine possible effects of new herbicides and other phytotoxic chemicals upon non-target submersed macrophytes. It would be a beneficial addition to the existing pesticide testing requirements and help protect non-target aquatic environments.

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## 8. APPENDICES

### 8.1 ACRONYMS

Table 97: A list of acronyms used in the text of this thesis and their meanings.	
ACRONYM	MEANING
ae	Acid Equivalent
a.i.	Active Ingredient
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
CAM	Crassulacean Acid Metabolism
c.i.	Confidence Interval
DMF	N,N'-dimethylformamide
DMSO	Dimethyl Sulphoxide
D.O.	Dissolved Oxygen
DT50	Time for 50% of a Chemical to Dissipate
DT95	Time for 95% of a Chemical to Dissipate
EC50	Effective Concentration to Produce a 50% Effect on an Endpoint Parameter
EEC	Expected Environmental Concentration
ELISA	Enzyme Linked Immunoabsorbent Assay
EPA	Environmental Protection Agency
GC	Gas Chromatograph
GLP	Good Laboratory Practise
HPLC	High Pressure Liquid Chromatography
IC25	Inhibitory Concentration to Inhibit an Endpoint Parameter by 25%
IC50	Inhibitory Concentration to Inhibit an Endpoint Parameter by 50%
I.D.	Inside Diameter
LD50	Lethal Dose to Kill 50% of a Population
LOEC	Lowest Observable Effect Concentration
LOEC	Lowest Observable Effect Level
M & S	Murashige and Skoog Liquid Media
MLR	Maximum Recommended Label Rate
NOEC	No Observable Effect Concentration
NOEL	No Observable Effect Level
O.D.	Outside Diameter
OECD	Organisation for Economic Co-operation and Development
OMAF	Ontario Ministry of Food and Agriculture
PDA	Potato Dextrose Agar
PMRA	Pest Management Regulatory Agency
SAM	Standardised Aquatic Microcosm
SC20	Stimulatory Concentration to Simulate an Endpoint Parameter by 20%
s.d.	Standard Deviation
TEA	Triethylamine
TSA	Trypticase Soy Agar
WSSA	Weed Science Society of America

## 8.2 SCIENTIFIC AND COMMON NAMES

Table 98: A listing of all the microorganisms in this document.	
SCIENTIFIC NAME	
Algae	
<i>Anabaena cylindrica</i> Lemm. <sup>1</sup>	
<i>Anabaena flos-aquae</i> Bréb. <sup>2</sup>	
<i>Anabaena inaequalis</i> (Kuetz) Bornet & Fiahault <sup>3</sup>	
<i>Anabaena variabilis</i> Keutzing <sup>3</sup>	
<i>Ankistrodesmus</i> sp.	
<i>Aphanizomenon flos-aquae</i> (L.) Ralfs <sup>2,4</sup>	
<i>Aphanocapsa</i> sp.	
<i>Ascophyllum nodosum</i> (L.) Le Jolis	
<i>Asterionella formosa</i> Hass. <sup>2,4</sup>	
<i>Chara</i> spp.	
<i>Characium</i> sp.	
<i>Chlamydomonas moewusii</i> Gerloff <sup>6</sup>	
<i>Chlamydomonas eugametos</i> Moewus (= <i>C. sphagnophila</i> Pascher) <sup>5</sup>	
<i>Chlamydomonas reinhardtii</i> Dang. <sup>2</sup>	
<i>Chlorella ellipsoidea</i> Gerneck <sup>4</sup>	
<i>Chlorella pyrenoidosa</i> Chick.	
<i>Chlorella sorokiniana</i> Shihira and Krauss	
<i>Chlorella vulgaris</i> Beij. <sup>4</sup>	
<i>Chlorococcum</i> sp.	
<i>Chlorococcum hypnosporum</i> Starr <sup>6</sup>	
<i>Cladophora glomerata</i> (L.) Kütz.	
<i>Cosmarium botrytis</i> (Menegh.) Ralfs <sup>7</sup>	
<i>Cryptomonas marssonii</i> Skija	
<i>Cyclotella meneghiana</i> Kütz. <sup>4</sup>	
<i>Ditylum brightwellii</i> (West) Grunow	
<i>Dunaliella tertiolecta</i> Butcher	
<i>Euglena gracilis</i> Klebs <sup>2</sup>	
<i>Franceia</i> sp.	
<i>Fucus serratus</i> L.	
<i>Fucus spiralis</i> L.	
<i>Fucus vesiculosus</i> L.	
<i>Gloecapsa alpicola</i> (Lyng.) Bornet. <sup>8</sup>	
<i>Isochrysis galbana</i> Parke	
<i>Laminaria digitata</i> (Huds.) Lamour.	
<i>Lyngbya</i> sp.	
<i>Lyngbya wollei</i> (Farlow in Gomont) Speziale & Dyck	

<i>Microcystis aeruginosa</i> Kütz. <sup>4</sup>	
<i>Navicula inserata</i> F. Hustedt <sup>9</sup>	
<i>Navicula pelliculosa</i> (Kützing) Hilse in Rabenhorst <sup>10</sup>	
<i>Nitzschia angularum</i> W. Smith <sup>10</sup>	
<i>Nitzschia kuetzingiana</i> Hilse (= <i>N. pusilla</i> Grunov) <sup>11</sup>	
<i>Nitzschia linearis</i> (Ag.) W. Smith <sup>10</sup>	
<i>Nitzschia palea</i> (Kützing) W. Smith <sup>10</sup>	
<i>Oscillatoria</i> sp.	
<i>Oscillatoria splendida</i> Grew.	
<i>Pelvetia canaliculata</i> (L.) Dec. et Thur.	
<i>Phaeodactylum tricornutum</i> Bohlin	
<i>Pseudoanabaena</i> sp.	
<i>Scenedesmus obliquus</i> (Turp.) Kützing <sup>3</sup>	
<i>Scenedesmus pannonicus</i>	
<i>Scenedesmus quadricauda</i> (Turp.) Bréb. <sup>2,4</sup>	
<i>Selenastrum capricornutum</i> Printz.	
<i>Skeletonema costatum</i> (Greville) Cleve	
<i>Spirogyra</i> sp.	
<i>Stigeoclonium</i> sp.	
<i>Thalassiosira weissflogii</i> Grun.	
<i>Ulothrix</i> sp.	
Bacteria	
<i>Pseudomonas maltophilia</i>	
Fungi	
<i>Penicillium</i> spp.	
<i>Fusarium</i> spp.	

- 1 Taxonomic information obtained from Desikachary (1959).
- 2 Taxonomic information obtained from Pentecost (1984).
- 3 Taxonomic information from Prescott (1951).
- 4 Taxonomic information from Prescott (1970).
- 5 Taxonomic information from Ettl (1983).
- 6 Taxonomic information obtained from Ettl and Gärtner (1988).
- 7 Taxonomic information obtained from Prescott *et al.* (1981).
- 8 Taxonomic information obtained from Whitford and Schumacher (1973).
- 9 Taxonomic information obtained from Round *et al.* (1990).
- 10 Taxonomic information from Hartley (1996).
- 11 Taxonomic information obtained from Krammer (1988).

Table 99: A listing of the scientific and common names for all the higher organism mentioned in this document.

Aquatic Macrophytes (Floating, Submergent and Emergent)	
SCIENTIFIC NAME	COMMON NAME
<i>Alisma plantago aquatica</i> L.	water plantain
<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	alligator weed
<i>Cabomba caroliniana</i> Gray	fanwort, cabomba
<i>Callitriche cophocarpa</i> Sendtner	water-starwort family
<i>Callitriche heterophylla</i> Pursh. <sup>1</sup>	
<i>Callitriche stagnalis</i> Scop.	
<i>Carex</i> spp.	sedge
<i>Carex retrorsa</i> Schwein <sup>2</sup>	
<i>Carex rostrata</i> Stokes.	beaked sedge
<i>Ceratophyllum demersum</i> L.	coontail, hornwort
<i>Crassula</i> spp.	
<i>Echinochloa crusgalli</i> (L.) Palisot de Beauvois	
<i>Echinodorus tenellus</i> (Mart.) Buchenau. <sup>2</sup>	burhead, Amazon sword plant
<i>Echinodorus paniculatus</i> P. Micheli. <sup>2</sup>	Amazon sword plant
<i>Egeria</i> spp.	elodea
<i>Egeria densa</i> Planch.	Brazilian elodea, egaria
<i>Eichhornia crassipes</i> (Mart.) Solms.	water hyacinth
<i>Eleocharis acicularis</i> (L.) Roem. & Schult.	slender spikerush
<i>Elodea canadensis</i> Michx.	American elodea
<i>Elodea densa</i> (Planch.) Caspary <sup>3</sup>	
<i>Elodea nuttallii</i> (Planch.) St. John	
<i>Fontinalis antipyretica</i> Hedw.	aquatic moss
<i>Hydrilla verticillata</i> (L.f.) Royle	hydrilla, Florida elodea
<i>Hydrocotyle</i> spp.	pennywort
<i>Iris versicolor</i> L.	blue water iris, blue flag
<i>Isoetes bolanderi</i> Engelm. <sup>4</sup>	Quillwort family
<i>Isoetes howellii</i> Engelm. <sup>4</sup>	
<i>Isoetes lacustris</i> L. <sup>1</sup>	
<i>Isoetes macrospora</i> Dur. <sup>5</sup>	
<i>Isoetes orcuttii</i> A.A. Eaton	
<i>Juncus effusus</i> L.	soft rush
<i>Lemna minor</i> L.	common duckweed, lesser duckweed
<i>Lemna gibba</i> L.	inflated duckweed
<i>Lemna paucicostata</i> Hegelm. ex Engelm. (= <i>L. perpusilla</i> Torrey) <sup>6</sup>	
<i>Lemna perpusilla</i> Torr.	
<i>Lemna polyrrhiza</i> L. (= <i>Spirodela polyrrhiza</i> (L.) Schleid.) <sup>4,5</sup>	
<i>Lemna trisulca</i> L.	

(Table 99 continued)	
<i>Lemna valdiviana</i> Philippi.	
<i>Littorella</i> spp.	
<i>Littorella uniflora</i> (L.) Asch. <sup>1</sup>	
<i>Ludwigia natans</i> Ell. <sup>5</sup>	false loosestrife family
<i>Lythrum salicaria</i> L.	purple loosestrife
<i>Myriophyllum aquaticum</i> (Vell.) Verdc.	parrotfeather
<i>Myriophyllum brasiliense</i> Cambess.	parrotfeather
<i>Myriophyllum exalbescens</i> Fernald	northern watermilfoil, American watermilfoil
<i>Myriophyllum heterophyllum</i> Michx.	
<i>Myriophyllum hippuroides</i> Nutt. <sup>4</sup>	
<i>Myriophyllum pinnatum</i> (Walt.) BSP	
<i>Myriophyllum sibiricum</i> Komarov <sup>1</sup>	northern watermilfoil, common watermilfoil
<i>Myriophyllum spicatum</i> L.	Eurasian watermilfoil
<i>Myriophyllum verticillatum</i> L.	whorled watermilfoil
<i>Najas</i> spp.	naiad
<i>Najas guadalupensis</i> (Spreng.) Mangus	southern naiad
<i>Nelumbo lutea</i> (Willd.) Pers.	American lotus
<i>Neptunia oleracea</i> Lour.	aquatic legume
<i>Nuphar lutea</i> (= <i>N. microphyllum</i> (Pers.) Fern.) <sup>1,4</sup>	water-lily family
<i>Onobrychis viciaefolia</i> Scop.	Sainfoin
<i>Oryza sativa</i> L.	domestic rice
<i>Panicum miliaceum</i> L. <sup>2</sup>	millet
<i>Phalaris arundinacea</i> L.	reed canary grass
<i>Polygonum muhlenbergh</i> G.	nodding smartweed
<i>Potamogeton crispus</i> L.	curlyleaf pondweed
<i>Potamogeton foliosus</i> Raf.	leafy pondweed
<i>Potamogeton lucens</i> (= <i>Potamogeton illinoensis</i> Morong.) <sup>1,5</sup>	
<i>Potamogeton nodosus</i> Poir.	American pondweed
<i>Potamogeton pectinatus</i> L.	sago pondweed
<i>Potamogeton perfoliatus</i> L.	redhead-grass, perfoliate pondweed
<i>Potamogeton polygonifolius</i> Pourret <sup>6</sup>	
<i>Potamogeton pusillus</i> L.	small pondweed
<i>Potamogeton richardsonii</i> (A. Benn.) Rydb.	clasping leaf or Richardson pondweed
<i>Potamogeton schweinfurthii</i> A. Benn.	
<i>Potamogeton thunbergii</i> Cham. & Schlecht.	
<i>Proserpinaca palustris</i> L.	mermaidweed
<i>Ranunculus aquatilis</i> L. <sup>6</sup>	
<i>Ranunculus subrigidus</i> W.B. Drew	water buttercup
<i>Ranunculus trichophyllus</i> Chaix	white buttercup
<i>Riccia fluitans</i> L. <sup>2</sup>	crystal wort
<i>Ruppia cirrhosa</i> (Petagna) Grande	
<i>Ruppia maritima</i> L.	widgeon grass

