Use of the Biolog system to characterize size fractionated components of an estuarine heterotrophic bacterial community

by

E. Scott Moffitt
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ABSTRACT

A review of the present state of microbial ecology with emphasis on the Biolog system of community profiling: conventional topics, fatty acid profiling and nucleic acid techniques are discussed.

Microbial communities from the Cornwallis estuary, Atlantic Canada, (45^0 06'N, 63^0 24'W), were characterized from late June to late September 1995, using Biolog GN community profiles. Communities were separated into three details by either being left whole (crude) or size fractionated by filtration to obtain the attached and free-floating portions.

The continuous variable slope was used for principal component analysis (PCA). Slope is the result of curve fitting the optical density (OD) vs time graphs with the appropriate function for its shape. The PCA appears to indicate a shift in the structural and functional diversity midway through the summer. The June and July samples group in a similar, loose, fashion on all three axes of the PCA plot, with a noticeable shift occurring in the late July samples. The August and September samples are grouped in a tight cluster, indicating a high degree of similarity.

The supporting data are in agreement with these observations. Plots of the mean corrected optical density (optical density minus the A1 control well) show a distinction between the free-floating portion and the others.

The metabolic profiles based on the Biolog system indicate a shift in the functional ability of the community, however they do not indicate if this functional shift is a result of physiological adaptation or a structural shift in community members. Additional data (total counts, total and fecal coliform counts, tritiated thymidine and viable counts) indicates both structural and functional involvements in the shift.

The interpretation of the Biolog profile is ongoing. Determination of structural or functional influences on metabolic potentials are necessary before functional diversity measurements can be facilitated.

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ACKNOWLEDGMENTS

This thesis took longer than expected! After changing my project three times in my first year, I was a little disinterested to say the least. I must thank Colin for his patience during that time and for his words of encouragement-- "just do it" is more accurate--or maybe even something politically incorrect!! I'm glad that he gave me the push, my interest in microbial ecology has certainly been peaked by it. The time spent kayaking was a welcome reprieve from the lab.

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INTRODUCTION

Estuarine environments have long been probed for their microbiological information in order to better understand and to realize the importance of the system. For the most part these studies have not been focusing on the most important aspect of the microenvironment, but have focused on that part which has traditionally been the easiest and most accepted to examine. From a system's perspective these studies are limited in that they do not address the questions from a community or system's point of view; these analyses based on species' perspectives are inappropriate unless attempts to duplicate critical features of the natural environment are attempted (21).

Microbial ecology has been defined as the study of the relationships of microorganisms with their natural environments (21). Studying assemblages of bacteria *in situ* (i.e., natural environments) has been a problem throughout the infancy of microbial ecology mainly because of a lack of methodological support. Until recently the pure culture orientation of microbiologists has been the norm, thus neglecting ecological research. Macroecological observation and research do not befit the small size, limited morphological detail and deficient behavioral differentiation of the microbial world (6). The species concept as a basis for the study of microbial communities becomes a confusing circumstance, as the species detection methods are all highly selective. Thus, any species list utilized will have two characteristics: the list will be (i) incomplete and (ii) biased towards those species with a physiological ability selected for by the method. Most isolation techniques can not distinguish between the viable but non-culturable individuals (101). Therefore if we are to understand the interactions and associations of communities,

we must study them at the community level. Graduation from autecology to synecology is the natural evolution for microbial ecologists.

The emphasis of this research has two main objectives aimed at expanding the understanding of microbial ecology in an estuarine environment.

The first objective was to move away from taxonomic and genetic diversity studies and apply a community based methodology, in order to acquire primary microbiological information about the functional diversity. The Biolog system for community analysis is an attempt to measure functional potentials by getting as close to *in situ* conditions as presently possible.

The second goal was to investigate the characteristics of size fractionated components of an estuarine community based on the evidence gained from the Biolog system. The results of the Biolog assessment were compared to presently accepted evaluations of this type of environment in the hope that this relatively new technique might provide some insight into the functional abilities of each fraction, while at the same time overcome the shortcomings of presently employed conventional methodologies.

The Environment

Estuaries are sites of mixing for large volumes of marine and fresh water, along with the vast amounts of organic and inorganic materials carried by both. The large amounts of substrate entering the estuarine environment makes it a more productive area than oceanic or freshwater habitats. Typical estuaries are areas of highly variable environmental conditions with respect to temperature, organic content, pH, salinity and others.

Efficient nutrient trapping leads to rapid recycling of nutrients with little loss to offshore areas. The base of this recycling is the microbial remineralization of organic carbon derived primarily from phytoplankton. Phytoplankton are usually responsible for the majority of primary production. However, in turbid estuaries, with low phytoplankton production, more reliance is placed on benthic diatoms and vegetative inputs for primary production. The vegetation, with their associated microbiota, will be relied upon to a greater degree as carbon sources.

The Cornwallis Estuary (45^0 06' N, 63^0 24' W) is fed by the relatively small Cornwallis River, which meanders through agricultural land before emptying into the Southern Bight of the Minas Basin. The estuary covers an area of approximately 23 km^2, of which, 80% is intertidal. Surrounding salt marshes consist primarily of *Spartina alterniflora*, and cover approximately 5.5 km^2 (Acadia Centre for Estuarine Research, unpublished data). There are two *Spartina* peak inputs, one in early May due to leaf loss by new growth and a larger one in September due to fall die-off (22). Tides are semi-diurnal with a 4 hour flood period and an 8 hour ebb (22). The water within the estuary behaves as a discrete mass, oscillating with each tidal cycle through the estuary with a net movement towards the Minas Basin. Aerial photos show sharp boundary lines between the estuarine water and the water of the Minas Basin (22). Large tidal height differences, 0.2 m to 15 m, ensure vigorous mixing with currents often exceeding 1 m/s (22, 42). Therefore the water column is homogenous with high levels of suspended particulate

matter (SPM) (> 5000 mg/L reported in the upper estuary). Low levels of SPM (< 10 mg/L) were recorded at or near high slack tide; flows approximate 0 m/s at this time (Acadia Centre for Estuarine Research, unpublished data).

As a result of high SPM loads, the phytoplankton activity within the estuary is low, with no evidence of a phytoplankton bloom. Phytoplankton production rates indicate that the total carbon fixation rarely exceeds 5% of the total. Zooplankton densities are high as compared to other coastal systems. Grazing pressure on the microbial community is high; when the bacterial numbers are lowest, production per bacteria is highest (32), as expected for a grazer controlled system.

Community Analysis: Methods for Study

Conventional Approaches: Outdated Approaches

Pure culture studies involving individual natural environmental isolates and mixed culture studies involving a sample of the natural environment grown under controlled conditions in rich culture media have been discredited as methods for ecological analysis. These types of studies are closer to physiology than ecology (21).

Viable counts may result in colony formation by cells which were dormant in the environment. It is selective against filamentous organisms. Plating is inefficient, it is common for viable counts to be two orders of magnitude lower than direct microscopic counts (21).

Species diversity based on taxonomic differentiation of pure cultures isolated from natural systems are either categorized as species or an OTU (operational taxonomic unit). The species diversity from this type of study will not be representative of the natural community for the same reasons that pure culture/mixed culture studies were discredited.

These types of investigations are inappropriate for the study of microbial ecology because of the intense focus on individuals and the extreme distance that the experimental conditions are away from natural systems.

Direct Methods of Community Analysis

For the most part the studies in this area have been molecular in their approach and I will discus these in some detail. But before I do, I would like to point out a few other techniques that are useful.

Microscopic studies of organisms within their natural environments; the emphasis in this area has been placed on the use of microscopes to reveal important ecological features. Specifically, scanning electron microscope analysis of interactions between microbe/microbe and microbe/environment. Formation of biofilms and the interactions of microbes within them has been a main area of study.

Transmission electron microscopy has been used to a lesser degree but a few studies have been carried out for measurement of dry matter and elemental content within individual bacteria.

Epifluorescent microscopy techniques in order to detect, enumerate and size microbes are becoming more useful. Fluorescent antibody approaches are intended to target specific groups of microbes while autoradiographic methods give an indication of activity (21).

Scanning confocal laser microscopy (SCLM) is a relatively new method aimed at alleviating the problems asociated with traditional light microscopy (depth of focus) and epifluorescent microscopy (autofluorescence). The specimen is scanned with a focused laser beam at a specific plane. A confocal pinhole eliminates fluorescence originating from areas other than the focused plane. The fluorescence is detected by a photomultiplier and images are developed with image analysis computers. This method offers a nondestructive

method for investigating natural environments such as microbial biofilms and the rhizosphere.

This is a brief outline of the more prevalent microscopic approaches. It is not intended to be a critical review but more of a prologue towards community analysis.

Molecular Approaches: A Review

The focus in this section will be on the more predominant areas of interest for community study, the use of biomarkers for structural analysis. In order for a biomarker to be useful in terms of community structural analysis it must have structural diversity coupled with high biological specificity (88).

Fatty Acid Profiles

The fatty acids ester-linked in the polarlipid fraction (phospholipid ester-linked fatty acids or PLFA) of the total lipid allow for the selection required to define the bacterial community structure. Endogenous storage lipids and anthropogenic contaminants are thus avoided because they are found in the neutral and glycolipid fractions of the total lipids (88).

The relatively rapid turnover of phospholipids ensures that the phospholipid represents viable cells. This also enables it to be used as a better measure of the viable cellular biomass than enzyme activity, muramic acid levels and total ATP (10, 89).

The use of PLFA analysis for the archaebacterial component of the bacterial community is not possible for they consist of lipids formed with ether linkages and isoprenoid branching. In order to identify this section of the community it is necessary to use a slightly different protocol intended to quantify the phospholipid-derived ether lipids (PLEL) (74, 89). Fatty acids in the range C12 to C19 are considered to be bacterial in

origin (85, 88). Terrestrial inputs are considered to be C20 to C30, thus making the distinction between bacterial and terrestrial relatively easy (85).

Biomarker fatty acids were used by Findlay (34) to classify microorganisms into four distinct groups based upon the types of fatty acids present.

Group 1: microeukaryotes (polyunsaturated fatty acids).

Group 2: aerobic prokaryotes and eukaryotes (monounsaturated fatty acids).

Group 3: anaerobic bacteria and gram positive prokaryotes (saturated and branched fatty acids ranging from C14 to C16).

Group 4: sulfate-reducing bacteria (SRB) and other anaerobic prokaryotes (branched fatty acids, including the methyl-branching fatty acids ranging from C16 to C19) (9, 74, 89, 90, 99).

Gram negative bacteria have straight chain saturated and unsaturated acids in the range C12 to C19 (85).

Rajendran (90) indicates that the cyclopropyl fatty acids are indicative of SRB and other anaerobic prokaryotes. This is in conflict with Parkes and Taylor (85) who found significant levels of cyclopropyl fatty acids in an aerobic community. They indicated that cyclopropyl acids may be more indicative of aerobic than of anaerobic bacteria. Haack found cyclo fatty acids in some anaerobic species: *Clostridium* spp., some sulfate reducing bacteria, *Vibrio cholerae*, and some members of the family *Enterobacteriaceae* under some physiological conditions. They were also found in some aerobic species: *Pseudomonas*. From this, it would seem that the cyclopropyls are not exclusive to any one group (53).