(Table 99 continued)	
<i>Sagittarius latifolia</i> Willd. <sup>5</sup>	broad-leaved arrowhead, duck-potato
<i>Salvinia</i> spp.	salvania
<i>Salvinia natans</i> (L.) All.	salvania, water fern
<i>Scirpus</i> spp.	bulrush
<i>Scirpus acutus</i> Muhl.	hardstem bulrush
<i>Sparganium simplex</i> Huds.	
<i>Spartina alterniflora</i> Loisel. <sup>4</sup>	salt-water cord-grass
<i>Spartina pectinata</i> Link.	prairie cordgrass
<i>Spirodela polyrhiza</i> (L.) Schleiden	giant duckweed, greater duckweed
<i>Thalassia testudinum</i> Konig <sup>7</sup>	seagrass
<i>Typha</i> spp.	cattail
<i>Typha latifolia</i> L.	narrowleaf cattail
<i>Utricularia</i> spp.	bladderwort
<i>Utricularia purpurea</i> Walt.	bladderwort
<i>Utricularia vulgaris</i> L.	bladderwort
<i>Vallisneria americana</i> Michx.	wild celery, tapegrass, eeigrass
<i>Vallisneria spiralis</i> L. <sup>6</sup>	
<i>Zannichellia palustris</i> L.	horned pondweed
<i>Zinania aquatica</i> L.	giant wild rice
<i>Zostera marina</i> L.	eelgrass
	paragrass
Terrestrial Macrophytes	
<i>Agropyron repens</i> (L.) Beauv.	couch grass, quackgrass
<i>Allium cepa</i> L. <sup>8</sup>	onion
<i>Amaranthus</i> spp.	pigweed
<i>Ananas comosus</i> Merr.	pineapple
<i>Arachis hypogaea</i> L.	peanuts
<i>Avena sativa</i> L.	oats
<i>Beta vulgaris</i> L.	sugar beets
<i>Brassica</i> spp.	mustard
<i>Cucumis sativus</i> L.	cucumber
<i>Glycine max</i> (L.) Merr.	soybeans
<i>Hordeum vulgare</i> L.	barley
<i>Gossypium hirsutum</i> L.	cotton
<i>Linum usitatissimum</i> L.	flax
<i>Medicago sativa</i> L.	alfalfa
<i>Brassica</i> spp.	canola
<i>Petroselinum hortense</i> Hoffm.	parsley
<i>Pinus resinosa</i> Ait.	red pine
<i>Saccharum officinarum</i> L.	sugar cane
<i>Solanum tuberosum</i> L.	potatoes
<i>Sorghum vulgare</i> Pers.	sorghum
<i>Tradescantia</i> sp.	
<i>Trifolium</i> spp.	clover

(Table 99 continued)	
<i>Trifolium repens</i> L.	white clover
<i>Triticum aestivum</i> L.	wheat
<i>Zea mays</i> L.	maize or corn
<i>Zinnia elegans</i> Jacq. <sup>2</sup>	common zinnia
Invertebrates	
<i>Cypridopsis</i> sp.	
<i>Ceriodaphnia dubia</i>	
<i>Daphnia magna</i> Straus. <sup>9</sup>	cladocera, daphnia
<i>Hyalella azteca</i> Strassure <sup>9</sup>	amphipods
<i>Philodina</i> sp.	
Fish	
<i>Pimephales promelas</i>	fathead minnow

1 Taxonomic information was obtained from Gleason and Cronquist (1991).

2 Taxonomic information for some cultivated plants was obtained from Hortus Third (1976).

3 Taxonomic information was obtained from Fassett (1957).

4 Taxonomic information was obtained from Scroggan (1978).

5 Taxonomic information was obtained from Fernald (1950).

6 Taxonomic information was obtained from Flora Europaea (1980).

7 Taxonomic information was found in Adams (1972).

8 Scientific and common names of cultivated plants were found in Bailey (1971) and Bailey and Bailey (1976).

9 Taxonomic information from Pennak (1978).

Other taxonomic information was obtained from the papers where the species were cited.

### 8.3 D.O. CURVES: MINIMISING THE ERROR BETWEEN MEASURED DATA AND ANALYTICALLY DERIVED ESTIMATES

#### 8.3.1 Oxygen Production by Healthy *Myriophyllum sibiricum* Plants - Mathcad® Equations and Dissolved Oxygen Curve

##### Dissolved Oxygen Experiment Using Healthy Plants

data1 = READPRN(do)

t1 = data1<1>      dol = data1<2>

x1 = length(t1)      x1 = 39      il = 1..x1

$$\text{dol}_{\text{avg}} = \frac{\sum_{il} \text{dol}_{il}}{x1} \quad \text{dol}_{\text{avg}} = 116.077$$

Initial Guesses:

A1 = 45      t1<sub>o</sub> = 0      T1 = 24

$$\text{DOI}(A1, T1, t1_o, t1) = A1 \cdot \sin\left(\frac{2 \cdot \pi}{T1} \cdot t1 - t1_o\right) - \text{dol}_{\text{avg}}$$

$$\text{SSE}(A1, T1, t1_o, t1) = \sum_{il} (\text{dol}_{il} - \text{DOI}(A1, T1, t1_o, t1_{il}))^2$$

Given

$$\text{SSE}(A1, T1, t1_o, t1) = 0 \quad 1=1 \quad 2=2$$

$$\begin{bmatrix} A1 \\ T1 \\ t1_o \end{bmatrix} = \text{Minerr}(A1, T1, t1_o)$$

$$A1 = 49.582 \quad T1 = 24.516 \quad t1_o = 0.704 \quad \text{SSE}(A1, T1, t1_o, t1) = 1.506 \cdot 10^4$$

### DO vs. Time for Healthy Plants

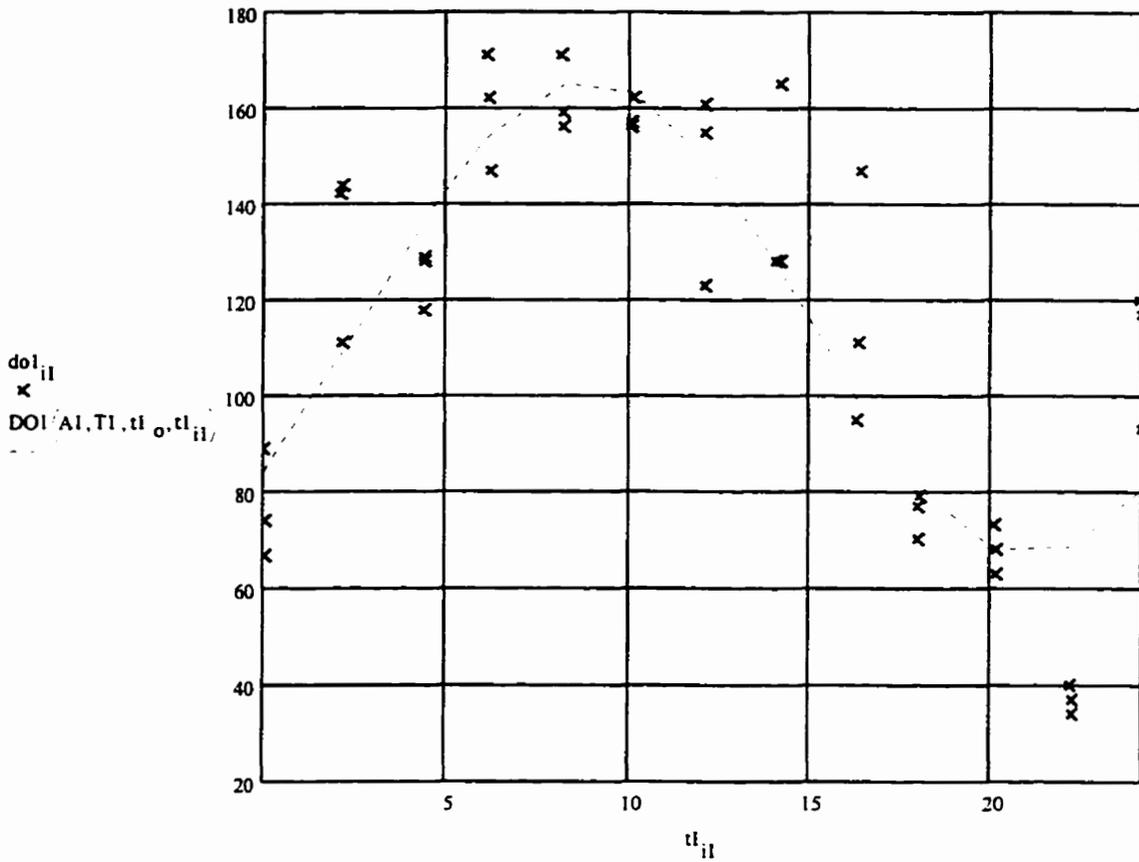


Figure 93: Oxygen (% dissolved oxygen) produced by healthy *Myriophyllum sibiricum* plants over a twenty-four hour period, during which the plants were exposed to 16 hours of light and 8 hours of dark. The plants were grown in full strength modified Andrews' medium for fourteen days prior to being harvested.

### 8.3.2 Oxygen Production by Unhealthy *Myriophyllum sibiricum* Plants - Mathcad® Equations and Dissolved Oxygen Curve

#### Dissolved Oxygen Experiment Using Unhealthy Plants

data2 = READPRN(do2)

t2 = data2<1>      do2 = data2<2>

x2 = length(t2)      x2 = 36      i2 = 2..x2

$$\text{do2}_{\text{avg}} = \frac{\sum_{i2} \text{do2}_{i2}}{x2} \qquad \text{do2}_{\text{avg}} = 92.5$$

Initial Guesses:

A2 = 45      t2<sub>o</sub> = 0      T2 = 24

$$\text{DO2}(A2, T2, t2_o, t2) = A2 \cdot \sin\left(\frac{2 \cdot \pi}{T2} \cdot t2 - t2_o\right) - \text{do2}_{\text{avg}}$$

$$\text{SSE}(A2, T2, t2_o, t2) = \sum_{i2} (\text{do2}_{i2} - \text{DO2}(A2, T2, t2_o, t2_{i2}))^2$$

Given

$$\text{SSE}(A2, T2, t2_o, t2) = 0 \qquad 1=1 \qquad 2=2$$

$$\begin{matrix} A2 \\ T2 \\ t2_o \end{matrix} = \text{Minerr}(A2, T2, t2_o,$$

$$A2 = 13.271 \qquad T2 = 47.677 \qquad t2_o = 5.005 \qquad \text{SSE}(A2, T2, t2_o, t2) = 1.69 \cdot 10^3$$

### DO vs. Time for Unhealthy Plants

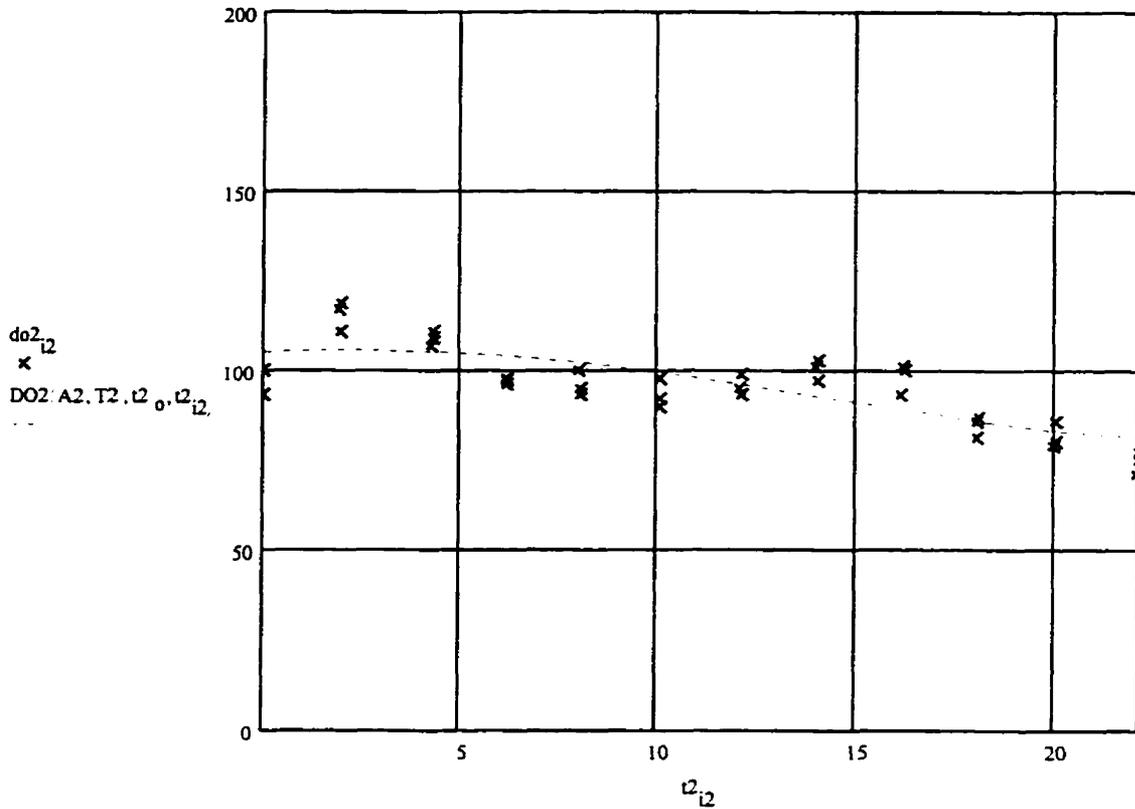


Figure 94: The percent of oxygen produced by *Myriophyllum sibiricum* plants grown in axenic culture without an external carbon source. The plants were cultured in modified Andrews' medium without sucrose for fourteen days. The plants were harvested over a 24 hour period during which time the plants were exposed to a 16:8 hour photoperiod.

## 8.4 SPECTROPHOTOMETER PROGRAMS

### 8.4.1 Calibration of the Spectrophotometer

000:	Strt	
001:	estX	
002:	MSG	cINS
003:	MSG	ERT
004:	MSG	BLAN
005:	MSG	K
006:	R/S	
007:	CALL	FILL
008:	MSG	cABS
009:	MSG	AT
010:	MSG	470
011:	CALL	COUT
012:		4.0000
013:	CALL	BLNK
014:	MSG	cABS
015:	MSG	AT
016:	MSG	647
017:	CALL	COUT
018:	CALL	BLNK
019:		4.0000
020:	MSG	cABS
021:	MSG	AT
022:	MSG	663
023:	CALL	COUT
024:	CALL	BLNK
025:		4.0000
026:	MSG	cCHL
027:	MSG	ORO
028:	MSG	A
029:	CALL	COUT
030:	CALL	BLNK
031:		4.0000
032:	MSG	cCHL
033:	MSG	ORO
034:	MSG	E
035:	CALL	COUT
036:	CALL	BLNK
037:		4.0000
038:	MSG	cCAR
039:	MSG	OTEN
040:	MSG	GIDS
041:	prt	
042:	CALL	CRLF
043:	ABS	
044:		470.00
045:	LMDA	
046:	CALB	
047:		647.00
048:	LMDA	
049:	CALB	
050:		663.00
051:	LMDA	
052:	CALB	
053:		2.0000
054:	CALL	CHAN
055:	rtn	
056:	CALL	C
057:		9.0000
058:	MSG	B
059:	MSG	B
060:	CALL	B4K
061:	CALL	S
062:	CALL	C
063:		5.0000
064:	MSG	N
065:		70.000
066:	MSG	N
067:		7.0000
068:	MSG	B
069:	MSG	NB
070:	MSG	4F?
071:	MSG	4F?
072:	MSG	N
073:		7.0000
074:	MSG	c
075:	MSG	c'
076:		470.00
077:	MSG	E
078:		470.00
079:	ENTR	
080:	f	
081:	MSG	N
082:		70.000
083:	MSG	w
084:		470.00
085:	ENTR	
086:		470.00
087:	MSG	E
088:	MSG	N
089:		700.00
090:	MSG	c
091:	ABS	
092:	MSG	c/
093:	CALL	F
094:	RCL	001
095:	CALL	FIL
096:	Rv	
097:	rtn	

## 8.4.2 Sampling and Calculating Pigment Content of Samples

```

000:  Strt
001:  lbl  LOOP
002:  MSG  cINS
003:  MSG  ERT
004:  MSG  SAMP
005:  MSG  LE
006:  R/S
007:  CALL FILL
008:      470.00
009:  LMDA
010:  READ
011:  STO 000
012:  CALL FOUT
013:      8.0000
014:  CALL BLNK
015:      647.00
016:  LMDA
017:  READ
018:  STO 001
019:  CALL FOUT
020:      8.0000
021:  CALL BLNK
022:      663.00
023:  LMDA
024:  READ
025:  STO 002
026:  CALL FOUT
027:      8.0000
028:  CALL BLNK
029:  RCL 001
030:      2.7900
031:  *
032:  STO 003
033:  RCL 002
034:      12.250
035:  *
036:  RCL 003
037:  -
038:  STO 004
039:  CALL FOUT
040:      6.0000
041:  CALL BLNK
042:  RCL 002
043:      5.1000
044:  *
045:  STO 005
046:  RCL 001
047:      21.500
048:  *
049:  RCL 005
050:  -
051:  STO 006
052:  CALL FOUT
053:      7.0000
054:  CALL BLNK
055:  RCL 006
056:      85.020
057:  *
058:  STO 007
059:  RCL 004
060:      1.8200
061:  *
062:  STO 008
063:  RCL 000
064:      1000.0
065:  *
066:  RCL 007
067:  -
068:  RCL 008
069:  -
070:      198.00
071:  /
072:  STO 009
073:  CALL FOUT
074:      7.0000
075:  CALL BLNK
076:  MSG  c
077:  prt
078:  GOTO LOOP
079:  rtn
080:  rtn
081:  rtn
082:  rtn
083:  rtn
084:  rtn
085:  rtn
086:  rtn
087:  rtn
088:  rtn
089:  rtn
090:  rtn
091:  rtn
092:  rtn
093:      5.2347E-39
094:  FILL
095:      1.7510E-30
096:  rtn

```

## 8.5 EQUIPMENT LISTS AND DATA TABLES

The following equipment lists and data sheets are recommended for use with the *Myriophyllum sibiricum* axenic bioassay.

---

Table 100: The reusable equipment required to execute a *Myriophyllum sibiricum* bioassay.

- adjustable environmental cabinets
- Barnstead NANOpure II ultrapure water purification system, Millipore Super - Q water purification system (or equivalent)
- Laminar Airflow Cabinet
- cold room (4 0C)
- burner and gas source
- autoclave
- light meter
- pH, conductivity and dissolved oxygen meter
- analytical balance and weighing spatula
- glass Petri plates (20 x 150 mm)
- scalpel handles (No. 3 or equivalent)
- extra long stainless steel forceps (250 mm)
- fine dissecting forceps (115 mm)
- 25 mL graduated pipettes
- 1 mL volumetric pipettes
- volumetric flasks: 10, 50, 100, 200, 500, and 1000 mL capacities
- glass graduated cylinders: 100 and 250 mL capacities
- 500 mL Erlenmyer flasks
- 2800 mL Fernbach flasks or 4L Erlenmyer flasks
- round bottomed test tubes (25 x 150 mm)
- flat bottomed test tubes (25 x 100 mm)
- test tube closures: clear plastic (I.D. = 25 mm, 37 mm in height)
  - plain
  - with a 35 mm section of tygon tubing (I.D. = 7 mm, O.D. = 10 mm) glued with epoxy into the inside centre of the cover
- test tube racks (29 x 12 x 9 cm with 40 spaces)
- 1 L amber bottles
- wash bottles
- flat 15 cm ruler
- magnetic stirrer and stirring bars
- hot plate

- flat bottomed enamel container
- callipers
- Westergren blood sedimentation tubes cut into 150 mm lengths

Optional

- photocopier
  - black construction paper circle of a known area
- 

---

Table 101: Consumable equipment required to execute a *Myriophyllum sibiricum* bioassay.

- sterile stainless steel scalpel blades (No. 10)
- aluminium foil
- glass Pasteur pipettes
- Turface®
- 20 mL Borosilicate scintillation vials
- sterile disposable Petri plates (15 x 100 mm)
- epoxy glue
- weighing boats and weighing paper
- storage envelopes (64 x 109 mm)
- parafilm
- marking pens
- label tape

Optional:

- photocopier acetates
-

---

**Table 102: Reagents required to execute the *Myriophyllum sibiricum* toxicity test.**

- reagent water:
    - Barnstead NANOpure II ultrapure water, Millipore Super Q water or equivalent water (e.g., must be free of ions, organic molecules, particles, and microorganisms greater than 0.45  $\mu\text{m}$  diameter)
  - technical chemicals to be used in all tests
  - reference toxicant(s)
  - cleaning reagents:
    - commercially available detergent, hydrochloric acid, and acetone
  - absolute ethanol
  - reagent alcohol
  - certified buffer solutions of pH 4,7, and 10 for calibration of the pH meter
  - certified conductivity standard solution (1413 TS) for calibration of the conductivity meter
  - certified zero oxygen solution for calibration of the D.O. meter
  - stock nutrient solutions
  - sucrose
  - Trypticase Soy Agar (TSA)
  - sodium hypochlorite
  - Tween-20<sup>®</sup>
  - inoculum of *M. sibiricum* from stock culture that is 10 to 12 days old and at least 3 cm in length
-

Table 103: A sample *Myriophyllum sibiricum* growth inhibition toxicity test worksheet.

Test Chemical: \_\_\_\_\_

Date Test Begun: \_\_\_\_\_ Completion Date: \_\_\_\_\_

(Every second day, record length in mm and number of nodes per plant. Make observations on the production of roots and branches)

Treatment	Rep	Day 0 0 h	Day 2 48 h	Day 4 96 h	Day 6 144 h	Day 8 192 h	Day 10 240 h	Day 12 288 h	Day 14 336 h
Control	1	length (mm)							
	2								
	3								
	4								
	5								
Chemical Rate 1	1								
	2								
	3								
	4								
	5								
Chemical Rate 2	1								
	2								
	3								
	4								
	5								
Chemical Rate 3	1								
	2								
	3								
	4								
	5								
Chemical Rate 4	1								
	2								
	3								
	4								
	5								

Table 104: A sample worksheet containing all the possible endpoints for a *Myriophyllum sibiricum* growth inhibition toxicity test.

Test Chemical: \_\_\_\_\_

Date Test Begun: \_\_\_\_\_ Completion Date: \_\_\_\_\_

Observations: \_\_\_\_\_

Treatment	Rep	Area under the growth curve	Calliper Length (mm)	Root Number	Total Root Length (mm)	D.O. (%)	Total Fresh Weight (mg)	Chloro a Content	Chloro b Content	Carotenoid Content	Membrane Integrity	Plant area (cm <sup>2</sup> )
Control	1											
	2											
	3											
	4											
	5											
Chemical Rate 1	1											
	2											
	3											
	4											
	5											
Chemical Rate 2	1											
	2											
	3											
	4											
	5											

## 8.6 DRAFT OF THE AMERICAN SOCIETY FOR TESTING AND MATERIALS PROTOCOL

The following document has been submitted to the American Society for Testing and Materials (ASTM) for publication in the Annual Book of ASTM Standards. This document was submitted for comments to the Subcommittee E47.11 (Plant Toxicology) and then balloted at the subcommittee level. It was then balloted concurrently at the subcommittee and main committee level (E47 - Biological Effects and Environmental Fate). During the latest ballot (April, 1997), the document received 4 negatives, 3 comments, 50 affirmatives and 190 abstentions. One of the negatives was withdrawn. The following version contains the corrections recommended by the voters in the latest ballot. Following ASTM recommendations, any text that is to be removed from the document is marked with ~~strikeout~~ and any new text is double underlined. This version will be balloted to the subcommittee, main committee and all of ASTM in August 1997 and the results will be announced in November 1997 at the ASTM E47 meeting in San Francisco.

Citation of this document is not allowed without the approval of the Chairperson of Committee E47.11 (Nancy Lane) and the President of the Society (ASTM, 100 Barr Harbor Drive West, Conshohocken, PA, U.S.A. 19428-2959). Please refer to the latest edition of Vol. 11.05 of the Annual Book of ASTM Standards to determine if this document has been published.

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~~Test~~ Guide for Conducting Static, Axenic, 14-day Phytotoxicity Tests in Test Tubes with the Submersed ~~Submerged~~ Aquatic Macrophyte, Myriophyllum sibiricum Komarov<sup>1,2</sup>

ASTM Designation: E

## 1. Scope

1.1 Submersed ~~Submerged~~ rooted aquatic macrophytes are important components of aquatic systems. They contribute to primary productivity, improve water quality, cycle nutrients, generate oxygen, affect flow patterns, provide habitat and food for other organisms, and stabilize the sediment. These plants can be adversely affected when pesticides are sprayed to control aquatic weeds and algal blooms or when

---

<sup>1</sup> This guide is under the jurisdiction of ASTM committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.11 on Plant Toxicology.

<sup>2</sup> A standard guide is a document, developed using the consensus mechanisms of ASTM, that provides guidance for the selection of procedures to accomplish a specific test but which does not stipulate specific procedures.

phytotoxic chemicals enter the waterway through atmospheric fallout, soil erosion, industrial effluent, sewage discharge, spills or drift from aerial or ground applications.

1.2 This ~~test~~ guide is designed to give guidance for assessing the potential phytotoxicity of a test material added to a sterile liquid growth medium on a certain species of freshwater submersed ~~submerged~~ macrophyte (*Myriophyllum sibiricum* Komarov) during a 14-day static exposure. A sterile system is recommended to determine the direct effect of the test chemical upon individual parameters of the submersed ~~submerged~~ macrophyte. Overall environmental impact can not be directly determined. These procedures could possibly be useful for conducting toxicity tests with other species of submersed ~~submerged~~ macrophytes, although modifications might be necessary (1, 2, 3,4, 5)<sup>3</sup>.

1.3 The procedures in this ~~test~~ guide are applicable to most chemicals, either individually or in formulations, commercial products, or known mixtures. These procedures might be used to conduct tests for dependency on temperature, light, nutrients and pH. With appropriate modification, these procedures might be used to conduct tests for contaminated surface waters and aqueous effluents (see Guide E 1192). This static, axenic toxicity test might not be applicable to materials that contain microorganisms unless the sample can be filter sterilized without removing the toxicant. If the test materials are highly volatile, care should be taken to ensure that the test chambers are isolated. It might be necessary to replace the test material on a regular basis if the test material is rapidly biologically or chemically transformed in aqueous solution, or is removed from the test solutions in substantial quantities by the test chambers or organisms during the test. This toxicity test bioassay is not suitable for testing interactions between aquatic plants and other organisms, such as plant pathogens.

1.4 Results from the toxicity test outlined in this ~~test~~ guide can be reported in terms of a 14-day IC25, IC50 or NOEC. This parameter may be based on several endpoints including inhibition of plant growth during the 14-day period, inhibition of shoot length, inhibition of root number and length, inhibition of fresh or dry weight (see Guide E 1415), inhibition of oxygen production, change in membrane permeability, and change

---

<sup>3</sup> The boldface numbers in parentheses refer to the list of references at the end of this guide ~~test method~~.

in chlorophyll *a*, chlorophyll *b* and carotenoid content extracted from sections of the plants (see Practice D 3731 and Guide E 1218) (6, 7, 8, 9, 10, 11, 12). All or some of these endpoint parameters may be examined depending upon the mode of phytotoxic action or researcher preference. It might be necessary to conduct the toxicity test at only one concentration to determine whether or not that specific concentration is inhibitory to plant growth and development.

1.5 This test guide is arranged as follows:

	Section
Referenced Documents	2
Terminology	3
Standard Terminology	3.1
Terminology Specific to this Document	3.2
Summary of Test Guide	4
Significance and Use	5
Interferences	6
Hazards	7
Apparatus	8
Facilities	8.1
Test Chambers	8.2
Equipment	8.3
Cleaning	8.4
Acceptability	8.5
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1.6 This test guide does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and to determine the applicability of regulatory limitations prior to use. This standard may involve hazardous materials, operations, and equipment. See Section 7 for specific hazard statements.