A similar conflict occurs with the branched chain fatty acids. It has been stated that these are characteristic of gram positive bacteria (34). This appears to be true, but it is not exclusive. Gram negative bacteria have also been shown to contain these fatty acids (*Cytophaga* and *Flavobacterium*) (53). These bacteria are not considered to compose a

significant part of bacterial communities, therefore the large proportion of these fatty acids can be considered to come from gram positive bacteria (61).

Parkes and Taylor (85) also observed the relationship between fatty acid distribution and bacterial respiratory types from sediments by using a multiple chemostat system, in an attempt to obtain bacterial cultures representative of the sediment. The multiple chemostat system would seem to overcome the difficulties associated with the selection pressures of culture conditions as only the input for the first pot must be determined. As all inputs into subsequent chemostats are predetermined by the previous metabolic processes, the multiple chemostat system mimics the natural conditions.

The PLFA analysis gave a good correlation with the *in situ* conditions and enabled them to characterize the respiratory types present. Aerobic, facultative aerobic, facultative anaerobic, anaerobic and SRB populations were noted. The results indicate that the fatty acids present support the groupings devised by Findlay with the exception of the cyclopropyls noted above. It was also concluded that the identification of all of the respiratory types (high species diversity) was indicative of a 'healthy' estuary (85). PLFA profiles which indicate a dominance of one or more of the fatty acid groups can indicate a dominance of the community by that group. This low species diversity is also an indication of possible types of environmental stress (90).

Rajendran (90) studied the community structure of Osaka Bay, Japan, and found areas of the bay that differed in its community structure according to the types of pollution in those corresponding areas. Two distinct clusters were noticed. One contained predominantly branched PLFA (anaerobic bacteria), the other monounsaturated PLFA (aerobic bacteria). Reported levels of pollution were higher in the area dominated by branched PLFA. Chemical oxygen demand was also greater in this area. In an earlier study Rajendran (89) found that the areas with pollution and reduced oxygen availability had reduced numbers of fatty acids indicative of aerobic bacteria and an increase in the levels of anaerobic indicative fatty acids. The reduction of microeukaryotic polyenoic

fatty acids as a direct result of organic contamination was also noted (100). Low levels of polyenoic fatty acids longer than 19 carbon atoms is characteristic of organically polluted areas. This correlates to a low contribution of terrigenous input to the total lipid content (100).

The community structure has been shown to change under conditions of environmental stress (51, 66). The fatty acid biomarker profile of groups and individuals of those groups can also change under these same conditions. During nutrient deprivation *Vibrio cholerae* undergoes a change in the amounts of *cis* and *trans* fatty acids as well as the amounts of cyclopropyls (51). A shift from predominantly *cis*-monoenoic acids in the range C14 to C18 to *trans*-monoenoic occurred as starvation progressed. There was also a subsequent progressive increase in the amounts of cyclopropyls in the range of C15 to C18 (10, 74, 81, 88). The shifts in proportions of these fatty acids could be linked to membrane integrity. There is a preferential loss of *cis*-monoenoic acids under starvation conditions. When this occurs there is a reduction in the membrane fluidity, which reduces the likelihood of long term survival under starvation conditions (51). The proportion of *trans*-monoenoic fatty acids will increase. *Trans* fatty acids are more stable than *cis* fatty acids which may stabilize the membrane. The cyclopropyls are formed by a transmethylation of *cis*-monoenoic fatty acids. This requires an input of ATP, but the result is an acid less likely to be degraded for cell maintenance, as *trans* fatty acids are thought to be unavailable for enzyme degradation (51). This will also stabilize the membrane, maintaining membrane fluidity (86).

The degree of unsaturation plays a role in determining how sensitive a cell is to conditions of nutrient depletion (75). *Escherichia coli* AK7 was provided with two acids with differing degrees of saturation (oleic acid and linoleic acid, both are 18 carbon atoms long) in an attempt to alter the unsaturated fatty acid membrane component, thus altering the fluidity or microviscosity of the membrane. The more saturated acid (linoleic) resulted in an increased level of susceptibility to starvation conditions. In this case a predominantly

unsaturated fatty acid membrane would appear to increase survival probabilities. *E. coli* would fit under Findlay's grouping as being an aerobic prokaryote (34) with monounsaturated fatty acids. Parkes and Taylor's classification of gram-negative bacteria includes characteristic straight chain saturated and unsaturated fatty acids in the C12 to C19 range (85). It may be more valid if a bacterial type were used which does not contain predominantly unsaturated fatty acids, perhaps a group three or group four isolate (branched chain and saturated fatty acids) would give further information to determine if an unsaturated membrane is favorable.

This *cis/trans* and cyclopropyl relationship is still in the theoretical stage and further research is required before a complete understanding is reached.

These *cis/trans* and cyclopropyl changes have been noted in other communities as well (10, 50, 51, 74, 81, 88). This may be a partial explanation for the observations of Haack, Parkes and Taylor and Rajendran (53, 85, 90) who noticed the presence of cyclopropyls in a wide variety of community groups.

Nutrient contamination and physical environmental factors are not the only conditions altering the biomarker signature of a community. Petersen (86) studied soil samples to assess post-sampling factors, the effects of incubation temperature and storage. Storage of the samples for up to six weeks did not alter the profile significantly. Storage preservation is thought to be linked to the overall variation between the collection temperature and the storage temperature. A large difference between the two, theoretically, will cause dramatic changes in the PLFA profile (86). The collection temperature and the storage temperature of the Petersen study were quite similar, thus potentially reducing the amount of variation between the 'new' and 'old' PLFA profiles.

Temperature is the environmental factor most responsible for changes in PLFA composition (86), because of its effect on membrane function. An increase in temperature will increase the membrane fluidity, thus affecting membrane permeability. Once optimal membrane fluidity has been compromised the cell will modify its membrane to

compensate. By modifying the membrane it will change the PLFA profile. Petersen noticed a decrease in the degree of unsaturation, branched fatty acids increased and cyclopropyls increased. It is possible to say that the community may be shifting towards a different dominating group (i.e.,. structural diversity is changing) or that the community is unchanged with respect to structure, thus changes are due to temperature compensating factors (physiological or functional shift) within each cell (86).

When assessing temperature adaptation it is necessary to take the following nutrient influences into account: the temporal pattern is probably influenced, since starved cells would not be able to adapt as quickly as well-fed cells. Different PLFA changes may occur within a given bacterial type, depending on the quality of the carbon source. The inability of some organisms to adapt may lead to cell death and thereby induce successional changes fueled by substrates released from compromised cells (86).

Nucleic Acid Techniques

The diversity of microbial communities based on nucleic acid heterogeneity has proven that most of the microbial diversity within the community is located in the fraction which cannot be cultured (20, 104), justifying the efficacy of a molecular approach.

For extraction and probe targeting, RNA has been favored over DNA for two basic reasons: the RNA content increases intracellularly with increased microbial activity, so the RNA content is dominated by active portions of the community. DNA is more likely to demonstrate random chance probe binding because of its extreme length and diversity (36).

Ribosomal RNA has been selected as the source of nucleic acids because of the high number of ribosomes per cell. It has been shown that a single *E. coli* cell contains between 10^4 and 10^5 ribosomes (2, 44). The rRNA therefore provides a naturally amplified source of nucleic acids available for hybridization probes. For the most part 16S

rRNA has been the preferred choice because the molecule evolves slowly enough that it contains conserved regions which are common to all phylogenetic groups but also contains regions with enough variability that distinctions can be made between closely related groups (36, 77, 115). Analysis of the variable regions can give indications of the phylogenetic relationships among most organisms, the questionable relationships being among highly related organisms.

Nucleic Acid Techniques: Hybridization Probes

Hybridization probes have been developed to accurately identify bacterial types, mainly because they pose a public health risk and must be quickly and easily identified from a community environment. Wang (109) was able to develop a 16S rRNA probe that is specific for the detection of *Listeria monocytogenes*. Other *Listeria* species were tested for detection but all were negative.

The specificity is good if you are trying to isolate or identify one component of a community, but in order to understand the community structure a broader approach is necessary. Probes designed to identify groups of organisms which are evolutionarily related instead of individual bacterial types are more appropriate. Related organisms are expected to have characteristics in common. Giovannoni (44) designed a phylogenetic group-specific probe to distinguish the archaebacteria, eubacteria, and eukaryotes. By polymerase chain reaction (PCR) amplification and comparison of the complete 16S rRNA sequences of members of these three groups it was noticed that members of each group had sequences which were the same. These sequences were different when compared group to group. Designing the probe around these sequences clearly distinguished one group from another.

Group-specific 16S rRNA hybridization probes were developed and used by Raskin (92) to describe natural communities of methanogens. Raskin was able to identify the presence of the methanogenic population within the community. Raskin used this

technique to analyze the microbial community structure of communities growing under anaerobic conditions in chemostats (91). The 16S rRNA probe made it possible to follow changes in the community profile as environmental conditions changed. The chemostats initially used glucose as a carbon source. This was changed so that acetate was the only available carbon source. Raskin observed a gradual decline in the amounts of eubacterial 16S rRNA while observing an increase in the archaebacterial component. Raskin was able to follow the change in the methanogenic population as well as follow the structural community shift.

Hybridization techniques are by no means exhausted in this review; many other approaches including fluorescent hybridization probes and autoradiographic probes are common place (3, 4, 13, 18, 20, 26, 27, 36, 43-45, 67, 68, 70, 83, 84, 87, 93, 111).

Ward (110) used an oligonucleotide primer and PCR for a universally conserved region of the 16S rRNA to study a hot spring. By comparing the 16S rRNA sequences to sequences isolated from known microorganisms from similar habitats, Ward discovered sequences which were previously unknown. The unknown sequences represented unculturable microorganisms (61). A study of the lipid cellular components also indicated the presence of undescribed community members (61). Fuhrman (37) found similar previously undescribed groups while studying sites in the Atlantic and Pacific oceans, while Weller (112) noted a similar trend during study of hot spring cyanobacterial mats.

Jensen (62) used a primer and PCR to amplify spacer regions between the 16S and the 23S regions of prokaryotic rRNA. There are conserved regions within the 16S and 23S regions to which the primers anneal. The spacer regions of over 300 strains of bacteria were amplified. Unique elements within the regions allowed for the distinction of all of the strains. Amplification of the 16S-23S ribosomal spacer region would appear to be suitable for the identification of community members.

Nucleic Acid Techniques: Restriction Fragment Length Polymorphism (RFLP)

Genotypic diversity assessment by RFLP analysis is a result of the random distribution of restriction enzyme cleavage sites located within the microbes is under investigation. Genomic DNA is cut with restriction enzymes and electrophoresed on an agarose gel. A labeled piece of DNA is then hybridized to the genomic DNA fragments. The molecular weights of the fragments are then compared to known standards, any differences in banding patterns can be analyzed statistically to determine similarities.