## 2. Referenced Documents

### 2.1 ASTM Standards:

D 1129 Standard Definitions of Terms Relating to Water<sup>4</sup>

D 1193 Standard Specification for Reagent Water<sup>4</sup>

D 1871 Test Guide for Conducting Renewal Phytotoxicity Tests with Freshwater  
Emergent Macrophytes<sup>5</sup>

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<sup>4</sup> Annual Book of ASTM Standards, Vol 11.01

- D 3731 Standard Practices for Measurement of Chlorophyll Content of Algae in Surface Waters<sup>5</sup>
- D 3978 Standard Practice for Algal Growth Potential Testing with Selenastrum capricornutum<sup>5</sup>
- E 729 Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians<sup>5</sup>
- E 943 Standard Terminology Relating to Biological Effects and Environmental Fate<sup>5</sup>
- E 1023 Standard Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses<sup>5</sup>
- E 1192 Standard Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians<sup>5</sup>
- E 1218 Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae<sup>5</sup>
- E 1415 Standard Guide for Conducting Static Toxicity Tests with Lemna gibba G3<sup>5</sup>
- E 1598 Standard Practice for Conducting Early Seedling Growth Tests<sup>5</sup>
- E 1733 Standard Guide for the Use of Lighting in Laboratory Testing<sup>5</sup>
- E 1847 Practice for Statistical Analysis of Toxicity Tests Conducted under ASTM<sup>5</sup>
- ~~D 1871 Test Guide for Conducting Renewal Phytotoxicity Tests with Freshwater Emergent Macrophytes~~

### 3. Terminology

#### 3.1 Standard Terminology:

3.1.1 The words must, should, may, can, and might have very specific meanings in this test guide. Must is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified conditions, unless the

purpose of the test requires a different design. Must is only used in connection with factors that directly relate to acceptability of the test (see Section 15). Should is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one should is rarely a serious matter, violation of several will often render the results questionable. Terms such as is desirable, is often desirable, and might be desirable are used in connection with less important factors. May is used to mean is (are) allowed to, can is used to mean is (are) able to, and might is used to mean could possibly. Thus the classic distinction between may and can is preserved, and might is never used as a synonym for either may or can.

3.1.2 ~~3.1.5~~ For definitions of other terms used in this test guide, refer to Terminology D 1129, Guide E 729, Terminology E 943, and Practice E 1598 and Practice E 1847.

~~3.1.2 IC25~~ a statistically or graphically estimated concentration of a test material that under specific test conditions is expected to cause a 25% inhibition in a biological process (such as growth or development) and for which the data are not dichotomous.

~~3.1.3 IC50~~ a statistically or graphically estimated concentration of a test material that under specific test conditions is expected to cause a 50% inhibition in a biological process (such as growth or development) and for which the data are not dichotomous.

~~3.1.4 NOEC~~ the statistically or graphically estimated highest concentration of a test material that under specified test conditions is expected to cause no observable effects on a biological process (such as growth or development).

### 3.2 Terminology Specific to this Document:

3.2.1 apex - the uppermost portion of a plant containing the actively growing tissue or apical meristem.

3.2.2 axenic or sterile - free from other organisms, both active and dormant.

3.2.3 culture - the stock of organisms that is raised under controlled conditions to produce test organisms through asexual reproduction.

3.2.4 submersed submerged macrophyte - a rooted freshwater vascular plant that remains covered with water during the growing season.

3.2.5 toxicity test - a standardized procedure that measures the concentration at which a test material has a defined effect upon the test organism.

3.2.6 turion - an asexual reproductive structure formed on lateral branches in response to lower autumn temperatures. Turions develop into new plants when environmental conditions become favorable (13, 14, 15, 16, 17).

#### 4. Summary of Test Guide

4.1 Axenically cultured apices of Myriophyllum sibiricum are exposed in a static system to a single concentration or a dilution series of a test substance. At the end of a 14-day test period under standardized conditions, growth and development of plants exposed to the test material is compared with the growth and development of plants in an appropriate control. A test substance is considered ~~toxic~~ biologically active when a statistically significant dose-dependent inhibition of plant growth occurs.

4.2 The axenic toxicity testing technique involves exposing the test organism to selected concentrations of the test chemical in individual tubes. Each test tube contains a rooting substrate ~~3 g of Turface®<sup>6</sup>~~ and 40 mL of nutrient medium previously spiked with the test chemical. In this axenic testing system, 3 g of Turface®<sup>6</sup> has proven successful as an artificial rooting medium (6). ~~The Turface® acts as an artificial rooting medium without which the plant's roots push the plant segment upwards. Turface® is the only artificial rooting medium that has proven successful (6).~~ Without a rooting substrate, the plant's roots may push the plant segment upwards and out of the nutrient medium. The use of another rooting medium would need to be validated. A 3 cm apical segment of M. sibiricum is added to the tube. The tubes are incubated (16 h light (fluence rate = 100 - 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 8 h dark at 25 °C during the light and 20 °C during the dark phase) for 14 days, during which time the increase in plant height over time may be measured and growth curves established. On Day 14, other possible test bioassay endpoints that may be measured include final shoot length, root number and

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<sup>6</sup> The Turface® has been found satisfactory for the purpose of an artificial rooting medium but is not endorsed by ASTM. Other non-opaque rooting medium, such as silica sand or glass beads, allow too much light to reach the rooting area and the roots begin to store/produce photosynthetic pigments. Fine grained substrates, such as mineral soil, organic soil, natural sediment, or formulated sediment (18), may reduce light penetration by adhering to the test tube or plant or by remaining suspended in the nutrient medium (6).

total root length, total fresh weight, chlorophyll *a*, chlorophyll *b* and carotenoid content, membrane integrity, and oxygen evolution.

## **5. Significance and Use**

5.1 Protection of aquatic areas is currently being emphasized by several agencies including the U.S. Environmental Protection Agency, the Pest Management Regulatory Agency of Health Canada and Environment Canada. For pesticide registration, these agencies are beginning to require data regarding the toxicity of test chemicals to aquatic vascular plants (19, 20, 21).

5.2 Recently, toxicological research with terrestrial and aquatic vascular plants has been initiated (22) including the development of a protocol for testing with emergent macrophytes (ASTM Guide D 1841) (23). However, protocols for phytotoxicity testing with freshwater submersed ~~submerged~~ plants still require development. Toxicological research has been conducted using submersed ~~submerged~~ macrophytes (1, 2, 3, 4, 5, 24, 25, 26, 27, 28) but standardization of the test-methods is required.

5.3 This test guide is designed to assess the phytotoxic effects of chemicals upon a selected freshwater species of submersed ~~submerged~~ aquatic macrophyte, Myriophyllum sibiricum Komarov. This species is an ecologically important submersed ~~submerged~~ aquatic dicotyledon with a north temperate distribution. It is readily cultured in test tubes in the laboratory (29). Lower temperatures in autumn initiate the formation of turions on lateral branches that develop into new plants when environmental conditions become favorable (13, 14, 15, 16, 17). Toxicological testing with this species has demonstrated that it is an ideal species for laboratory testing since it grows readily under laboratory conditions, the toxic response is reproducible and there is very little variation between experimental replicates (6, 7, 8, 9, 10, 11, 12).

5.4 It is a common practice to use sterile plant culture when testing the direct effects of test materials upon a plant species. Sterile plant culture and toxicity testing have been conducted with algae (ASTM D 3978, 30, 31, 32), floating aquatic macrophytes (ASTM E 1415, 30, 33) and submersed ~~submerged~~ aquatic plant species (2, 3, 4, 5). An axenic testing system is designed to determine the direct effect of the test material upon the test species. There is nothing except the plant within the test system that could degrade or otherwise change the test chemical. Hydrolysis or phytolysis may occur but degradation studies can determine the rate of degradation by

these means. Axenic tests are especially valuable during the initial stages of examining a new compound (e.g., pesticide evaluation and registration (Tier 1 and Tier 2)) (19, 20, 21). In studies with other species of aquatic macrophytes, it has been shown that the presence of filamentous algae can cause a reduction in new shoot growth, fresh weight and chlorophyll *a* content of the macrophytes when compared to macrophytes grown in the absence of algae (34). The test tubes are recommended for testing because they require a small incubation area, small amount of plant tissue, small volume of test material and allow for the maintenance of a sterile culture (2, 3, 29). Furthermore, test tubes permit height measurements *in situ* (29).

5.5 There are numerous possible physiological and morphological endpoints that can be utilized to assess the toxicity of chemicals to this aquatic plant species. The test material effect is assessed as a change in total plant height, growth rate, fresh or dry weight, number and total length of roots, chlorophyll *a*, chlorophyll *b*, carotenoids, membrane integrity or oxygen evolution, or any combination of these parameters. Peroxidase activity might be another endpoint that could be explored (24, 25, 26, 27, 28).

5.6 This toxicity test may be utilized during the pesticide registration process, to provide an early warning of potential ecosystem problems, identify hazardous chemicals before contamination of aquatic systems occurs, and help establish "margins of safety" for specific chemicals within wetlands (see Guide E 1023).

5.7 This test is not designed to replace field assessments of test material damage or other aquatic testing procedures, but should be used as a screening tool. It should compliment other testing so that a more complete environmental assessment is possible. It is difficult to interpret effects observed in the lab in reference to those which could be found in the environment. Currently, there is a need for additional field data to validate the results obtained in laboratory plant toxicity tests. Since this toxicity test bioassay can detect non-lethal physiological endpoints as well as morphological changes, this toxicity test bioassay could act as an early warning system for possible environmental effects. If effects are noted in this toxicity test bioassay, it could indicate that further lab and field testing may be required.

## **6. Interferences**

6.1 Since this test is designed as an axenic testing system method, there is the possibility of microorganism contamination that could render the test results invalid. This microorganism contamination can reduce the nutrient content of the liquid nutrient solution, interfere with light intensity and interact with the test chemical. During a test, contamination (bacterial, fungal or algal) can be assessed by visually examining every test plant within each test system for the presence of a cloudy or fuzzy appearance that could indicate the presence of contamination. This contamination is usually evident within 6 days but definitely by the end of the 14-day test period. To help identify any potential contamination, random sampling of plants and media should be conducted during stock plant transfer and test initiation. Place approximately 1 mL of liquid nutrient solution or an unused plant segment onto an agar plate (e.g. Trypticase Soy Agar (TSA), Potato Dextrose Agar (PDA) or other suitable agar medium). Incubate for a minimum of seven days and microscopically or macroscopically check for bacterial or fungal contamination. Contaminated plants and media should be autoclaved and disposed. They must not be used in a test. Maintaining sterile test conditions ensures that any change in the condition of the test plant is the direct effect of the unaltered test material and is not caused by an interaction between the test material and the contaminating organism.

6.2 Autoclaving may cause precipitation of certain constituents in the liquid growth medium or test material and may change the pH (33). The pH should be monitored after autoclaving and adjusted if necessary. These precipitates are not necessarily irreversible or unavailable as nutrients. Irreversible precipitation renders the growth medium or test material unusable, unless the precipitate and its effect on the test endpoint(s) are known. The test material, medium or constituents that precipitate upon autoclaving may be filter sterilized.

## **7. Hazards**

7.1 It is recommended that the material safety data sheets (MSDS) be reviewed for safety, storage, and disposal precautions for each test substance.

7.2 Many materials can affect humans adversely if precautions are inadequate. Therefore, contact with all test materials and solutions should be minimized by wearing

protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, glasses, and respirators if necessary. Information on toxicity to humans (35, 36, 37, 38, 39), recommended handling procedures (40, 41, 42, 43), and chemical/physical properties of the test material should be studied before a test is started.

7.3 Dispose stock solutions, test solutions, test organisms and artificial substrate in a manner appropriate to the test material. Health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

7.4 Cleaning of equipment with a volatile solvent, such as acetone, should be performed only in a well-ventilated area where no smoking, open flame, such as a pilot light, or sparking electrical equipment are present.

## 8. Apparatus

8.1 Facilities - Stock plants should be cultured in and experiments should be conducted in environmentally controlled growth chambers in which light and temperature can be manipulated. Culturing facilities should be isolated from the toxicity testing facilities to minimize the risk of stock culture contamination by volatile chemicals released from test solutions. Light should be provided by either fluorescent or incandescent lights or a mixture of both. If an application of the spectrum of natural sunlight is required, see ASTM Standard Guide E 1733. Minor changes in humidity are not of concern because the plants are within a closed system.

8.2 Test Chambers - All sterile stock and test plants are maintained in borosilicate glass test tubes (15 cm x 2.5 cm in diameter) (Figure 2, Appendix X1). For stock plants, each test tube is covered with a clear plastic test tube closure (I.D. = 25 mm, 38 mm in height). The test tubes are recommended for testing because of the small area required for incubation and the ease in maintaining a sterile culture. The test tube culture also encourages a vertical growth habit that facilitates height measurements during the test run. When the plant is being utilized for experimentation, each test tube contains ~~3g of Turface® (or other artificial a~~ rooting substrate medium). Turface® (3 g) has proven successful in this axenic testing system. When culturing Hydrilla verticillata, a fine sand covered with a Teflon barrier between two layer of agar

has been used as an artificial rooting substrate (3). Fine silica sand, glass beads, mineral soil, organic soil, natural field collected sediment and a formulated sediment (18) are not as effective ~~ineffective~~ for culturing M. sibiricum in this sterile test system (6). After addition of the plant segment, the test chamber is covered with a clear plastic test tube closure (I.D. = 25 mm, 38 mm in height) fitted with a 35 mm section of Tygon®<sup>7</sup> tubing (I.D. = 7 mm, O.D. = 10 mm) (or other nontoxic, nonabsorbent, autoclavable material such as clear polyethylene or other polymer tubing) glued with epoxy into the inside center of the closure. The Tygon® tubing section supports a measuring rod (15 cm section of a Westergren Blood Sedimentation Tube) within the test chamber so that plant height measurements can be made during the 14-day test period. It is important to ensure that neither the Tygon® tubing nor the epoxy glue contact the liquid nutrient solution, the test solution or the plant segment during the testing period.

8.3 Equipment - Some or all of the following equipment will be needed:

8.3.1 Autoclave - To ensure that all dry material and liquid solutions are sterile, they must be autoclaved in an autoclave for 20 min. at 121 °C and  $1.31 \cdot 10^5$  Pa. The liquid cycle should contain a slow exhaust portion. Guide E 1218 recommends microwaving as an acceptable alternative to autoclaving and this technique has been used for the sterilization of soil (44) and phytoplankton culture medium (45). Microwaving times and temperature cycles should be investigated before being used in this test method because microwaving might not acceptably eliminate the microbial population (46).

8.3.2 Laminar Airflow Cabinet - All manipulations of plant material, liquid media and test solutions must be conducted within a sterile environment. A laminar airflow cabinet is most commonly used to maintain sterile conditions. The sterility of the cabinet must be maintained and the filters cleaned on a regular basis. A UV sterilization hood may also be acceptable but the sterility of the work space should be determined prior to experimentation.

8.3.3 Analytical Balance - capable of accurately weighing to 0.01 mg

8.3.4 Erlenmeyer Flasks - borosilicate glass, numerous sizes

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<sup>7</sup> Tygon® tubing has been found satisfactory for the purpose of keeping the measuring rod centered in the tube. It is chemically inert and autoclavable. This product is not endorsed by ASTM.

8.3.5 Fernbach Flasks - borosilicate glass, 2800 mL

8.3.6 Volumetric Flasks - borosilicate glass, various sizes

8.3.7 Pipettes - Eppendorf® or equivalent

8.3.8 Volumetric Pipettes - 1 to 50 mL, graduated. Pipette bulbs and filters - Brinkman Pipette Helper™ or equivalent

8.3.9 pH Meter and calibrating solutions

8.3.10 Conductivity Meter and calibrating solutions

8.3.11 Light Meter

8.3.12 Spectrophotometer

8.4 Cleaning - Test chambers and equipment used to prepare and store growth medium, stock solutions and test solutions must be cleaned thoroughly before and after use. Residues on the glassware can adversely affect the Myriophyllum growth. To remove all trace metals and organics, all reusable glassware (test tubes, Erlenmeyer, Fernbach and volumetric flasks, pipettes, etc.) should be cleaned in warm water with a non-phosphate detergent, triple rinsed with tap water, triple rinsed with deionized water, rinsed with 10% HCl (v/v), rinsed three times with deionized water, rinsed with acetone, and triple rinsed with deionized water. Equipment should be dried, capped with appropriate closures or covered in aluminum foil and autoclaved for 20 min at 121 °C and  $1.31 \cdot 10^5$  Pa.

8.5 Acceptability - To determine the acceptability of new testing facilities, it is desirable to conduct a preliminary growth test, in which plants are grown in test chambers containing growth medium with no added test material. This is to determine before the first toxicity test whether the plants will grow acceptably in the new facilities, whether the growth medium, handling procedures, sterility, etc., are acceptable, whether there are any location effects on growth, and the magnitudes of the within-chamber and between-chamber variances.

## 9. Reagents

9.1 Reagent grade (or better) chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available. Other grades may be used, provided it is first ascertained

that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. The test material should be reagent grade or better unless a test on a formulation, commercial product, or technical-grade material is specifically required.

9.2 References to water shall be understood to mean reagent water as defined by Type 1 A or equivalent, as recommended in Specification D 1193.

## 10. Nutrient Solution

10.1 The nutrient solution is full strength modified Andrew's medium. It is prepared by adding specified amounts of nutrient salts to reagent water. Then, appropriate volumes of these nutrient salt stock solutions are added to reagent water (Appendix X2) (5) to produce the liquid nutrient medium (modified Andrew's medium). This liquid nutrient solution is used for culturing stock plants, for growing the test plants and for preparing the test chemical solutions (29). The nutrient medium must support healthy *M. sibiricum* growth through 14 days without the stock or control test plants showing signs of stress.

10.2 A constant source of reagent water, acceptable to the test organism and available in adequate supply, should be used to make the modified Andrew's medium. The reagent water and nutrient solution must be free of microorganisms after autoclaving.

10.3 Chlorinated or dechlorinated municipal tap water should not be used as the reagent water because it may be toxic to the plants.

10.4 The water source should be analyzed semiannually ~~several times a year~~ (Guide E 729) for physical and chemical factors including metals and other inorganic chemicals, and organic chemicals including pesticides. The concentration of these potentially harmful factors in the reagent water should be below (a) detection limit or (b) the lowest concentration that is adversely toxic to the test species (47).

## 11. Test Material

11.1 General - The test material should be reagent grade or better, unless a specific formulation, commercial product, or technical grade material is under examination. Before a test is initiated, the following information should be obtained about every test material:

11.1.1 Identities and concentrations of major ingredients and major impurities (i.e. ingredients or impurities constituting more than 1% of the material).

11.1.2 Solubility and stability of the test material in the reagent water and nutrient solution.

11.1.3 Stability of the test material if autoclaving or filter sterilization is required.

11.1.4 An estimate of the test material toxicity to the test organism under the conditions of this test-guide. A preliminary range-finding test may be conducted.

11.1.5 Precision and bias of the analytical method at the concentration(s) of test material to be tested.

11.1.6 Estimate of human toxicity and the toxicity to other organisms.

11.1.7 Recommended test material handling procedures (see Section 7).

## 11.2 Test Concentrations:

11.2.1 Chemical concentrations are expressed by weight of the test material per volume of nutrient solution. For each test concentration, the correct amount of test chemical may be added directly to the nutrient solution. A stock test chemical solution made with nutrient solution or a solvent may also be prepared and appropriate aliquots added to the different test dilutions.

11.2.2 The concentration of test material in each treatment should be measured at least at the beginning and end of the test. If the test is modified so that the test solution is renewed during the 14-day test period, the concentration of test material in the original medium and the replacement ~~renewal~~ solution may also be determined. Test solutions may be combined from the different replicates within each treatment.

11.2.3 The number of selected test concentrations should be based upon the study goals (see Section 13). Testing at a single level or multiple concentrations may be conducted. Multiple concentrations allows for the calculation of an IC<sub>25</sub>, IC<sub>50</sub> or NOEC value but a percent inhibition may still be calculated if only one test concentration is used.

11.2.4 If calculation of an IC<sub>25</sub>, IC<sub>50</sub> or NOEC value (see Section 13) is anticipated, the test concentrations should bracket the expected IC<sub>25</sub>, IC<sub>50</sub> or NOEC value. The expected value might be based upon the results of a test on the same or a similar test material with the same or a similar test organism. If there are no literature

values available, then it is desirable to conduct a range-finding test in which the test species is exposed to the control and three to five concentrations of the test material that differ by a factor of ten. As the similarity between the range-finding test and the actual test increases, the more useful will be the information obtained from the range-finding test.

11.2.5 Aquatic macrophytes may be exposed to concentrations of formulated chemicals above the reported water solubility of the chemical so it may be informative to test at these concentrations. ~~The use of concentrations greater than ten times water solubility may be of limited value. With some test materials, it may be found that concentrations above water solubility do not affect survival or growth any more than the concentration at the water solubility limit.~~ A true concentration cannot exist above solubilities and the term "loading" is used. Testing materials at levels above their water solubility presents several difficulties. At loading levels above solubility, test materials exist in a variety of aggregate forms (e.g., particulates, crystals, liquid crystals, etc.). Relatively little is known about the uptake of aggregated compounds into biological membranes and the expression of this toxicity. In fact, toxicity may be due to certain physical effects, such as a reduction in light penetration or interference with nutrient uptake by test material particulates. For materials tested at loadings in excess of solubility, the use of data in risk assessments or for comparison with other test materials is complicated by the lack of knowledge as to whether the effect is due to a physical effect or true toxicity. These difficulties suggest that toxicity testing at loadings above solubility should be discouraged. To ensure that solubility has been achieved in the toxicity test, it may be appropriate to test concentrations up to approximately twice the solubility limit in the nutrient medium. Any observed toxicity above the solubility limit should be clearly identified as such. For test materials of limited aqueous solubility, analytical verification of the solubility under the test conditions can be important.

11.2.6 When the object of a test is to determine (a) the effect of a specific concentration of test material on the growth and development of a test species or (b) whether or not the IC<sub>25</sub>, IC<sub>50</sub> or NOEC value is above or below a specific concentration, only that one concentration (see Section 13.1) and the controls (see Section 11.4) are required.

11.2.7 The pH and conductivity of all the concentrations of the test solution should be measured at the beginning of the test. At the end of each test, pH and conductivity of the solution in each test chamber may be measured. Other physical parameters such as water hardness and salinity may also be measured.

### 11.3 Test Material Stock Solutions:

11.3.1 If the test material has a high water solubility, a test chemical stock solution may be made by dissolving the test material in the nutrient solution. For test materials with low water solubility, a solvent can be used to make a stock solution that can then be aseptically added to the nutrient solution.

11.3.2 If a solvent other than the nutrient solution is necessary, its concentration in the test solution should be kept to a minimum and should be low enough that it does not adversely affect either survival or growth of the test plant. Reagent grade or better organic solvents should be used and their concentration in the test solution should not exceed 0.5 mL/L (48, 49). These limitations do not apply to any ingredients of a mixture, formulation, or commercial product unless an extra amount of solvent is used in stock solution preparation.

11.3.3 When a solvent other than the nutrient solution is used, a solvent control must be employed in the test (see Section 11.4).

11.3.4 If the solvent has an unknown toxicity to the organism, a test using a dilution series of the solvent must be conducted. This will determine if the survival or growth of the test species is affected by the solvent and what concentration of solvent is non-toxic to the test organism. If a solvent test has already been conducted with the same solvent on the same test species using the same reagent water, then the dilution series solvent test does not need to be repeated. Choose another solvent if the solvent test affects the organisms growth or survival. Methanol is non-toxic to M. sibiricum up to a concentration of 0.4% (v/v) (6).

11.3.5 It may be of interest to determine if the chemical and solvent interact at different concentrations. If there is an interaction, this should be taken into account when choosing solvent concentrations (50, 51).

11.3.6 The test material is added to the autoclaved nutrient solution or solvent under sterile conditions (e.g., laminar airflow cabinet or UV sterilization hood). It is not

necessary to autoclave the test material/nutrient solution or solvent mixture. Liquid test materials may be filter sterilized.

#### 11.4 Controls:

11.4.1 If no solvent other than the nutrient solution is used in the test, then only a nutrient solution control must be included in the test.

11.4.2 If a solvent other than water is used, two controls must be included in the test. One control would be the nutrient solution control and the other control would be a nutrient solution/solvent control containing solvent from the same batch used to make the stock solution.

11.4.3 The concentration of solvent in the nutrient solution/solvent control should be equivalent to the concentration used in the test solutions and should be no greater than 0.5 mL/L (48, 49).

11.4.4 The percentage of organisms that show signs of stress, such as necrosis, chlorosis, stem disfigurement, etc., must be 10% or less for each control type (52, 53) (ASTM D 1841).

11.4.5 If the test contains both a nutrient medium control and a nutrient solution/solvent control, the growth and development of the plants in the two controls should be compared using a t-test or a non-parametric test such as the Mann-Whitney U-test. Another method of comparing the control groups would be to perform an analysis of variance including all treatment and control groups followed by an LSD comparison of the control group means. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

11.4.6 If a statistically significant difference in growth or development is detected between the two controls, only the solvent control can be used for meeting the requirements of Section 15 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of Section 15 and as the basis for the calculations.

11.4.7 Two reference toxicants (positive controls) (54) have been tested at this time. Zinc chloride is a more effective reference toxicant than phenol (6). If space and time permit, positive controls may be conducted with each test. Other positive controls may be used after validation in this axenic testing system.

## 12. Test Organism

12.1 Recommended Species - It is recommended that Myriophyllum sibiricum Komarov (northern watermilfoil) be used for testing. M. sibiricum is ecologically important since it provides food and shelter for other organisms (55). This species is readily available from laboratory sources or it can be easily collected from field sites and sterilized. It is easy to culture and can produce new growth within 10 to 12 days. In this test system, asexual reproduction allows the plant to produce numerous experimental plants from a small number of stock plants. Possible sterile sources of stock plants are listed in Appendix X1. This species was previously named M. exalbescens Fernald (13, 55, 56).

12.2 Alternate Species - Other test species may also be tested following this test guide but more research needs to be conducted. Bioassay Toxicity tests with other species of Myriophyllum are currently under development. This bioassay may be modified to screen new chemicals for the control of invasive aquatic plants, such as Myriophyllum spicatum (Eurasian watermilfoil).

### 12.3 Culturing:

12.3.1 If starting from field collected, non-sterile plants, collect M. sibiricum turions in the autumn. Place the turions into a 20-L aquarium containing 5 cm of sterile sediment that is covered with silica sand or Turface® and 18-L of reagent water. Aerate the aquarium and maintain at a temperature of 15 °C and a fluence rate of 200 - 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 16 h per day (see ASTM E 1733 for conversion to other fluence rate units). The plant culture in the aquarium may be maintained as a backup source of plants in case the sterile plant cultures are destroyed by mechanical malfunction in the growth cabinet, contamination, or other reason. The plants grown in the aquarium are not sterile and sterile cultures cannot be maintained in a batch culturing system. To sterilize the culture, plants are removed from the aquarium and rinsed under flowing deionized water for about 0.5 h. Under aseptic conditions in a Laminar Airflow Cabinet, the plants are disinfected for 20 min in a 3% (w/v) sodium hypochlorite solution containing 0.01% Tween®-20 or other suitable surfactant. Agitate the disinfectant and plant material. Segments with several nodes are transferred into sterile culture tubes containing 45 mL of sterilized modified Andrew's medium (Appendix X2) and capped with plain culture tube closures. Only one plant segment is placed into each test

chamber. Laboratory sealant film is used to secure the closure to the culture vessel. Once a sterile culture has been established, plant segments containing several nodes should be transferred to new test chambers containing fresh liquid nutrient media every 10 to 12 days. As demonstrated by culturing on agar plates, the plants must be sterile and remain sterile for 8 weeks before testing can be initiated.

12.3.2 If starting with a sterile culture, all transfers must be conducted using aseptic techniques. The stock plants are segmented so that each section contains several nodes and visible buds. The test species must be cultured for 8 weeks in the new facilities before testing can be initiated. If the plants transferred are going to be utilized for an experiment in 10 to 12 days, they should contain only one visible bud no longer than 1 cm. Each segment is placed into a sterile culture tube containing 45 mL of modified Andrew's medium and covered with a sterile plain culture tube closure. Laboratory sealant film is used to secure the closure and the culture tube. Sterile stock plants are maintained by transferring plant segments containing several nodes and visible buds to new test chambers containing fresh liquid nutrient media every 10 to 12 days. Only one plant segment is placed in each test chamber. Always leave a few sterile plants untransferred to ensure the continuation of sterile plants in case a batch of freshly transferred plants becomes contaminated.

12.3.3 The stock plant tubes should be alternated in test tube racks (12 x 30 cm with 40 spaces) and placed in an environmental chamber set at 16/8 hour photoperiod and a 25/20 °C temperature regime. The temperature is lowered during the dark period to simulate natural conditions in temperate climates. Other temperature regimes may be used if it can be demonstrated that they promote healthy *Myriophyllum* growth. The fluence rate at the base of the test tube rack should be 100 - 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

12.3.4 Ten to 12 days before each experiment, double the number of plants necessary for the experiment should be transferred. This permits the selection of healthy plants of similar size for the experiment while leaving the additional plants for new stock plant creation. Apical shoots are ready for experimental use when they are at least 3 cm in length.