RFLP analysis of amplified 16S rRNA genes was done by Moyer (77, 78) to assess the community structure. A universal primer was used to anneal to conserved regions of the 16S rRNA. After amplification of the region between the conserved areas it was digested with restriction enzymes. The result was a variety of RFLP patterns, each of which was labeled an OTU (operational taxonomic unit), analogous to a bacterial species. A total of 12 OTUs were identified from a hydrothermal vent which would indicate 12 populations within the community. Determination of the more productive OTU is correlated to the amount of amplified 16S rRNA (77), which in turn gives an indication of the physiological status of the community.

DeLong (26) and Britschgi (20) found similar characteristics while studying attached vs. free-floating and marine bacterioplankton, respectively.

The use of 16S rRNA for community analysis comes with a few precautions. There are four main problems with *in situ* hybridization approaches: (1) low cell numbers and less common organisms often mean that there will be no signal; an intense search must be undertaken to find the cells if numbers are low, (2) low signal intensity, usually directly related to (1) and to target accessibility, (3) rRNA content, slowly growing cells have reduced amounts of rRNA which makes detection difficult, (4) hybridization difficulties, usually associated with rapidly growing cells with cell exteriors which limit the diffusion of the probe. Also, structures within the ribosomes (RNA-RNA or RNA-protein complexes) hinder probe hybridization: both result in low intensity levels (5, 20, 26, 27, 72).

These problems can be rectified by using sequence amplification (PCR); low numbers and intensity are therefore eliminated. However, some caution must be taken with this approach as well. There is a possibility of the formation of chimeric rRNA sequences during PCR amplification (72), but guidelines for their avoidance are presently accepted (5).

Nucleic Acid Techniques: Randomly Amplified Polymorphic DNA (RAPD)

Genotypic diversity can also be assessed by RAPDs. Arbitrary primers are used to create DNA fragments from the genomic DNA via PCR. Polymorphisms within the newly created fragments can then be detected by electrophoretic techniques. Direct comparison of patterns will lead to community differentiation. Community studies with this approach have thus far been limited (20, 26).

RAPD patterns have been used for intraspecies discrimination. Czaka (25) used these to discriminate among strains of *Listeria monocytogenes*. By using randomly chosen primers a wider variety of patterns is possible because they are not focused on any one area of the DNA, but on the entire DNA molecule. Strains with identical 16S rRNA patterns would be indistinguishable from one another if only the 16S region were examined. By looking at the entire chromosome it is possible to identify a higher number of discriminating sequences and therefore identify differing strains. This would appear to be useful for the identification of community members but it may be problematic. The RAPD could tell if the community had a wide variety of individuals or groups of individuals (community profile), but it would be difficult to determine the members of that community. Essentially, the RAPD will allow for the differentiation among community profiles but has difficulty in determining which members of the community are responsible for the differences. The RAPD is not targeting the 16S conserved regions, so it has no basis for determining the relatedness of bacterial community members. If the RAPD were

to target the 16S region it would not necessarily increase the discriminatory power. RAPD analysis requires the primer to be about 10 base pairs (bp) long (25), the probability of the primer finding an analogous sequence within the 16S region is low because of the relatively short length of the region (1.5-3.0 kb) (77). The RFLP restriction enzymes target sequences of approximately four bp, improving the chances of finding a match.

Nucleic Acid Techniques: Denaturing Gradient Gel Electrophoresis (DGGE)

Gradient electrophoresis allows for the separation of DNA fragments of equal length based on their characteristic melting properties. DNA is electrophoresed through a linearly increasing gradient of denaturants (urea and formamide @ 60^0 C) within a polyacrylamide gel. As the DNA reaches a point where the denaturant can not be tolerated, the DNA branches or melts into single stranded fragments. Melting is based upon stretches of base pairs with identical melting conditions: the melting domain. These fragments have drastically reduced mobilities and are thus slowed, allowing for differentiation among the DNA samples. Because each species will have DNA with a characteristic melting property, it is possible to fingerprint each variant within the community (80). The DNA of choice is most often the 16S rRNA encoding DNA fragments.

In one of the earliest applications of the technique, Muyzer *et al.* (80) PCR amplified genes coding for 16S rRNA. DGGE analysis indicated a variety of bands, each of which likely corresponds to an individual species (80). By probing (oligonucleotide probe specific for the V3 region of 16S rRNA of SRB) the DGGE profiles of each population, Muyzer *et al.* (80) identified a population of sulfate-reducing bacteria from an environment in which they were previously thought to be unable to survive.

Ferris *et al.* (33) were able to differentiate among cyanobacterial populations from a hot spring. The DGGE profile exhibited essentially identical profiles for regions of the same temperature, whereas regions of differing temperatures had a corresponding variation in the profile. By comparing the profiles to previously described samples it was noted that several novel populations had been characterized.

Other studies have used this technique to follow community shifts with a corresponding shift in the chemocline of a stratified fjord (102); identify bacteria not previously thought to be responsible for the biodeterioration of cultural heritage pieces (94); support the results of previous studies which indicated that bacterial communities from two estuaries were metabolically distinct (79).

Estuarine review: attached and free-floating bacterial dynamics

External controls on bacterial ratios

The preference for attached or free-floating lifestyles within the community will depend solely upon the characteristics of that system. It has been shown that the relationship between being attached or free-floating is directly related to the level of SPM (14, 16, 23, 47, 54, 55, 57, 95, 103, 105). As the level of SPM increases, there is a larger percentage of attached bacteria, presumably because SPM provides zones of enriched organic matter and nutrients (66). Attachment makes it easier to deal with complex carbon sources (12). Attachment to inert particles enhanced the ability of bacteria to utilize organic matter at low concentrations (73).

Seasonal differences are noted to cause shifts in community compositions. Free floating bacterial numbers are affected by long term seasonal changes whereas attached bacterial numbers are affected by short term seasonal changes, such as the SPM variations explained above (47, 82, 113). Free floating numbers covary with temperature and are

directly related to the concentration of dissolved nutrients; free floating numbers correlate with phytoplankton production (14, 105).

Alterations in carbon sources affect the attached : free floating ratio; the numbers of free floating bacteria are regulated by phytoplankton production, zooplankton exudates, sloppy feeding by zooplankters, and egestion of fecal pellets (103, 105). Therefore in a highly turbid estuary, like the Cornwallis Estuary, with low phytoplankton productivity (Acadia Centre for Estuarine Research, unpublished data), there will be more attached bacteria and a greater reliance on zooplankton derived carbon sources.

The quality of SPM as a carbon source may indirectly accentuate seasonal differences with respect to free floating numbers. Estuaries with good quality SPM from salt marshes appear to show more free floating seasonal variation than estuaries with lower quality SPM (more suspended inorganic solids) (47, 113). The hydrolytic activity of attached bacteria will release more dissolved organic compounds from SPM with high initial carbon content than from SPM with poor carbon content. It has been suggested that there is a net release of dissolved organic compounds from organic particles via extracellular polymer hydrolysis (76). These compounds are then available for use by the free floating fraction. This net release of dissolved organic compounds is important when considering that sinking aggregates have been proposed to be an inhospitable habitat for bacteria (8). Also, based upon the low carbon demands of attached bacteria, aggregate decomposition by bacteria would take months or years to turnover the available carbon (8, 29, 65, 98). But as stated by Azam *et al.* (8), the particle environment does support rapid bacterial growth. The extent of the growth will depend upon the size of the particle. Small particles are active as growth sites for a longer duration than large particle because they do not develop areas of low oxygen concentration, decreased pH and increased levels of toxic metals (8). Larger particles will rapidly develop an attached colony until the above factors limit the growth. But these larger particles are still degraded at a relatively fast rate; the longer times (months to years for degradation) are far surpassed. The

explanation for this is the extracellular polymer hydrolysis which is not linked to usage by attached bacteria (8, 76, 98). This uncoupled solubilization of dissolved organic compounds supplies the free floating bacteria with an added carbon supply.

The numbers of attached bacteria are also affected by flushing, sedimentation and grazing (7, 82). The effects of flushing and sedimentation will depend on the flow rates and tidal currents within the estuary.

Grazing is a major source of biomass limitation in turbid estuaries because of the increased number of attached cells. Free floating cells experience less grazing pressure due to their typically small size. Small cells are more advantageous; the mean cell size decreases with grazing. Bacterial numbers and biomass are limited by grazing pressure, but are maintained at a minimum level by small possibly dormant cells (66). There are detritus quality influences on grazing as well. Within organically enriched sediments protozoa may be unable to control microbial activity; bacterial growth rates may be too fast and numbers too high to be impacted (1).

Biological differences between attached and free floating bacterial cells

The activities of each fraction, most often measured by radioisotope incorporation, are not well understood. In an early study, Hanson and Wiebe (54) concluded that most of the heterotrophic activity was associated with SPM and thus the attached fraction. Their findings have been supported by others (12, 59, 64, 65, 82, 96, 97, 105). These studies base their results on incorporation rates or the percent of total radioisotope taken up by that fraction. This is a bit misleading considering that in most of these studies the numbers of attached bacteria were higher than free floating bacteria. In situations where free floating bacteria were more numerous, eutrophic systems characterized by high concentrations of dissolved nutrients, they also incorporated a larger amount of radiolabeled substrate (103). It is apparent now that most of the results are simply a result

of the higher numbers; however, Simon (96) and Unanue *et al* (105) normalized the incorporation to represent incorporation per cell. Their results indicate that the differences between attached and free floating activities were less than if normalization was not undertaken. Attached bacterial cells were still more active in the incorporation of radiolabeled substrates. Unanue noticed substrate dependent variation within the activities; when substrates were changed the attached cells were two to five times as active as free floating cells (105).

It has also been noted that the attached cells were larger than free floating cells (57, 64, 97). Larger cells mean larger volumes, thus incorporation which is normalized for volume/biomass would seem to be a better estimate of activity. Hodson *et al* (57) noted the importance of taking into account the cell as well as the biomass when discussing metabolic activity. They found uptake rates of dissolved ATP (DATP), based on per unit volume, were about the same for attached and free floating cells.

Simply stated the specific growth rate is the production estimate divided by the standing crop biomass (28), and is an indicator of the ability of the bacterial population to replace its biomass (59). Ducklow and Kirchman (30), Simon (97) and Iriberri (59) showed that specific growth rates calculated from production data and biomass data were similar for attached and free floating bacteria. Contradictory results indicating that attached bacteria are more active than free floating have been pointed out by Kirchman and Mitchell (65) and Iriberri *et al* (60); the differences were noted with specific substrates. It has been theorized that the growth rates are similar but attached bacteria require more of these substrates to synthesize extracellular attachment complexes (64). Vandevivere *et al* (107) has demonstrated that cells which attach and subsequently synthesize exopolymers show no changes in specific growth rate, growth stage or limiting nutrient.

Community Characterization: the Biolog System

Functional diversity has received little attention when investigating biodiversity. Taxonomic and genetic diversity have been the focus, but a community level approach may provide greater insight into microbial roles in ecosystems. Translation of the genetic diversity into taxonomic diversity is not well understood; how genetic and taxonomic diversity affects functional diversity or ecosystem properties is less well understood (117).

The Biolog microplate system was originally intended for the identification of bacterial isolates, based on the resultant carbon source utilization profile of the 95 substrates. Oxidization of the substrate leads to reduction of the tetrazolium dye (via NADH), forming a highly insoluble formazan crystal which stains the cell (15). The degree of substrate oxidation is determined colorimetrically at a wavelength of 590 nm. Profiles are then compared to a database within the software in order to identify strains.

Initial Studies:

Garland and Mills (40) inoculated the plate with whole environmental samples in order to assess the functional differences among and within an assortment of microbial communities (aquatic, soil and rhizosphere). Based on the substrate usage pattern from respiring cells, they were able to differentiate among samples from different habitats as well as differentiate among samples from related habitats. Differentiations were concluded to be a result of functional differences, which are ecologically relevant classifications of heterotrophic microbial communities.

Community profiling has been adopted by others as well. Bossio and Scow (17), Gorlenko and Kozhevin (46), Zak *et al* (117) and Wünsche *et al* (116) used the Biolog system to differentiate among soil communities of differing origins or treatments. Winding (114) was able to differentiate between different size fractions of the same soil, possibly indicating the fine scale applicability. Differences in functional abilities of rhizosphere

microbial communities were noted by Ellis *et al.* (31) while Victorio *et al.* (108) was able to differentiate among various types of wastewater treatment facilities.

Garland and Mills (40) noted the influence of inoculum density on the rate of colour development. In an attempt to normalize the data for numbers of microorganisms they developed the average well colour development (AWCD). AWCD is a means of measuring an overall colour development in order to compare among samples. The initial use for the AWCD was to give an indication of the microbial density by taking an early absorbance reading. A high initial reading could possibly indicate a large inoculum density. This appears applicable for short incubation times, but for longer incubation times it was not suggested because of an asymptotic effect with colour development. All of the above mentioned studies did not attempt to correct for inoculum density as Garland and Mills (40) did. Also, as Garland and Mills (40) and Haack *et al* (52) have pointed out, the colour development is not linear. This nonlinearity poses some problems when interpreting fixed-time readings, as used by all of the above studies (with the exception of Haack *et al* (52)). Responses of communities will differ during the incubation time, thus fixed-time readings will not take into account the lag phase, exponential phase or stationary phase exhibited by colour development (52).

Grayston *et al* (48) and Insam *et al* (58) controlled inoculum densities by doing direct microscopic counts and then diluting to ensure that all plates had the same inoculum density. Both of these studies used fixed-time readings, Grayston *et al* (48) followed the procedure of Garland and Mills (40) while Insam *et al* (58) standardized reading times at 12 hour intervals; only substrates which exceeded an average absorption value of 1-1.2 were selected for analyses, excluding less utilized substrates.

Further studies which normalized for inoculum density by AWCD calculation were done by Lehman *et al* (69), Colwell *et al* (24), Garland (38, 39) and Garland and Mills (41). Each of these studies used multiple variable time readings, with AWCD values for each reading time. Garland (39) modified the initial AWCD analysis to include four

arbitrarily set AWCD values (0.25, 0.50, 0.75 and 1.0 absorbance units). The 0.75 value is a mid-point in AWCD colour development used to compare relative rates of pattern development. This estimate of rate is a function of both the lag time and the linear rate of colour development among samples. Distinct patterns were noted at each of the four points in AWCD development, with patterns becoming more distinct at the 0.75 and 1.0 reference points. This study emphasized the influence of inoculum density. Plates with differing rates of colour development had variation in the overall colour development (AWCD). Classification using principal component analysis (PCA) was influenced by the variation in AWCD, thus the classification is based on the density of the inoculum. Garland (39) was able to eliminate the effect by normalizing the data (net absorbance values divided by the AWCD) prior to analysis or by using detrended correspondence analysis (DCA). Variations in the AWCD did not significantly affect the ordination of samples with DCA.

Heuer and Smalla (personal communication, SUBMECO 1996) found that the applied standardization could not correct for differences in initial cell densities. They used a standardization value which was slightly altered from the AWCD. The net absorbance value (OD) was divided by the number of utilized substrates. Nonutilized substrates were wells where the average corrected OD (substrate minus control) over all microplates was less than 0.03 abs. units.

Haack *et al* (52) used a similar approach with respect to multiple time readings, however, inoculum density was not normalized, possibly explaining the lack of similarity among soil sample replicates. However, the pattern of positive and negative results was highly reproducible with model communities when the inoculum density was controlled.

Vahjen *et al* (106) used another variation of the AWCD procedure. Instead of calculating an average color development for the entire plate, they calculated one (mean absorbance value), which included only substrates which had positive values. This value was then used to determine substrate utilization; if the absorbance value of the individual

substrate was greater than the mean absorbance value the substrate was regarded as utilized and vice versa. The utilized or not utilized data were transformed to binary data for analysis. The binary data are limiting in that it loses the ability to incorporate the strength of substrate utilization. They also noted that formazan formation was not dependent upon initial cell concentration; which conflicts with the above mentioned studies, nor was it dependent upon the growth rate. Formazan formation not being correlated with inoculum density is likely the result of the soil samples used. The same type and amounts were used for all plates and known concentrations of added cells were used. Therefore the inoculum density of the samples were inclined to be the same. Growth rate data were based on colony forming units (CFU counts), which may be too selective to give an accurate representation, considering the different environmental conditions between the initial plating and the subsequent plating.

Alternative sources of information

Other means of analyzing the carbon source utilization profiles were implemented by Zak (117) and Vahjen (106): (i) substrate richness: total number of substrates with positive absorbance values after background correction (control well) (again this does not consider nonutilization as being distinguishing) (ii) total plate absorbance: sum of the absorbance of all 95 substrates, is an indicator for the overall sample activity. (iii) substrate diversity or evenness: these describe the impact of the single substrates on the overall profile. Substrate evenness measures the equitability of activities across all substrates. Substrate diversity encompasses substrate richness and evenness.

The nonlinear nature of colour production was noted by Garland and Mills (40) and Haack (52). The substrate oxidation profile exhibits a lag phase, exponential phase and a stationary phase, similar to but not a direct representation of bacterial growth curves. Haack *et al* (52) have shown that the C sources responsible for the differences between

samples vary with reference point time readings (0.25, 0.5, 0.75 and 1.0 AWCD) and have suggested that the kinetic/sigmoidal profiles could be characterized by fitting them with an appropriate function. The resulting parameters could then be analyzed, providing greater analytical power.

Sigmoidal curve equations contain mathematical parameters that describe the various curve characteristics, but these parameters lack biological meaning. Parameters a, b, c, and d represent the maximum asymptote, slope, inflection point, and the minimum asymptote, respectively. These parameters were reworked according to Zweitering *et al.* (118) to get microbiologically relevant parameters; (a) was changed to (A) and represents the stationary growth phase where the slope equals zero; (b) was changed to (μ), the slope, which is represented by a tangent to the inflection point, (c) remained as the inflection point and (d) represents the initial inoculum density. The lag phase (λ) is the x-axis intercept of this tangent. The amount of information derived from one Biolog plate has now been vastly increased; 95 individual curves with, in this case, four microbiologically relevant parameters.

Profile Differences and Understanding their Causation

The results from this method have shown that it can consistently discriminate spatial and temporal gradients (40, 41, 69, 114) and elucidate fine shifts in rhizosphere communities with respect to plant development (38) especially when inoculum densities are controlled or accounted for (39, 52). The methodology seems to be well on its way, but the basis for the differences in community level physiological profiles (CLPP) remains unclear (i.e. what does the profile measure?). Some have used CLPP's as indicators of *in situ* abilities (58) and functional biodiversity (117). These are likely a bit premature considering the selective enrichment which occurs, it is more a measure of functional potential/metabolic potential. The use of functional potentials as a means of assessing

community abilities or ecological relevance are powerful, but the changes in functional potentials must be investigated to determine their cause. The functional potential of the community can be altered by either a structural or functional shift within that community. The significance of changes in the functional potential should therefore be examined with prudence before giving weight to ecological relevance. Garland and Mills (41) determined that differences in colour production for rhizosphere communities with identical functional requirements indicated that the assay reflected changes in community structure. Haack *et al* (52) found that different samples exhibited variation in the pattern of positive and negative responses to the 95 substrates, probably reflecting differences in community composition. Garland *et al* (personal communication, SUBMECO 1996) illustrated this point when they noticed that changes in CLPP were produced only when community structure was varied during rhizosphere inoculation experiments.

Differences in the rate and extent of colour development can not be interpreted with respect to the number of utilizers or to the metabolic potential of the community. The reasoning behind this: (i) inoculum density effects, (ii) different microorganisms oxidize the same substrates to different extents and (iii) substrate oxidation profiles are not summations of the individual profiles of the members (52). The fact that different microorganisms oxidize substrates differentially is important, but at the community level, these individual activities are superficial. The point of community assessment is to look at community function as a result of the interaction of all its members, not the behavior of individuals.

Garland *et al* (personal communication, SUBMECO 1996) have stated that the relative rate of utilization does not appear to provide useful functional information. This conclusion was based on bioreactor studies which were amended with asparagine. After amendment with asparagine, there was no noticeable difference in the response of the Biolog well containing asparagine, even though it represented from 30-50% of the total respired carbon *in situ*. The fact that asparagine utilization did not change with respect to

rate, does not necessarily mean that the functional information is limited. The functional information seems to indicate that there is no difference between the samples. Further carbon source amendment studies should be undertaken to resolve this, as suggested by Garland *et al* (personal communication, SUBMECO 1996).

Concerns

Numerous disadvantages have been forwarded regarding the usefulness of the Biolog system. Most of these are direct criticisms concerning the biological information as it is related to ecosystem relevance and methodological anomalies.

The problems associated with inoculum density have been discussed.

The Biolog system does not ascertain the proportion of the total bacterial community responsible for the utilization patterns. From a community perspective this is not a concern: Haack *et al* (52) have shown that the rate of substrate oxidization is not a function of the number of utilizers nor of their individual activities.

The Biolog system is selective, it only detects activities from bacteria capable of growing or metabolizing in this environment. Presently there is no way around this problem when trying to investigate functional potentials. The effect must be minimized in order to gain useful information; the Biolog system is an attempt to do this.

The Biolog system is an enrichment situation, resulting in the increase of a given type of organism, while minimizing the growth of others. This criticism must be studied further to be fully understood.

The Biolog system is unable to detect rare species contributions (hyperdispersal). Rare community members may be detected by the presence of wells that occasionally give a positive result.

The AWCD/mean absorbance calculations used in the above studies should be standardized and further evaluated for their usefulness. AWCD as a means of normalizing

for inoculum density may be inappropriate. When comparing two plates of differing inoculum densities, the carbon substrate responses will differ as a result. If the differences are more highly linked to abilities than numbers, the AWCD value may be biasing the comparison. For mean absorbance calculations which exclude the use of non-positive or zero scores, it will bias the value in that it does not consider nonutilization of a substrate. This results in lowly oxidized substrates being classed as not utilized and excluded from analyses.