## **13. Procedure**

**13.1 Experimental Design** - Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and number of test chambers per treatment should be based on the purpose of the test and the type of result calculations to be performed (see Section 15). One of the following two types of experimental designs will probably be appropriate in most cases.

**13.1.1** A growth test intended for the calculation of treatment differences (IC25, IC50 or NOEC) based on a measurable endpoint usually consists of one or more controls and a geometric series of five to seven concentrations of test material. In the nutrient solution control and, if necessary, a nutrient/solvent control (see Section 11.4), the plants are exposed to nutrient solution to which no test material has been added. Except for the control(s) and the highest concentration of test material, each test concentration should be at least 50% of the next higher one (**48, 57**), unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5, five to seven properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too low or too high (**48**).

**13.1.2** If it is only necessary to determine whether a specific concentration unacceptably affects growth and development or whether the IC25, IC50 or NOEC is above or below a certain concentration, only that concentration and the control(s) are necessary. However, two additional concentrations at about one-half and two times the concentration of concern are desirable for increased confidence in the results.

**13.1.3** With respect to factors that might affect results within test chambers and the results of the test, all test chambers in the test should be treated similarly. Test chambers are arranged alternately in the test tube racks in up to four rows per rack for a maximum of twenty tubes per rack. Treatments must be randomly assigned to individual spaces and may be randomly reassigned during a test. A randomized block design (with each treatment being present in a block, which may be a row within the test tube racks or a test tube rack) is preferable to a completely randomized design.

**13.1.4** A minimum of five replicate test chambers is recommended for use in each treatment of an experiment. Because of the importance of the control(s) in the

calculation of results, it might be desirable to use more test chambers for the control(s) than for the other treatments.

13.2 Temperature - Tests with M. sibiricum should be conducted at 25 °C during the light period and 20 °C during the dark phase. Temperature should be controlled by placing the test chambers in an environmental chamber. Other temperatures may be used to study the effect of temperature on this species or the effect of temperature on the toxicity of a material to M. sibiricum.

13.3 Illumination - Light should be provided by either fluorescent or incandescent lights or a mixture of both and provide a fluence rate between 100 - 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  when measured at the base of the test chamber (6). The fluence rate at each position in the growth cabinet should be measured and should not differ by more than 15% from the selected fluence rate. Other light intensities and wavelengths, especially that of UV radiation found in sunlight, may be used to examine the effect of light on the toxicity of a test material (ASTM E 1733).

#### 13.4 Beginning the Test:

13.4.1 A large enough batch of nutrient solution should be prepared so that (a) the desired volume can be placed in each control test chamber, (b) the necessary volume of each test solution can be prepared, (c) evaporative loss during autoclaving is accounted for, and (d) all desired analyses can be performed (see Sections 13.6 and 12). Enough test solution of each concentration should be prepared so that the desired volume can be placed in each test chamber and all desired analyses of water quality, test material, etc. can be conducted (see Sections 13.6 and 12).

13.4.2 Uniform, healthy-looking plants should be removed from the stock culture for use in testing. Randomly select 10 to 12 day old shoots that are approximately 3 cm long. Use plants of the same age and from the same source for each experiment. Aseptically, cut 3 cm apical lengths from the stock plants and transfer them into randomly selected autoclaved test tubes containing 40 mL of sterile test medium. Ensure that the cut end of the apex is touching the sterilized Turface® to optimize rooting. Carefully add a 15 cm measuring rod. The top end of the measuring rod is inserted into the 3.5 cm length of Tygon® tubing (or tubing made from another appropriate material).

13.4.3 The test begins when all the test chambers contain an apical segment. Measure the length of each plant using the measuring rod. Length is measured from the cut end of the plant to the top of the apex.

13.4.4 The tubes should be randomized in alternating holes in test tube racks and placed into a growth cabinet maintained under the conditions outlined in Sections 13.2 and 13.3.

13.4.5 The number of roots and branches produced plus the number of nodes may be measured every second day or less frequently. Every second day, the plant length from the cut stem base to the tip of the apex (mm) may be measured. Exclude any leaves that extend above the apical meristem. The initial height of each plant segment may be subtracted from all subsequent plant height measurements. The plant length data (mm) are used to establish a growth curve and area under the growth curve is calculated by:

$$\text{Area under Curve} = \sum_{i=2}^n \frac{IH_{i-1} + IH_i}{2} \cdot (T_i - T_{i-1}) ,$$

where  $IH_i$  is the increase in height from the start of the experiment and  $T$  is the time at each subsequent measurement point, in hours from time zero (19, 29). An advantage to the test tube system described in this test guide is the valuable information obtained from the growth curves. Along with the control growth curve (curve 1), test materials may produce one of five types of growth curves (Figure 1). The test material can have an immediate toxic effect that does not change over time (curve 2). The test material may not inhibit growth but may or may not affect the other parameters examined (curve 3). In some cases, the test chemical may appear to be initially toxic but *Myriophyllum* might metabolize the test chemical and the toxic effect is reduced (curve 4). When there is recovery, the final plant height may not be significantly different from the control plant final height, which emphasises the importance of measuring plant growth during the 14-days. The test chemical can have a delayed toxic reaction wherein toxicity is not displayed until several days after test initiation (curve 5). The last scenario is that plant height may be stimulated but there could be an effect on weight or one of the other endpoint parameters (curve 6). This type of data may be important in examining chemical metabolism or possible plant recovery from the effects of the test chemical.

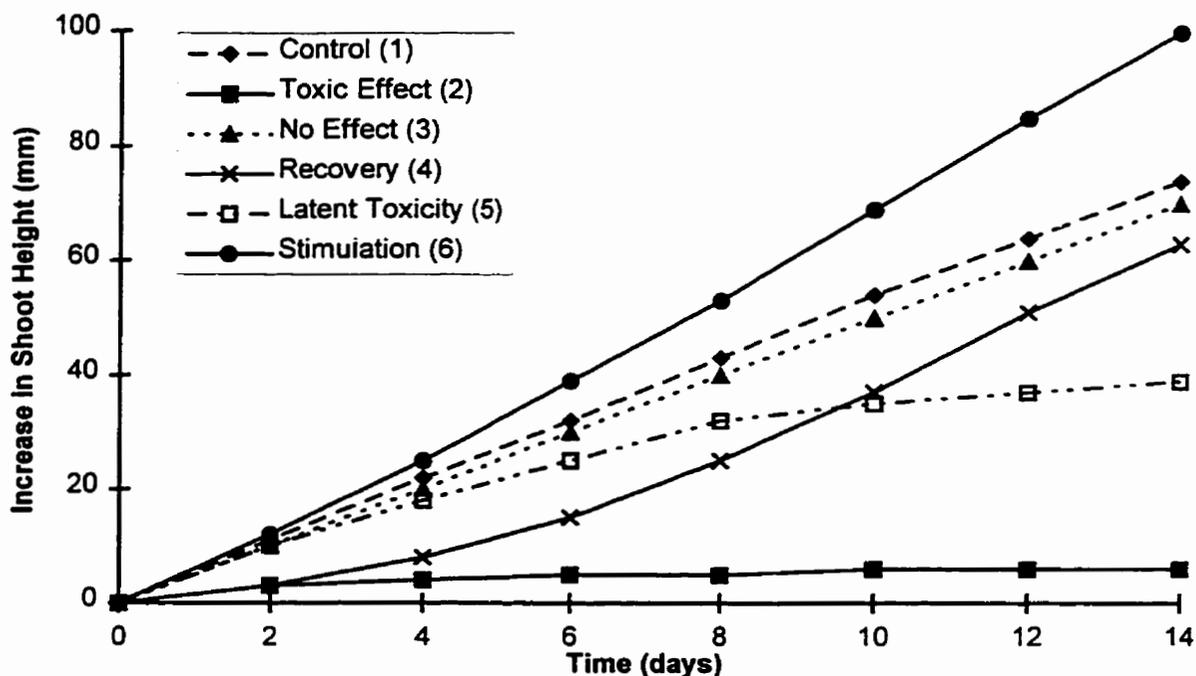


Figure 1: Hypothetical growth curves that may be obtained during a Myriophyllum sibiricum toxicity test bioassay when the plants are exposed for 14 days to test materials with different modes of action.

13.5 Duration of Test - The test ends 14-days after plants are initially placed into the test solutions. A shorter test duration might not be sufficient for toxicity to be demonstrated. A longer duration might allow the plants to adjust to the presence of the test material, produce excess growth that might make enumeration difficult or utilize all the nutrient resources in the medium, thus limiting control growth.

#### 13.6 Evaluation of Test:

13.6.1 Biological Data - Results of the toxicity tests with M. sibiricum should be calculated based on one or more morphological or physiological measurements on the plants in each test chamber (6). The steps listed below are the maximum number of endpoints that have been employed. If after preliminary testing, it is determined that the test chemical has a mode of action that does not affect one of the systems examined, that endpoint can be eliminated. It is recommended that the order of measuring the endpoints be followed but one or numerous endpoints need not be conducted, as determined by the researcher.

13.6.1.1 Visually record the plant length using the mm marks on the Westergren Blood Sedimentation Tube.

13.6.1.2 One at a time, remove the laboratory sealant film and measuring rod from each tube. Measure the D.O. immediately.

13.6.1.3 Remove the plant from the test chamber. Measure the caliper length of the shoot and roots (26), returning the plant to the test chamber to prevent desiccation.

13.6.1.4 Pat each plant dry on paper towels. Measure the fresh weight (4, 26).

13.6.1.5 Using an analytical balance and working quickly, cut off the apex to  $50 \pm 3$  mg. Record the actual fresh weight so that pigment content can be calculated on a weight basis (see Section 13.6.2.2). Place the apex into a glass scintillation vial containing 10 mL of 80% ethanol. Store the scintillation vials in a dark cold room (4 °C) for 24 h. These apices will be used to determine chlorophyll/carotenoid content.

13.6.1.6 To determine membrane permeability, the next  $100 \pm 5$  mg (fresh weight) of the shoot is triple rinsed in reagent water, placed into a flat bottomed tube containing 20 mL of reagent water, loosely covered and left at room temperature for 24 h. In order to avoid excess cellular leakage, this 100 mg sample should consist of only one section (58, 59).

13.6.1.7 The extra portion of the plant is weighed and dried at 80 °C for a minimum of 24 h. Weigh the dried plants.

13.6.2 The 24 h measurements are made 24 h after all the plants are weighed and segmented.

13.6.2.1 Measurement of Chlorophyll/Carotenoid Content of the Apices - After the apices have been soaking in 80% ethanol for 24 h, analyze for pigment content on a spectrophotometer at 470, 647, 663 nm. Calculate values for chlorophyll *a*, chlorophyll *b* and carotenoid content based on either the fresh or dry weight of the apices (ASTM Practice D 3731, 60, 61). Other extraction solvents such as DMSO, acetone and methanol have been used to extract pigments from other plant species (61, 62, 63, 64, 65) but ethanol has been successfully used to extract chlorophyll *a* from algae and terrestrial plants (61, 65, 66). Ethanol without maceration was efficient at extracting the photosynthetic pigments from Myriophyllum (6). As a submersed submerged

macrophyte, the leaves of Myriophyllum have a very thin cuticle (14). The chloroplasts are abundant throughout the epidermal and mesophyll cells of submersed submerged leaves (14, 15) so it is easy to extract the chlorophyll from the apical segments without maceration or other rupturing of the cells. Terrestrial and aquatic emergent leaves have a thick waxy layer and epidermal cells that do not contain chloroplasts (15) so the chlorophyll is much harder to extract.

13.6.2.2 Measurement of Membrane Integrity - Using a conductivity meter, measure the conductivity of the water/plant solution in the flat bottomed tubes. Boil the flat bottomed tubes for 20 min. Remove the tubes from the water. Allow to cool down to room temperature. Measure the conductivity of the solution again (58, 59). Membrane integrity is determined as percentage of total electrolyte leakage:

$$\% \text{ Membrane Leakage} = \frac{\text{Conductance before boiling}}{\text{Conductance after boiling}} \cdot 100.$$

#### 14. Analytical Methodology

14.1 The growth medium, stock solutions, or test solutions, or all three may be analyzed for chemical content at the beginning and end of a test. If these samples cannot be analyzed immediately, they should be handled and stored appropriately (67) to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, sorption, and volatilization. For example, the solutions may be frozen at -20 °C until analysis can be conducted.

14.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources (68).

14.3 The precision and bias of each analytical method used should be determined in the growth medium used. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

#### 15. Calculations

15.1 ~~After~~ Depending on the data to be analyzed and the purpose of the test, a variety of procedures can be used to calculate the results from a test.

15.2 The data may be examined for the presence of outliers and tested for heterogeneity before a randomized complete block analysis of variance (ANOVA) is conducted.

15.3 After the ANOVA, the treatments can be compared to the control using an appropriate mean comparison procedure (e.g., Dunnett's). The highest concentration not significantly different from the control is designated the no-observable-effect concentration (NOEC). The growth rate or the mean percent inhibition actually observed at the NOEC should be calculated.

15.4 If an IC50 is to be determined, first calculate the percent inhibition (% I) for each test chamber in each treatment other than the control(s). Percent inhibition is usually calculated:

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean}} \cdot 100 .$$

On occasion, it may be necessary to use a modified formula to calculate percent inhibition. This is useful for endpoint parameters, such as membrane integrity, where treatment values increase as toxicity increases.

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean} - \text{most toxic value}} \cdot 100 .$$

In situations where 100% inhibition is not equal to zero percent of the control, the following percent inhibition formula can be substituted:

$$\% I = \frac{\text{control mean} - \text{treatment value}}{\text{control mean} - \text{minimum value}} \cdot 100 .$$

The IC50 is then calculated using a regression model. Several statistical programs are available which assist with the analysis of data with a continuous response (69, 70). The type of model and estimation method should be described along with the 95% confidence intervals about the estimates (32).