The nonlinear characteristic of substrate oxidation patterns results in variations of the community response depending upon the reading time. The use of multiple reading times in conjunction with curve fitting will provide useful parameters which can avoid problems associated with reading times.

In conclusion it can be said that the problems associated with evaluating the functional potential of microbial communities is an area of opportunity ripe for further study.

This study examined the functional potential of the attached and free floating fractions of a heterotrophic estuarine community by using a relatively new community oriented technique. New information regarding estuarine ecology was produced by examining a range of parameters from the Biolog approach: (I) the slope of each curve, (2) the overall shape of each curve and (3) the corrected optical densities.

GENERAL MATERIALS AND METHODS

Sample Collection

Samples were collected from the Gladys Porter Memorial Bridge which crosses the Cornwallis river estuary (45° 06' N, 63° 24'W) at Port Williams, Nova Scotia, Canada. All samples were taken within a one hour period of high tide, over a period of approximately four months (from June 1995 to September 1995). *In situ* temperature (14-21° C) and salinity (28-29.7 ppt) were taken at the time of collection. Salinity was measured with a hydrometer and a density-salinity chart. Large sample volumes were taken (18-20L) in order to minimize environmental changes; storage in the dark at or near *in situ* temperatures was done for a maximum of 3 hr. All experimental procedures were conducted within this three hour period.

Biolog Microtitre Plate Inoculation and Incubation

Samples were prefiltered in order to remove large detritus by passing the sample through a 120 micron pore size mesh. The sample at this point contains attached and free floating bacteria and has been called the 'crude' sample. Size fractionation to separate the attached and free floating bacteria was done by filtering the crude sample through a 1.0 micron pore size Nuclepore (Nuclepore Canada Inc., Toronto, ON) filter. The 'filtrate' contains the free floaters. The filters were then agitated (Mistral Multimixer, Lab-Line Instruments, Inc. Melrose Park, ILL.) to release the surface materials in an equal volume of filter sterilized water (discussed later). The resuspended fraction contains the attached bacteria.

Samples were diluted 1/5 with filter sterilized estuarine water. A series of preliminary tests showed this to be the best dilution for colour development over the extended reading times (data not shown). Also, the fluctuations in salinity at high tide were minimal (28-29.7 ppt), thus the filter sterilized estuarine water was not adjusted for

salinity. Estuarine water (sampled at high tide) was sterilized by filtration through a 0.2 micron pore size Nuclepore (Nuclepore Canada Inc., Toronto, ON) filter and stored at 4^{0} C until just prior to use. At this point it was warmed to *in situ* temperature. Sterilization of the estuarine water was done the day before samples were to be taken. Biolog GN microtitre plates (Biolog INC., Hayward, CA) were inoculated in duplicate for each of the three fractions. Incubation was at 15^{0} C in a Conviron incubator (Conviron Controlled Equipment Ltd., Winnipeg, Manitoba) for up to 120 hours. Optical density readings were taken at approximate six hour intervals for the 120 hour duration with the Microlog 3N 3.01A Version DE (Biolog INC., Hayward, CA) computer program and a Cambridge Technology, Inc. (Cambridge Technology, Inc., Watertown, MA) series 750 Microplate Reader.

Tritiated Thymidine Incorporation

The methodological approach for this section was a modified version of the Fuhrman and Azam (35) technique. For each of the three treatments (crude, attached, and free-floating), 10 mL of sample was added to a sterile flask. For each of the three samples there were two controls and two tests. The controls were immediately fixed by adding 2 mL of 20% gluteraldehyde. Five µCi of tritiated thymidine (specific activity 7.0×10^{4} - 7.16×10^{4} µCi/mmole) were added and the samples were incubated at 20^{0} C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) for 2 hr. After incubation the test samples were fixed by adding 2 mL of 20% gluteraldehyde to stop thymidine uptake. Samples were cooled on ice for five minutes. An equal volume (12 mL) of ice cold 10% trichloroacetic acid (TCA) (Fisher Chemical/Fisher Scientific, Fairlawn, NJ) was added to extract proteins. Samples were cooled on ice for another 5 minutes before filtering through a 25mm, 0.45 micron pore size cellulose nitrate filter (Micro Filtration Systems, Dublin, CA). Samples were washed three times with 1mL of 5% TCA. Filters were placed in a scintillation vial with 10 mL of scintillation fluor

(Scinti-verse II or ScintiSafe Econo1 LSC Cocktail)(Fisher Chemical/Fisher Scientific, Fairlawn, NJ). The vials were stored for 24 hrs in the dark at 20^0 C to reduce the incidence of random coincidence events (chemiluminescence, bioluminescence, or photoluminescence). The tritiated thymidine uptake was measured with a Beckman LS 5000 TD Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA). Thymidine incorporation was calculated with a conversion factor of 2×10^{18} (Appendix A).

Biological Oxygen Demand

The biological oxygen demand (BOD) of only the crude sample was measured using a modified Winkler (63) approach over a four day period for each sample date. A total of 10 bottles were filled and sealed to prevent the infiltration of external oxygen. The bottles were incubated at 20^0 C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) until needed. Two of the bottles were evaluated 15 minutes after being filled to determine the initial oxygen concentration.

Two mL of magnesium sulfate plus 2 mL of alkaline iodide was added to the two initial time zero bottles. They were shaken and the precipitate was allowed to settle 1/3 of the way down the bottles; this was done twice. After the final settling, 2 mL of concentrated sulfuric acid was added and the bottle shaken until the precipitate completely dissolved. One hundred mL from each bottle was transferred to the flask and one eye dropper full of starch was added to each. The solutions were titrated with phenylarsine oxide (PAO). The volume of PAO necessary for the titration was used to calculate the amount of dissolved oxygen. The remaining 8 bottles were examined in the same manner over 4 days. The oxygen demand for each day was determined by subtracting the daily dissolved oxygen values from the initial time zero sample. The individual sample BOD change was plotted vs time with Sigmaplot and curve fitted (Appendix B) to get the L and K parameters, representing the ultimate BOD value or the amount of oxygen required to

degrade all nutrients and the BOD rate constant or rate of nutrient degradation, respectively.

Total Direct Microscopic Counts (AODC)

The total count, both viable and non-viable was done with the acridine orange direct count technique described by Hobbie *et al.* (56).

Dilutions were necessary to get a countable number of bacteria per field of view. Dilutions ranged from 1/10 to 1/20 for the crude and attached sample. The free-floating sample did not require dilutions. All dilutions were made with filter sterilized estuarine water. Sample were then fixed with gluteraldehyde (final concentration 2%) and stained for 4 minutes with filter sterilized acridine orange (AO) (final concentration 0.01%). Samples were filtered through a 25mm 0.2µm pore size Nuclepore filter stained with irgalan black (Nuclepore Canada Inc., Toronto, ON). The filter was pre-wetted with sterile distilled water to eliminate hydrophobic regions. Filters were placed on a slide with a drop of low fluorescence immersion oil (Fisher Scientific, Fairlawn, NJ) below the filter and on top of the filter. A cover slip sealed the preparation. Counting was done with a Nikon WFX-II fluorescence microscope (Nikon Canada Inc., Mississauga, ON) containing a 420-490nm excitation filter, 510nm dichroic mirror, and a 520nm barrier filter. Filters were first examined for hydrophobic regions and if present, the slide was discarded and the preparation was repeated. The green and red bacteria were counted for a total of 10 randomly chosen fields. The number of cells per mL of sample was determined by averaging the total number of cells counted for the 10 fields of view and entering that value into the formula n=YAd/av (Appendix A).

Coliform Counts

Total coliform counts for each sample and treatment (crude, attached and free-floating) were determined according to standard methods with mEndo broth plates (49).

The plates were made by adding 43.2g of mEndo broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) and 15g of agar (Becton Dickinson Microbiology Systems, Cockeysville, MD). The media was dissolved in 1000mL of distilled water along with 18 mL of 100% ethanol. The solution was heated to 80^{o} C with agitation and then cooled to 50^{o} C and dispensed.

5 mL of sample was used for the crude and attached treatments, 50 mL was used for the free-floating treatment; these volumes were determined by preliminary tests. The volumes were suction filtrated through 47mm diameter, 0.45 μm pore size cellulose filters (Micron Separations Inc., Westboro, MA). The filters were then rinsed with 20 mL of filter sterilized estuarine water. Each filter was placed on the surface of an mEndo broth plate , sealed with parafilm and incubated at 35^{0} C in a Queue incubator (Mandel Scientific Company, Inc.) for 18 to 24 hours. Duplicates were done for all tests. The plates were counted under fluorescent light with the plates at a 30 degree angle. Coliform colonies have a characteristic yellow-red metallic sheen; the total number of these colonies divided by the volume filtered gave the number of total coliforms per mL of sample (Appendix A).

Fecal coliform counts for each sample and treatment (crude, attached and free-floating) were determined according to standard methods with mFC agar plates (49). The plates were made by adding 29g of mFC broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) and 15g of agar (Becton Dickinson Microbiology Systems, Cockeysville, MD). The media was dissolved in 800mL of distilled water along with 8 mL of rosolic acid. The solution was heated to 80^{o} C with agitation and then cooled to 50^{o} C and dispensed.

20 mL of sample was used for the crude and attached treatments, 200 mL was used for the free-floating treatment; these volumes were determined by preliminary tests. Filtration was done as in the total coliform counts. The plates were incubated at 44.5^{o} C in an Isotemp incubator (Fisher Scientific, Fairlawn, NJ) for 18 to 24 hours. Fecal coliform colonies appear blue while all others appear grey. The total number of blue

colonies was divided by the volume filtered to give the number of fecal coliforms per mL of sample (Appendix A).

Plate Counts

A culturable/viable count for each of the three (crude, attached and free-floating) treatments was done with 1\10 tryptic soy agar which was adjusted to 6ppt and 28ppt salinity with Forty Fathoms Biocrystal MarineMix (Marine Enterprises International, Inc., Baltimore, Maryland).

The three undiluted treatments were plated using a Spiral Plater, Model C (Spiral Systems, Inc., Cincinnati, OH). The spiral plater varied the dilution from undiluted to a 1/100 dilution. Duplicate plates were incubated at 20^{o} C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) for 4-5 days. Counting of the Colonies was done as per the manufacture's instructions (Appendix A).

Data Treatment and Statistical Analysis

Each six hour reading was appended to the previous reading and stored in ASCII format. When the sampling time was complete the files were parsed (Appendix C) and reformatted so that each reading time was represented by one row, 96 values long.

The reformatted files, one for each plate, were imported into Sigmaplot For Windows Version 2.01 (Jandel Scientific Corporation, Corte Madera, CA). Corrected optical densities (OD of well A1 subtracted from OD of each of the 95 substrate containing wells; COD) were calculated for each plate.