15.5 If the test consisted of only one test concentration and the control(s), a %I for this concentration may be determined. A t-test or Mann Whitney U-test may be used on the raw or transformed data to determine if the treatment is statistically significantly different from the control(s).

15.6 If the test contains more than one control, such as nutrient solution and nutrient/solvent control, they should be compared and pooled if they are found not to be

significantly different (see Section 11.4). The ANOVA, NOEC or IC50 procedures, described in Sections 15.2 to 15.5, should be used.

15.7 All endpoints may be useful in risk assessment. ~~Based upon the IC25, IC50 and NOEC determined, t~~ Following traditional methods, the endpoint sensitivity may be ranked based upon the IC25, IC50 and NOEC. The mode of phytotoxic action of the test material often determines which endpoint(s) ~~is~~ are the most sensitive. The most consistently sensitive endpoint(s) for each test material is the one that may be used in environmental risk assessments. Currently, it is difficult to interpret effects observed in the lab in reference to those which could be found in the environment. Since this toxicity test bioassay can detect non-lethal physiological endpoints as well as morphological changes, this toxicity test bioassay could act as an early warning system for possible environmental effects. If effects are noted in this toxicity test bioassay, it could indicate that further lab and field testing may be required. The length of exposure to the toxicant would be an important consideration. If a physiological change is observed in the lab (e.g. chlorophyll content), this may be detrimental to a field population with a long term exposure.

## 16. Acceptability of Test

16.1 A test should be considered unacceptable if one or more of the following occurred:

16.1.1 All test chambers and covers were not identical in size, shape and composition.

16.1.2 Treatments were not randomly assigned individual test chamber locations.

16.1.3 A required nutrient medium or nutrient/solvent control was not included in the test or the solvent concentration affected the growth of the species.

16.1.4 The test organism had not been cultured in the nutrient solution and at the same temperature and fluence rate as used in the test for at least 8 weeks prior to the test.

16.1.5 M. sibiricum apices were not randomly assigned to test chambers.

16.1.6 The test lasted less than 14-days. It might be possible to present preliminary information if the test duration is less than 14-days.

16.1.7 Temperature and light were not maintained as specified in Sections 13.2 and 13.3.

16.1.8 At the beginning of the test, variation in apical height between test chambers was more than 6 mm.

16.1.9 Ten percent or more of the control organisms demonstrated some form of stress (chlorosis, necrosis, stem disfigurement) (52, 53).

16.1.10 One or more of the test chambers is contaminated with another organism (i.e. non-sterile conditions).

## **17. Report**

17.1 The record of the results of an acceptable M. sibiricum toxicity test should include the following information, either directly or by reference to available documents:

17.1.1 Name of test and investigator(s), name and location of the laboratory, and dates and times of initiation and termination of the test, plus dates that the stock plant cultures were initiated.

17.1.2 Source of the test material, its lot and CAS number, composition (identities and concentration of major ingredients and major impurities, if applicable), known chemical and physical properties, and whether it is a commercial product, formulation or active ingredient. The identity and concentration(s) of any solvent used.

17.1.3 Source and chemical characteristics (pH, hardness, conductivity, etc.) of the reagent water plus a description of any pre-use analysis to confirm the absence of pesticides, PCB's, toxic metals, etc.

17.1.4 The source, composition and lot number of the Turface® used.

17.1.5 Description of the preparation of the nutrient medium.

17.1.6 Source of the test species, scientific name, name of the person who identified the species and the taxonomic key used, and culture procedures used.

17.1.7 Description of experimental design, test chambers and covers, volume of solution in the chambers, and the average apical height at the beginning of the test.

17.1.8 Average and range of the measured temperature and fluence rate, plus the method of measuring both.

17.1.9 Schedule and methods for preparing test solutions.

17.1.10 Methods and results (with standard deviations or confidence limits) of chemical and physical analyses of water quality and test concentration(s). Include validation studies and reagent blanks.

17.1.11 Methods used for measuring the selected endpoints.

17.1.12 A table giving the endpoint data for each test chamber in each treatment including the control(s), in sufficient detail to allow independent statistical analysis.

17.1.13 Definition(s) of the endpoint(s) used for calculating IC50 and NOEC values. A summary of general observations on other effects.

17.1.14 The IC25, IC50 value and 95% confidence interval, the NOEC value, percent inhibition and the methods used to calculate them.

17.1.15 The most sensitive endpoint for each test material based upon the IC25, IC50 and NOEC.

17.1.16 The statistical procedures and computer programs used should be described in sufficient detail so that the calculations can be repeated. The statistical assumptions of, and the rationale for, the procedures used should be reported.

17.1.17 Any evidence of stimulation found in any treatment.

17.1.18 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

17.1.19 Published procedures should contain enough information to clearly identify the procedures used and the quality of the results.

## **18. Precision and Bias**

The precision and bias for this ~~Test-Guide E XXXX~~ for conducting static, axenic 14-day phytotoxicity tests with the submersed ~~submerged~~ aquatic macrophyte, Myriophyllum sibiricum are currently being determined (6).

## **19. Key Words**

19.1 ~~submerged~~ submersed aquatic macrophyte, phytotoxicity test, Myriophyllum sibiricum, aquatic toxicity testing

## 20. Appendixes

### X1. SOURCE OF MATERIALS<sup>8</sup>

#### Source of Supplies:

Turface® - Aimcor, Applied Industrial Materials Corporation, One Parkway North,  
Suite 400, Deerfield, IL, 60015.

Sigmaware™ Culture Tubes - 150 mm x 25 mm, Catalog # C 5916

Sigma Chemical Company, P.O. Box 14508, St. Louis, MO, 63178.

Other appropriate borosilicate glass culture tubes may be used.

Sigmaware™ Clear Closures for Culture Tubes - Catalog # C 5791

Sigma Chemical Company, P.O. Box 14508, St. Louis, MO, 63178.

Other test tube closures may be used if they are determined to be non-  
toxic and can maintain a sterile environment inside the culture tube.

Westergren Blood Sedimentation Tubes - Catalog # 2 - 676 - 15

Fisher Scientific Supplies.

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<sup>8</sup> These products and suppliers have been found satisfactory for the purposes outlined in this axenic bioassay but ASTM does not endorse them.

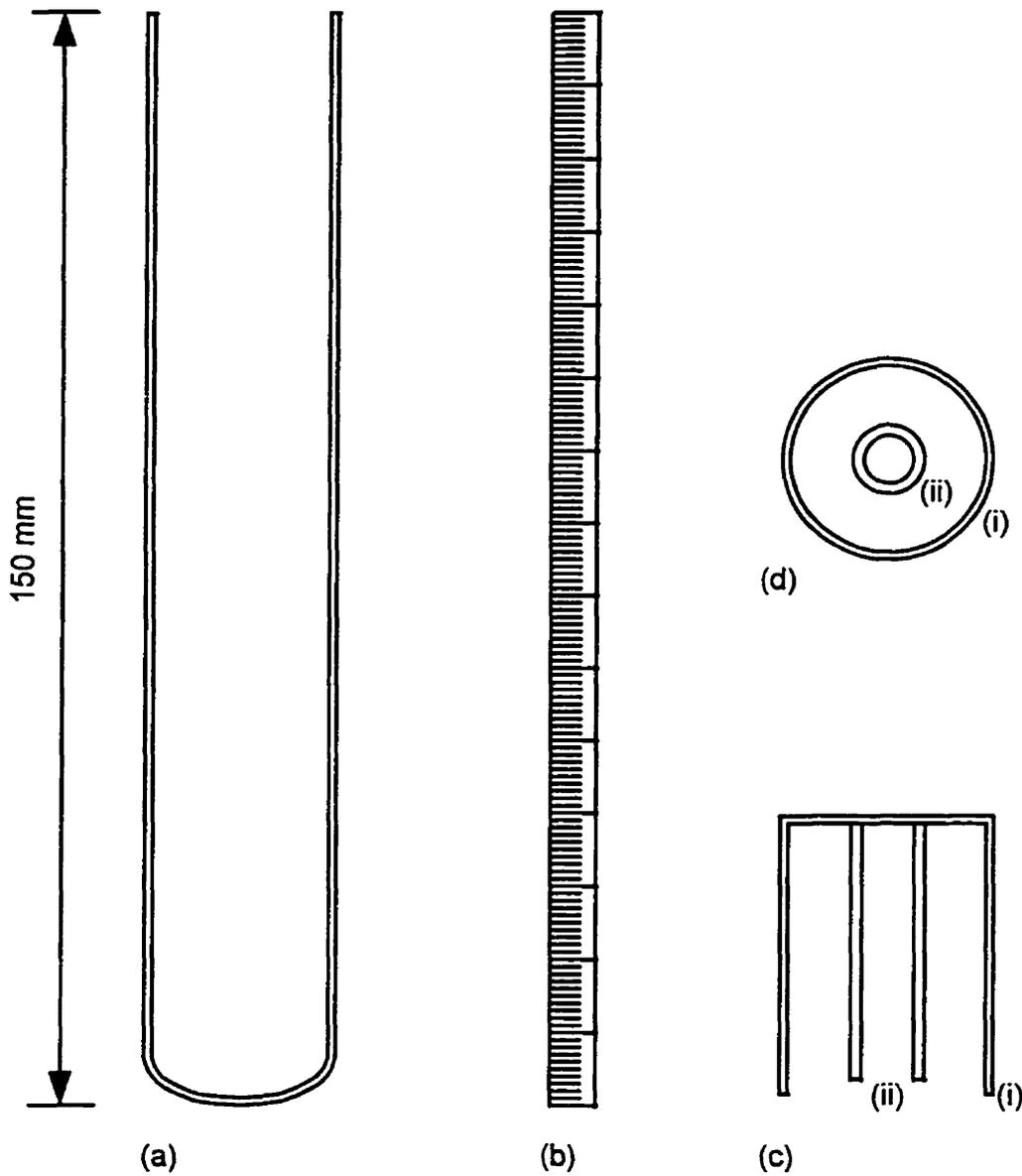


Figure 2: (a) Test chamber

(b) Measuring rod - a 15 cm section of a Westergren Blood Sedimentation Tube

(c) Clear plastic test tube closure (i) with segment of Tygon® tube (ii) (longitudinal view)

At the start of an experiment, a 3 cm apical segment of plant plus a measuring rod are placed inside the test chamber. The tip of the measuring rod fits inside the Tygon® tube as the clear plastic test tube closure is placed on top of the test chamber.

Source of Myriophyllum sibiricum<sup>9</sup>:

In Canada, M. sibiricum is currently being cultured by:

Department of Environmental Biology  
University of Guelph  
Guelph, Ontario  
CANADA, N1G 2W1  
Telephone: (519) 824-4120 ext. 4762  
Facsimile: (519) 837-0442

In the U.S., M. sibiricum is being cultured at:

Analytical Bio-Chemistry Labs, Inc.  
7200 E. ABC Lane  
Columbia, Missouri  
65202, U.S.A.  
Telephone: (314) 474-8579  
Facsimile: (314) 443-9089

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<sup>9</sup> These suppliers have been found satisfactory for the purposes outlined in this axenic bioassay but ASTM does not endorse them.

## X2. NUTRIENT SOLUTION

Modified Andrew's medium (5, 29) for experimental bioassay toxicity tests with Myriophyllum sibiricum.

Solution Number	Salt	Weight of salt per 1 L stock solution	mL stock solution per 2 L final volume
1	KNO <sub>3</sub>	16.16 g	10.0
2	Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	37.76 g	10.0
3	MgSO <sub>4</sub> •7H <sub>2</sub> O	19.72 g	10.0
4	KH <sub>2</sub> PO <sub>4</sub>	5.44 g	10.0
5	KCl	746 mg	2.0
6	H <sub>3</sub> BO <sub>3</sub>	155 mg	2.0
7	MnSO <sub>4</sub> •H <sub>2</sub> O	169 mg	2.0
8	ZnSO <sub>4</sub> •7H <sub>2</sub> O	115 mg	2.0
9	CuSO <sub>4</sub> •5H <sub>2</sub> O	12.5 mg	2.0
10	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> •4H <sub>2</sub> O	3.7 mg	2.0
11	FeEDTA	372 mg Na <sub>2</sub> EDTA 278 mg FeSO <sub>4</sub> •7H <sub>2</sub> O heat to 80°C	20.0

Stock nutrient solutions are made by dissolving the above salts into reagent water. Solution 11 (FeEDTA) is made by dissolving 372 mg Na<sub>2</sub>EDTA in 1000 mL reagent water. Once this is dissolved, add 278 mg FeSO<sub>4</sub>•7H<sub>2</sub>O and heat to approximately 80 °C (5). These stock nutrient solutions may be stored in the dark at 4 °C (31) for a maximum of 6 months. The cold storage room must be free from volatile compounds.

The liquid culture medium is prepared by adding the appropriate volume of each stock nutrient solution to 2L of sterile reagent water. Into each 2 L of liquid nutrient medium, 60 g of sucrose is added (71). Adjust the pH to 5.8 ± 0.1 with 1N KOH or HCl.

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8.7 SAMPLE STANDARD CURVE FOR THE TRICLOPYR GAS CHROMATOGRAPHIC ANALYSIS, TRICLOPYR RECOVERY DATA AND SELECTED GC OUTPUT CURVES

8.7.1 A Sample Standard Curve from the Triclopyr Gas Chromatographic Analysis

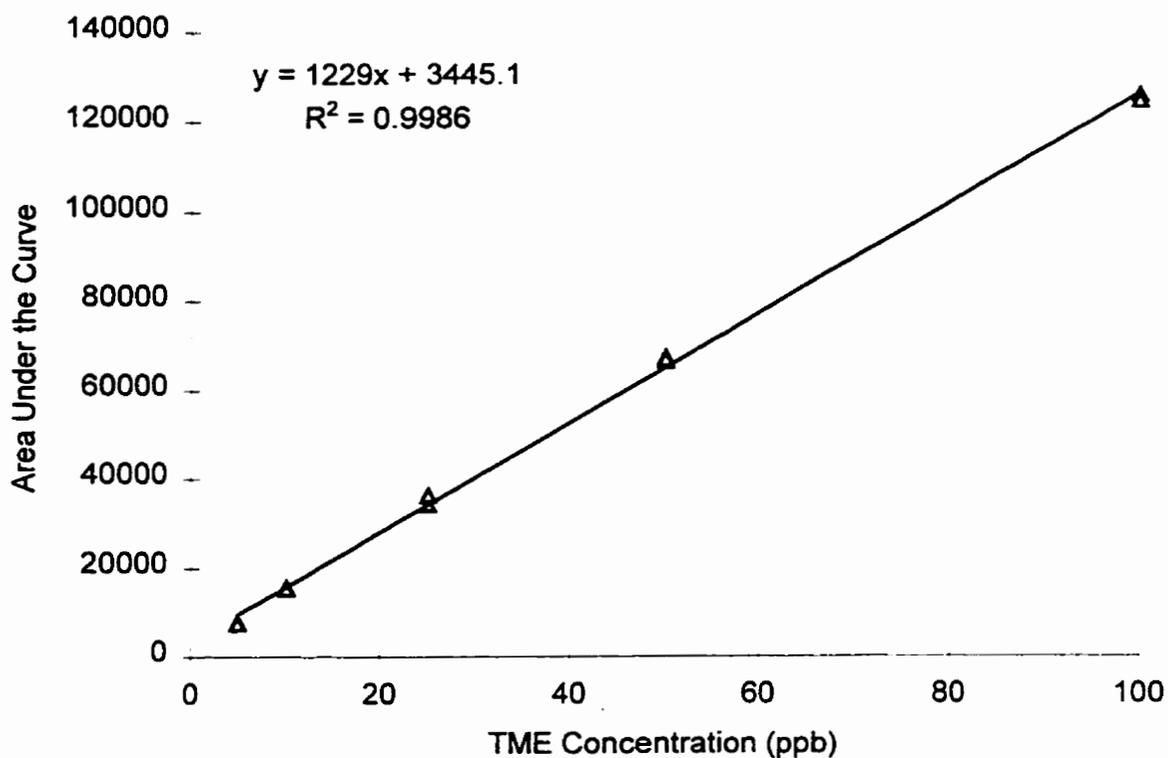


Figure 95: An example of a standard curve for triclopyr methyl ester (TME) recovery determined from autoclaved modified Andrews' medium spiked with triclopyr acid.

### 8.7.2 Triclopyr Recovery Data

Table 105: A sample of the triclopyr recovery data from autoclaved modified Andrews' medium that was spiked with triclopyr acid.							
Sample	Spike	Peak Area	Dilution Factor	TME Concentration	Acid Concentration	% Recovery	Average
control-1a	none	n.d.	1	n.d.	n.d.		
control-1b	none	n.d.	1	n.d.	n.d.		
control-2a	none	n.d.	1	n.d.	n.d.		
control-2b	none	n.d.	1	n.d.	n.d.		
control-3a	none	n.d.	1	n.d.	n.d.		
control-3b	none	n.d.	1	n.d.	n.d.		
9-1	9 ppb	10621	1	6.448579498	6.771008473	75.2334275	74.6810101
9-2A	9 ppb	10515	1	6.360525004	6.678551254	74.206125	
9-2B	9 ppb	10271	1	6.157833527	6.465725204	71.8413912	
9-3	9 ppb	10849	1	6.637979731	6.969878717	77.4430969	
82-1	82 ppb	74993	1	59.9225785	62.91870743	76.730131	78.801962
82-2	82 ppb	81194	1	65.07376641	68.32745473	83.3261643	
82-3a	82 ppb	75593	1	60.42100017	63.44205017	77.3683539	
82-3b	82 ppb	75983	1	60.74497425	63.78222296	77.7831987	
741-1a	741 ppb	61321	10	507.0209337	532.3719804	71.8450716	70.6852267
741-1b	741 ppb	65693	10	543.339259	570.506222	76.991393	
741-2a	741 ppb	58969	10	487.4828045	511.8569447	69.0765108	
741-2b	741 ppb	59206	10	489.45157	513.9241485	69.3554856	
741-3a	741 ppb	60646	10	501.41369	526.4843745	71.0505229	
741-3B	741 ppb	56179	10	464.306197	487.5215069	65.7923761	
6667-1A	6.67 ppm	65233	100	5416.549095	5687.376549	85.3063829	77.1081926
6667-1B	6.67 ppm	65401	100	5430.504901	5702.030146	85.5261759	
6667-2a	6.67 ppm	60092	100	4989.484798	5238.959038	78.5804565	
6667-2b	6.67 ppm	54760	100	4546.554079	4773.881783	71.6046465	
6667-3a	6.67 ppm	53414	100	4434.741485	4656.47856	69.8436862	
6667-3b	6.67 ppm	54900	100	4558.183918	4786.093113	71.7878073	

### 8.7.3 Selected Gas Chromatographic Output Curves

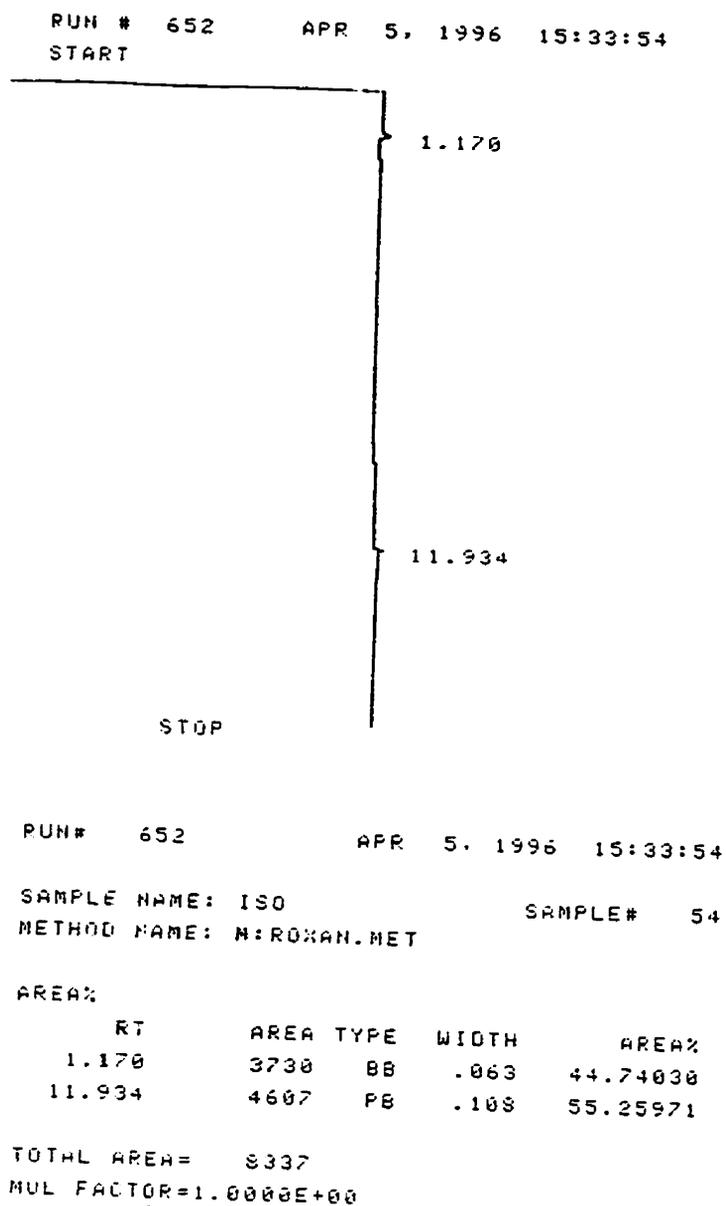
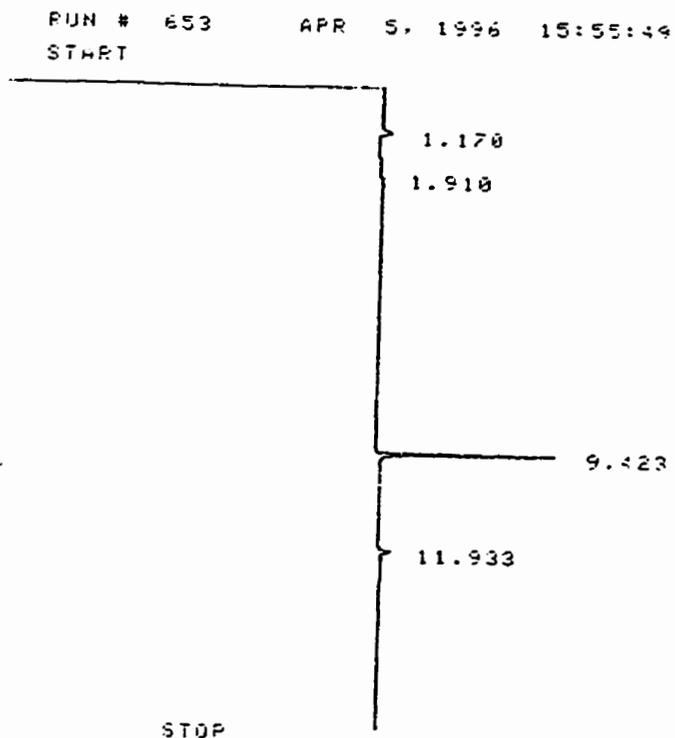


Figure 96: A sample gas chromatographic curve for an iso-octane blank.



RUN# 653      APR 5, 1996    15:55:49

SAMPLE NAME: DZ40A-3      SAMPLE# 55  
 METHOD NAME: M:ROXAN.MET

AREA:

RT	AREA	TYPE	WIDTH	AREA%
1.170	5564	BB	.080	8.25136
1.910	12116	PB	.469	17.97013
9.423	43622	BB	.038	64.69901
11.933	6121	BB	.095	9.07850

TOTAL AREA= 67423  
 MUL FACTOR=1.0000E+00

Figure 97: An example gas chromatographic curve for the EEC of triclopyr (6.67 sample. The sample was diluted before injection.

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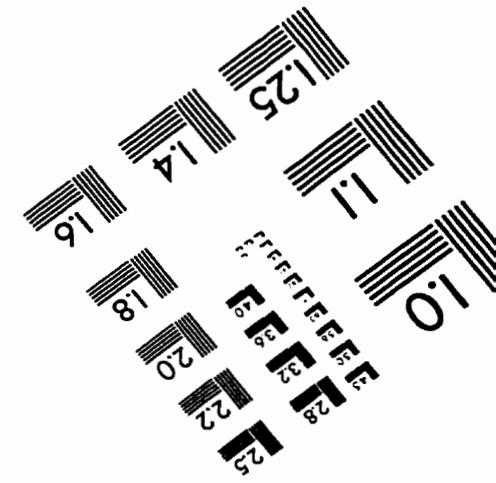
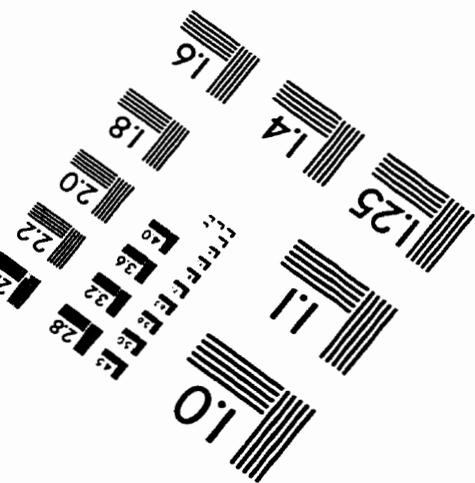
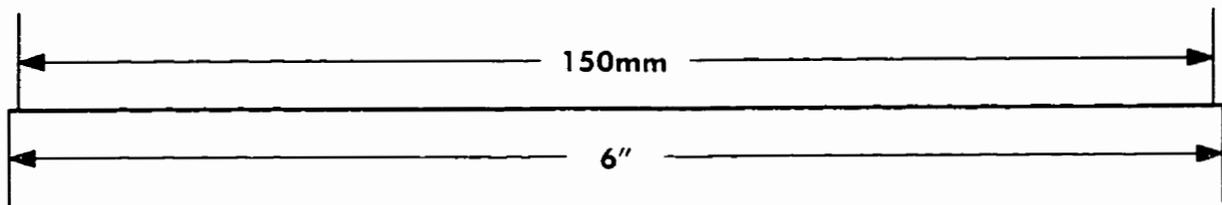
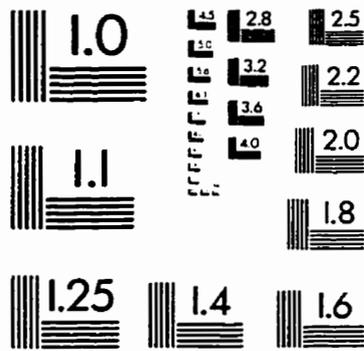
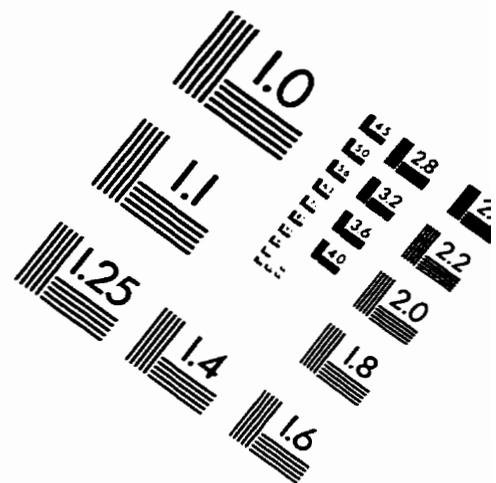
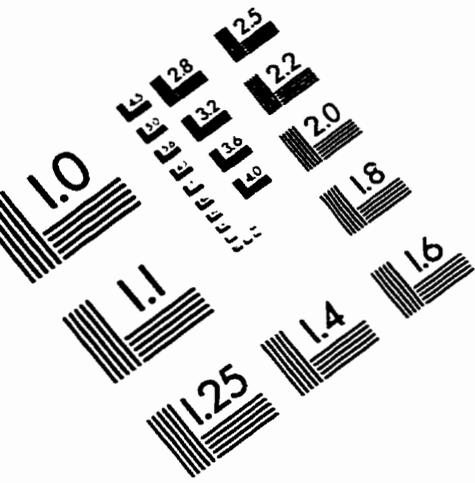
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