Using the MEAN.XFM transform (Appendix C) the mean changes in COD for the duplicate plates were calculated. Vertical error bars indicate the variance between the corresponding OD values. The means and ranges were plotted against time and curve fitted with a modified logistic function (Appendix B) within the SigmaPlot program to get a best-fit curve of the data. The curve fit program also provided relevant parameters,

defining the sigmoidal tendency of the optical density curve. Parameters a, b, c, and d represent the maximum asymptote, slope, inflection point, and the minimum asymptote respectively. These parameters were reworked according to Zweitering *et al.* (118) to get microbiologically relevant parameters; (a) was changed to (A) and represents the stationary growth phase where the slope equals zero; (b) was changed to (μ), the slope which is represented by a tangent to the inflection point; (c) remained as the inflection point; (d) represents the initial inoculum density; the lag phase (λ) is the x-axis intercept of this tangent. The PLATES1.XFM transform (Appendix C) calculated both μ and λ .

The OD reading times and the mean OD values were then imported into a new SigmaPlot file. The Biolog file gncolor.24 (Appendix D) was imported as well. This file is a list of the background percentage correction factors for each well; the percentage of the OD reading attributable to the interaction of substrate and tetrazolium dye. From this the PERCHAN.XFM transform (Appendix C) was executed to give the percent change in OD for each well. The AWPC.XFM (Appendix C) transform was then executed to give the average well percent change.

A mean plateau time for each treatment was calculated by examining all wells which resulted in a sigmoidal pattern. For all of these, a line tangent to the slope was drawn. The point at which this line would intersect the maximum asymptote (A) was used to determine the time to reach plateau.

The SigmaPlot files containing the microbiologically relevant parameters were then imported into SPSS (Statistical Package for the Social Sciences Professional Statistics 6.1, SPSS Inc., Chicago, IL) and arranged by month/day/row/well and detail. At this point each row represents one well. Additional columns were added to incorporate the supporting data and relevant supporting factors: asymptote, slope, inflection point, lag phase, y intercept, week, substrate (2-96), AODC, 6ppt and 28ppt salinity plate counts, total coliforms, fecal coliforms, tritiated-thymidine uptake, BOD L, BOD K, temperature, and class. The class was based on the 11 groups of substrates within the Biolog plate as

described by Garland and Mills (40), these include: (1) carbohydrates, (2) esters, (3) polymers, (4) esters, (5) alcohols, (6) amides, (7) phosphorylated chemicals, (8) amino acids, (9) aromatic chemicals, (10) brominated chemicals, and (11) amines. However, the class turned out to be of limited usage because of the complex differences in substrates which were classified within the same groups.

The slope (μ) data were incorporated into a new SPSS file containing the original month/day/row/well and detail variables and the supporting data. Ninety-five new variables were added to facilitate the slope values. The file contains 18 rows (one for each sample) and 116 columns; additional descriptive columns were added throughout the analysis. This file was used within SPSS for Principal Components Analyses (PCA). PCA were used to investigate similarities among the three fractions (crude, attached and free-floating) as well as similarities among the attached fraction and among the free-floating fraction.

SCIENTIFIC PAPER

ABSTRACT

Microbial communities from the Cornwallis estuary, Atlantic Canada, (45^0 06'N, 63^0 24'W), were characterized from late June to late September 1995, using Biolog GN community profiles. Communities were separated into three details by either being left whole (crude) or size fractionated by filtration to obtain the attached and free-floating portions.

The continuous variable, slope, was used for principal component analysis (PCA). Slope is the result of curve fitting the OD vs time graphs with the appropriate function for its shape. The PCA appears to indicate a shift in the structural and functional diversity midway through the summer. The June and July samples group in a similar, loose, fashion on all three axes of the PCA plot, with a noticeable shift occurring in the late July samples. The August and September samples are grouped in a tight cluster, indicating a high degree of similarity.

The supporting data is in agreement with these observations. Plots of the mean corrected optical density (optical density minus the A1 control well) show a distinction between the free-floating portion and the others. The metabolic profiles based on the Biolog system indicate a shift in the functional ability of the community, however it does not indicate if this functional shift is a result of physiological adaptation or a structural shift in community members. Additional data (total counts, total and fecal coliform counts, tritiated thymidine and viable counts) indicates both structural and functional involvements in the shift.

INTRODUCTION

The characterization of heterotrophic estuarine bacterial communities to explore differences between attached and free floating bacteria is an active area of research for microbial ecologists (3, 6, 23). Comprehension of the functional abilities of attached and free floating bacteria is paramount if a better understanding of microbial roles within turbid estuaries is to be gained.

For the most part, research involving attached and free floating bacteria has been limited to radioisotope incorporation (22, 26, 31) or biomass related approaches such as plating (6), ATP (22) and direct counts (AODC (7), DAPI (30)). The relationship between attached and free floating bacteria varies with the suspended particulate matter (SPM) concentration; as the SPM concentrations increase there is a greater proportion of attached bacteria (3, 4, 7, 20, 22, 34). Free floating bacterial numbers exhibit a temporal stability and are affected by only long term seasonal changes (i.e. summer vs. winter) (15, 36). Attached bacterial numbers are more variable, they are affected by short term environmental changes (15, 28, 36). Free floating numbers are directly related to the concentration of dissolved nutrients and correlate with phytoplankton production (3, 34). Zooplankton exudates, sloppy feeding by zooplankters, egestion of fecal pellets, SPM quality, flushing, sedimentation and grazing are other factors that control the attached:free floating ratio (1, 2, 15, 28, 33, 36).

The activities of each fraction appear to vary depending upon the type of analysis. Radioisotope incorporation studies of activity for each fraction have concluded that the

attached fraction is more active (19, 26, 28). The attached bacteria are often more numerous and have a larger average cell volume. On a per cell basis the attached are more active, but on a per unit volume basis there does not seem to be a difference with respect to growth rate (9, 23, 32) or uptake rates (22). In contrast, Kirchman and Mitchell (26) and Iriberri *et al.* (24) have noted differences depending on specific substrates. It has been theorized that the growth rates may be similar, but attached bacteria require more of these substrates to synthesize extracellular attachment complexes (25).

Prior studies have been limited to the above biomass and radiotracer studies, so the functional diversity of microbial systems has received little attention when investigating biodiversity. The Biolog system for community analysis is a quick, inexpensive and novel technique, introduced in 1991 by Garland and Mills (11). They instigated the use of carbon source utilization profiles as a means of comparison for microbial communities from a variety of natural environments (aquatic, soil and rhizosphere). Zak *et al.* (38) and Winding (37) successfully differentiated among soil samples. Colwell and Lehman (8) investigated zones within a basalt aquifer. The applicability of the Biolog community level profile to assess functional potential is becoming widespread (5, 12, 14, 16, 27).

Interpretation of the substrate responses has been the limiting factor thus far. Garland and Mills (11) developed the AWCD (Average Well Colour Development: sum of the OD of the test wells minus the OD of the control well divided by 95, [Σ(test well - control)]/95) value with fixed time readings to alleviate the inoculum density problems. Later they added multiple reading times to overcome the nonlinear nature of colour development within the wells. Variations of the AWCD approach have been applied by

Vahjen *et al.* (35) and Heuer and Smalla (personal communication, SUBMECO 1996). Diversity index studies have been tried by others (35, 38).

Garland and Mills (11) and Haack *et al.* (18) have recommended analyses based on the nonlinear relationship of colour production. The substrate oxidation profile often develops a pattern similar to the bacterial growth curve. These sigmoidal curves can be fitted to a 'best-fit' function, but the mathematical parameters lack biological meaning. These parameters, however, can be reworked as outlined by Zweitering *et al.*. (39) to get microbiologically relevant parameters: slope, lag phase and asymptotic maximum (stationary phase).

This study examined the attached and free floating fractions of a heterotrophic estuarine community by using this new technique. New information regarding estuarine ecology was produced by examining a range of parameters from the Biolog approach: (I) the slope of each curve, (2) the overall shape of each curve and (3) the corrected optical densities.

MATERIALS AND METHODS

Sample Collection

Samples were collected from the Gladys Porter Memorial Bridge which crosses the Cornwallis river estuary (45^{0} 06' N, 63^{0} 24'W) at Port Williams, Nova Scotia, Canada. All samples were taken within a one hour period of high tide, over a period of approximately four months (from June 1995 to September 1995). *In situ* temperature

(14-21° C) and salinity (28-29.7 ppt) were taken at the time of collection. Salinity was measured with a hydrometer and a density-salinity chart. Large sample volumes were taken (18-20L) in order to minimize environmental changes; storage in the dark at or near *in situ* temperatures was done for a maximum of 3 hr. All experimental procedures were conducted within this three hour period.

Size Fractionation

Samples were prefiltered in order to remove large detritus by passing the sample through a 120 micron pore size mesh. The sample at this point contains attached and free floating bacteria and has been called the 'crude' sample. Size fractionation to separate the attached and free floating bacteria was done by filtering the crude sample through a 1.0 micron pore size Nuclepore (Nuclepore Canada Inc., Toronto, ON) filter. The 'filtrate' contains the free floaters. The filters were then agitated (Mistral Multimixer, Lab-Line Instruments, Inc. Melrose Park, ILL.) to release the surface materials in an equal volume of filter sterilized water (discussed later). The resuspended fraction contains the attached bacteria.

Tritiated Thymidine Incorporation

The methodological approach for this section was a modified version of the Fuhrman and Azam (10) technique. For each of the three fractions (crude, attached, and free-floating), there were two controls and two tests. Five mCi of tritiated thymidine (specific activity 7.0×10^4 - 7.16×10^4 mCi/mmole) were added and the samples were

incubated at 20° C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) for 2 hr. After incubation the test samples were fixed by adding 2 mL of 20% gluteraldehyde to stop thymidine uptake. Samples were cooled on ice for five minutes. An equal volume (12 mL) of ice cold 10% trichloroacetic acid (TCA) (Fisher Chemical/Fisher Scientific, Fairlawn, NJ) was added to extract proteins. Samples were cooled on ice for another 5 minutes before filtering through a 25mm, 0.45 micron pore size cellulose nitrate filter (Micro Filtration Systems, Dublin, CA). Samples were washed three times with 1mL of 5% TCA. Filters were placed in a scintillation vial with 10 mL of scintillation fluor (Scinti-verse II or ScintiSafe Econo1 LSC Cocktail) (Fisher Chemical/Fisher Scientific, Fairlawn, NJ). The vials were stored for 24 hrs in the dark at 20° C to reduce the incidence of random coincidence events (chemiluminescence, bioluminescence, or photoluminescence). The tritiated thymidine uptake was measured with a Beckman LS 5000 TD Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA).

Biological Oxygen Demand

The biological oxygen demand (BOD) of only the crude sample was measured over a four day period for each sample date. A total of 10 bottles were filled and sealed to prevent the infiltration of external oxygen. The bottles were incubated at 20° C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) until needed. Two of the bottles were evaluated 15 minutes after being filled to determine the initial oxygen concentration.

Two mL of magnesium sulfate plus 2 mL of alkaline iodide was added to the two initial time zero bottles. They were shaken and the precipitate was allowed to settle 1/3 of the way down the bottles; this was done twice. After the final settling, 2 mL of concentrated sulfuric acid was added and the bottle shaken until the precipitate completely dissolved. One hundred mL from each bottle was transferred to the flask and one eye dropper full of starch was added to each. The solutions were titrated with phenylarsine oxide (PAO). The volume of PAO necessary for the titration was used to calculate the amount of dissolved oxygen. The remaining 8 bottles were examined in the same manner over 4 days. The oxygen demand for each day was determined by subtracting the daily dissolved oxygen values from the initial time zero sample. The individual sample BOD change was plotted vs time with Sigmaplot and curve fitted to get the L and K parameters, representing the ultimate BOD value or the amount of oxygen required to degrade all nutrients and the BOD rate constant or rate of nutrient degradation, respectively.

Total Direct Microscopic Counts (AODC)

The total direct count was done with the acridine orange technique described by Hobbie *et al.* (21).

Dilutions were necessary to get a countable number of bacteria per field of view. Dilutions ranged from 1/10 to 1/20 for the crude and attached sample. The free-floating samples did not require dilution. All dilutions were made with filter sterilized estuarine water. Samples were then fixed with gluteraldehyde (final concentration 2%) and stained for 4 minutes with filter sterilized acridine orange (AO) (final concentration 0.01%).

Samples were filtered through a 25mm 0.2mm pore size Nuclepore filter stained with irgalan black (Nuclepore Canada Inc., Toronto, ON). The filter was pre-wetted with sterile distilled water to eliminate hydrophobic regions. Filters were placed on a slide with a drop of low fluorescence immersion oil (Fisher Scientific, Fairlawn, NJ) below the filter and on top of the filter. A cover slip sealed the preparation. Counting was done with a Nikon WFX-II fluorescence microscope (Nikon Canada Inc., Mississauga, ON) containing a 420-490nm excitation filter, 510nm dichroic mirror, and a 520nm barrier filter. Filters were first examined for hydrophobic regions; if present, the slide was discarded and the preparation was repeated. The fluorescing bacteria were counted for a total of 10 randomly chosen fields. The number of cells per mL of sample was determined by averaging the total number of cells counted for the 10 fields of view as per Hobbie et al. (21).

Coliform Counts

Total coliform counts for each sample and fraction (crude, attached and free-floating) were determined according to standard methods with mEndo broth plates (17).

Fecal coliform counts for each sample and fraction (crude, attached and free-floating) were determined according to standard methods with mFC agar plates (17).

Plate Counts

A culturable/viable count for each of the three fractions (crude, attached and free-floating) was done with 1\10 tryptic soy agar which was adjusted to 6ppt and 28ppt salinity with Forty Fathoms Biocrystal MarineMix (Marine Enterprises International, Inc., Baltimore, Maryland).

The three undiluted fractions were plated using a Spiral Plater, Model C (Spiral Systems, Inc., Cincinnati, OH). Duplicate plates were incubated at 20^{o} C in a Caron Refrigerated incubator (Caron Products and Services, Inc., Marietta, OH) for 4-5 days. Counting of the colonies was done as per the manufacturer's instructions.

Biolog Microtitre Plate Inoculation and Incubation

Samples were diluted 1/5 with filter sterilized estuarine water. A series of preliminary tests showed this to be the best dilution for colour development over the extended reading times (data not shown). Also, the fluctuations in salinity at high tide were minimal (28-29.7 ppt), thus the filter sterilized estuarine water was not adjusted for salinity. Estuarine water (sampled at high tide) was sterilized by filtration through a 0.2 micron pore size Nuclepore (Nuclepore Canada Inc., Toronto, ON) filter and stored at 4^{o} C until just prior to use, at this point it was warmed to *in situ* temperature. Sterilization of the estuarine water was done the day before samples were to be taken. Biolog GN microtitre plates (Biolog INC., Hayward, CA) were inoculated in duplicate for each of the three fractions. Incubation was at 15^{o} C in a Conviron incubator (Conviron Controlled Equipment Ltd., Winnipeg, Manitoba) for up to 120 hours. Optical density readings were taken at approximate six hour intervals for the 120 hour duration with the Microlog 3N

3.01A Version DE (Biolog INC., Hayward, CA) program and a Cambridge Technology, Inc. (Cambridge Technology, Inc., Watertown, MA) series 750 Microplate Reader.

Data Treatment and Statistical Analysis

Each six hour reading was appended to the previous reading and stored in ASCII format. When the sampling time was complete the files were parsed and reformatted so that each reading time was represented by one row, 96 values long.

The reformatted files, one for each plate, were imported into Sigmaplot For Windows Version 2.01 (Jandel Scientific Corporation, Corte Madera, CA). Corrected optical densities (OD of well A1 subtracted from OD of each of the 95 substrate containing wells; COD) were calculated for each plate. Mean COD values were calculated for each plate and plotted for sample comparison.

The mean COD values for the duplicate plates were calculated, means were plotted against time and curve fitted with a modified logistic function within the SigmaPlot program to get a best-fit curve of the data. The curve fit program also provided relevant parameters, defining the sigmoidal tendency of the optical density curve. Parameters a, b, c, and d represent the maximum asymptote, slope, inflection point, and the minimum asymptote respectively. These parameters were reworked according to Zweitering *et al.*. (39) to get microbiologically relevant parameters; (a) was changed to (A) and represents the plateau phase where the slope equals zero, (b) was changed to (μ), the slope, which is represented by a tangent to the inflection point, (c) remained as the inflection point and

(d) represents the initial inoculum density. The lag phase (λ) is the x-axis intercept of this tangent.

The SigmaPlot files containing the microbiologically relevant parameters were then imported into SPSS (Statistical Package for the Social Sciences Professional Statistics 6.1, SPSS Inc., Chicago, IL) and arranged by month/day/row/well and treatment (crude, attached or free floating). At this point each row represents one well. Additional columns were added to incorporate the supporting data and relevant supporting factors: asymptote, slope, inflection point, lag phase, y intercept, week, substrate (2-96), AODC, 6ppt and 28ppt salinity plate counts, total coliforms, fecal coliforms and tritiated-thymidine uptake.

The slope (μ) data were incorporated into a new SPSS file containing the original month/day/row/well and treatment variables and the supporting data. Ninety-five new slope variables were thus added to the file. This file was used within SPSS for Principal Components Analyses (PCA). PCA were used to investigate similarities among the three treatments (crude, attached and free-floating) as well as similarities among the attached fraction and among the free-floating fraction. The PCA was varimax rotated and a three factor solution was chosen.

RESULTS

General Microbial Ecology of the Cornwallis Estuary

Based on the crude estuarine environmental sample (i.e. no fractionation), there appears to be a general depression in all of the general microbiological parameters during

mid to late summer (August) (Fig. 1). By the September 20 sampling period the activity, as measured by ^{3}H thymidine incorporation, and the total coliform counts have returned to the high levels of early summer (June and July). The fecal coliform numbers, and salinity plate counts show only marginal increases by Sept. 20.

The relative involvement of the free floating fraction in the overall bacterial community (Fig. 2), indicates that the free floating fraction represents a small proportion of the community involvement.

Biolog Data Analysis

PCA based on the crude sample and slope data, Fig. 3, shows a distinction between early and late summer samples. Differentiation is based, predominantly, on PCA 1 with its high correlations to salinity plate counts (28ppt; $p=0.06$, 6ppt; $p=0.05$) and fecal coliforms ($p=0.03$) (data not shown). The July 11 and July 19 samples differ on PCA 2 and PCA 3, respectively, and these components were not correlated with any of the parameters (data not shown).

The COD plots for the individual substrates were characterized into seven categories (Fig. 4) according to the strength of substrate response, primarily the plateau values. Fig. 5, represents the percentage distribution for the attached and free floating fractions. The free floating fraction has a low percentage of medium response curves and a higher percentage of no response and negative curves when compared to the attached fraction. Neither the attached nor the free floating fraction have any category 1 responses (strong sigmoidal). Category 6 and 7 curves (no response and negative) were adjusted to

a slope of zero for the slope parameter calculations, representing non-utilization of the substrate.

This overall community transition from early to late summer is also seen in the mean COD plots, Fig. 6 and Fig. 7. During the early portion of the summer the attached and free floating fractions differ. However, the mean COD plots for the late summer samples indicate that the attached fractions are not significantly different from the free floating fractions.

PCA based on the attached and free floating fractions with slope data supports the idea of a shift between early summer to late summer (Fig. 8). The lines connecting the attached and free floating fraction scores on a given sample date show a more marked shift for the early summer (June 28, July 11 and July 19) than the late summer. The plots for the August 8, August 14 and September 20 are tightly clustered with small shifts.

The non parametric Mann-Whitney U-test (MWU) (Table 1) was used to compare early versus late summer samples for both the attached and free floating fractions. Significant differences between early and late summer were found only for the attached fraction with fecal coliforms and ^{3}H thymidine.

MWU comparisons of attached and free floating fractions (Table 2) indicated significant differences during early summer as well as late summer.

DISCUSSION

Shift in the Attached Fraction

The incubation of environmental samples from a turbid estuary in Biolog microtitre plates resulted in profiles representative of their metabolic potential. The metabolic potential has been evaluated with the rate of substrate response; the slope of the colour development curves, extent of substrate response; based on graph shape and COD. Examining the nonlinear nature of colour production, as suggested by Garland and Mills (11) and Haack (18) has revealed additional information about the attached and free floating bacterial fractions.

The Cornwallis Estuary is a turbid estuary with high SPM concentrations (> 5000 mg/L in the inner estuary at high tide) and low primary productivity (22). The abundance of SPM within the Cornwallis Estuary results in a greater proportion of the community being attached, resembling other turbid estuaries (3, 4, 7, 26). Because the proportion of free floating bacteria is low, their contribution to the estuarine community involvement is expected to be low (Fig. 2); the free floating fraction accounts for 3.5 to 13.3% of the counts and 5% of the activity. Goulder (15) has noted high numbers of attached bacteria in a turbid estuary, but that it gives no indication of the distribution of metabolic potential.

Changing environmental conditions during summer are likely responsible for the temporal shift in the general microbiological parameters (Fig. 1); environmental stability affects the diversity of microbial communities (21). The Biolog slope data (Fig. 3) appears to indicate a seasonal nature as well; the late summer samples group in factor space and are separate from the early summer grouping of samples. Seasonal differences (ie. summer to winter) and within season differences (hydrographic conditions and particle composition) are noted to cause shifts in community compositions (15, 28, 36). The

higher percentage of 'no response' shapes (Fig. 5) indicates a subtle difference between the free floating and attached fractions, when considering the entire sampling period.

Evaluating the Biolog COD data from Fig. 6 and Fig. 7, it is apparent that the early summer attached and free floating samples are different from one another. Non-overlapping error bars generally indicate significant differences at the 0.05 level. During late summer, the Biolog COD does not indicate a difference between the two fractions at the 0.05 level. The difference between the free floating fraction and attached fraction is accounted for by the increased metabolic potential of the early attached fraction, as is seen in the activity measure in Table 1. The shift from early to late summer is reinforced by the Biolog slope data (Fig. 8). Marked shifts over the summer are apparent. Early summer free floating fractions and associated attached fractions are separated in factor space more so than the late summer free floating fractions and associated attached fractions. Late summer samples (attached and free floating) are closely grouped in factor space, indicating similarities.

The reduction in the activity for the attached bacterial fraction during late summer, lack of a significant difference betweenn the late summer attached and free floating fractions (Table 2), may be linked to the SPM. Brown (22) has noted *Spartina alterniflora* input peaks within the Cornwallis Estuary. There are two *Spartina alterniflora* input peaks in the Cornwallis Estuary, a small peak in May and a larger one in September , which make up a major proportion of the SPM. It is possible that the SPM quality is declining (degradation) throughout the early summer, which results in a reduced rate of substrate oxidation during late summer. After each *Spartina* input peak, flushing

of the system will reduce the amounts of high quality *Spartina* SPM. The reduction in SPM quality may not result in a decrease in attached numbers, but may result in a decreased level of activity. Azam *et al.* (2) have noted that phytodetritus particles are sites of rapid colonization and growth, but agglomeration of particles can cause a reduction in growth as O_2 and pH levels decline and the concentration of toxic metals increases, however, agglomeration within the Cornwallis Estuary has not been investigated.

The shift in metabolic potential as measured with Biolog slope data is supported by the ^3H thymidine activities. Values for the attached fraction are significantly different from early summer to late summer (Table 1). The metabolic potential of the free floating fraction is similar throughout the study period; the activities for the free floating fraction are not significantly different from early summer to late summer (Table 1). The free floating fraction within the Cornwallis Estuary exhibits temporal stability, whereas the attached fraction is more variable. The free floating fraction exhibits seasonal stability, differences are noted with changes in season (ie. summer to winter) (15, 36). The variability of the attached fraction is related to hydrographic conditions and changes in the particulate matter (ie. SPM) (28). The temporal stability for the free floating fraction and variability for the attached fraction noted with the Biolog system have been seen by Bent and Goulder (3), using heterotrophic activity measures.

The lack of a significant difference between the counts (Table 1), with the exception of the fecal coliforms, for early and late samples of both attached and free floating bacteria supports the idea that the metabolic shift is a result of changes in the

activity level of the attached fraction and not a change in abundance. The significant reduction in fecal coliform numbers may also be directly related to SPM quality and abundance reduction. If the fecal coliforms are attached, and quality and abundance is reduced, the fecal coliforms may experience a more inhospitable environment as found by Azam *et al.* (2). Azam (2) states that larger particles will rapidly develop an attached colony until they develop areas of low oxygen concentration, decreased pH and increased levels of toxic metals. The smaller particles are less likely to have colonizers sequestered, thus the incidence of salinity related mortality (29) is likely to increase.

The early summer attached fraction has significantly greater numbers as measured by AODC, fecal coliforms and total coliforms than does the free floating fraction (Table 2), as would be expected in a community dominated by attached bacteria. The same trend holds true for the late summer attached fraction versus late summer free floating fraction. The activities (^{3}H thymidine) are not significantly different for the late summer attached versus the late summer free floating fraction. The biomass data (AODC and coliforms) support the functional data from the Biolog analysis in that the attached fraction is different from the free floating fraction. The Biolog slope of substrate response is a measure of the metabolic rate, thus measuring an activity/functional aspect of the community. The Biolog activity measure supports the ^{3}H thymidine activity, with one exception; the lack of a significant difference, with respect to activity, in the early summer attached fraction versus the early summer free floating fraction. This poses only a small concern, considering the Biolog slope data are measuring functional as well as structural shifts. The significant differences (Table 2) between early free floating fractions and early

attached fractions as well as late free floating fractions and late attached fractions indicate that a structural shift is involved with the change in functional potential.

A similar trend was noticed when comparing another activity measure, the Biological Oxygen Demand (BOD) L and BOD K (data not shown). The L factor is a measure of the potential for substrate degradation and the BOD K is a measure of the rate. MWU comparisons of early summer versus late summer for the crude sample, indicated no significant differences ($p = 0.05$) for either L or K factors (data not shown). The lack of a difference between early and late summer for these activity measures seems to contradict the Biolog data, possibly supporting the idea that the Biolog system is measuring structural changes as well functional changes. Clarification of this by comparing early versus late for the attached and free floating fractions is necessary.

Garland and Mills (11) have stated that responses produced in the Biolog community assay are a reflection of functional potential. Zak (38) has said that it may assess functional diversity. Haack (18) has shown that differences in community kinetic profiles were probably due to differences in community composition, Garland *et al.* (personal communication, SUBMECO 1996) supported this in saying that community level physiological profiles were describing community structure, whereas Garland and Mills (12), Zak *et al.* (38) and Vahjen *et al.* (35) have all stated that functional information can be obtained. Whether the functional potential is the result of structural or functional changes, or a combination of the two, must be ascertained before functional diversity interpretation becomes clear. Garland and Mills (12) proposed that the Biolog assay can make an evaluation of community structure (based on patterns of colour production) as

well as function (based on growth rates and degradation rates). Analysis based on slope of substrate response and COD appears to be an accurate indicator of the metabolic potential. The data available from only the Biolog system has demonstrated the ability to characterize a temporal community shift of a whole environmental sample as well as within two portions of that community. Other data support this and suggest that this temporal shift is due to a structural as well as a functional change within the attached fraction. Incorporation of supporting information will aid in determining the functional or structural inputs involved with functional shifts.

Inclusion of the lag phase and stationary phase data in the analysis may elucidate some fine differences between the fractions. The free floating fraction is approximately 10 fold lower with respect to inoculum density (AODC), however the mean COD plots do not indicate protracted rates of colour development as would be predicted for low inoculum densities (18). Lag phase data may indicate differences between the two fractions before the onset of colour development, while stationary phase data may indicate differences in the level of substrate utilization (maximum absorbance values). There are three distinct variables that describe the functional response to any given substrate; analyses based on all three will further increase the information received and will make interpretation of Biolog responses more accurate.

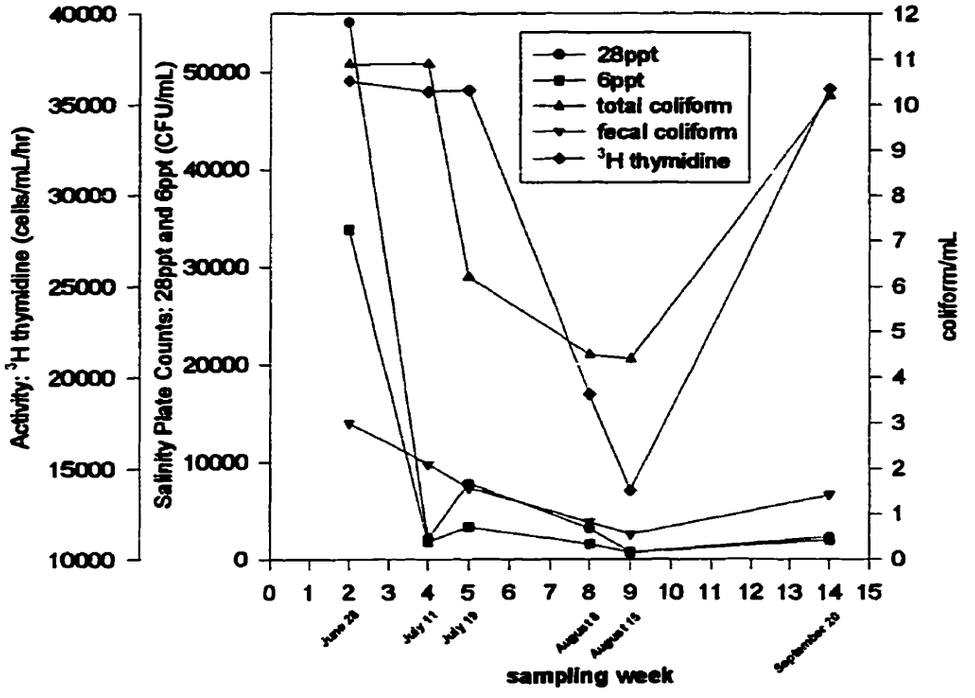


Fig. 1. General microbiological parameters, based on the crude estuarine environmental sample (i.e. no fractionation).

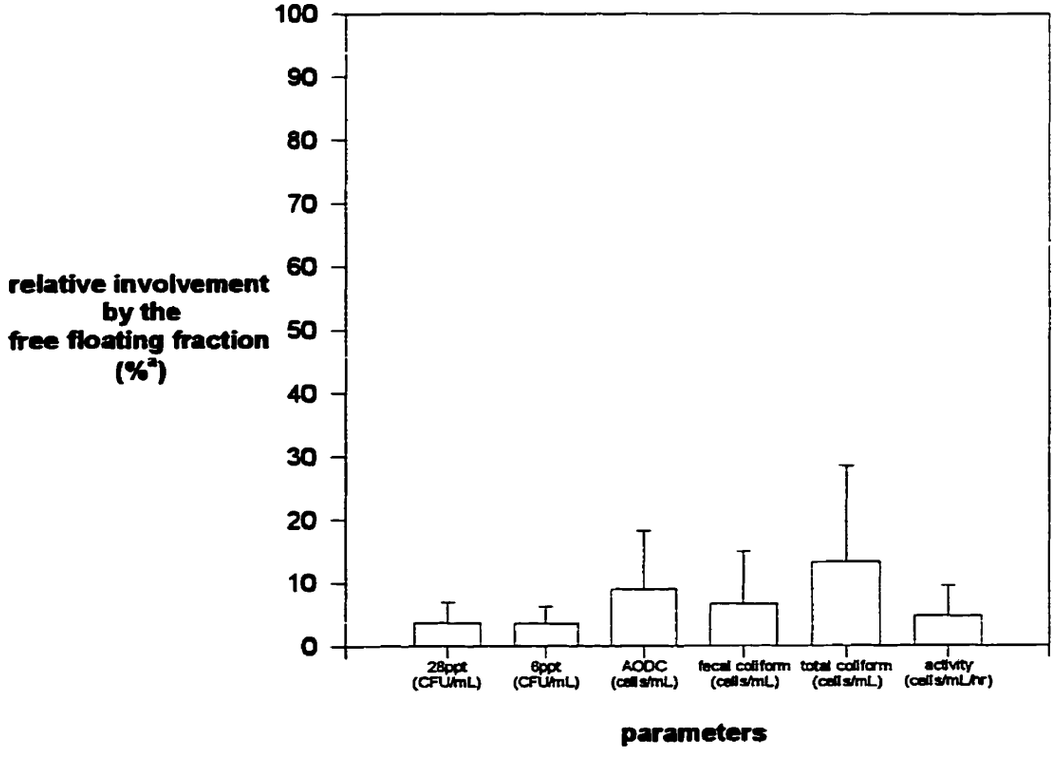


Fig. 2. Relative involvement (%), by the free floating fraction, in the overall bacterial community. %[a] represents (free floating/free floating + attached)×100. Error bars represent standard deviations.

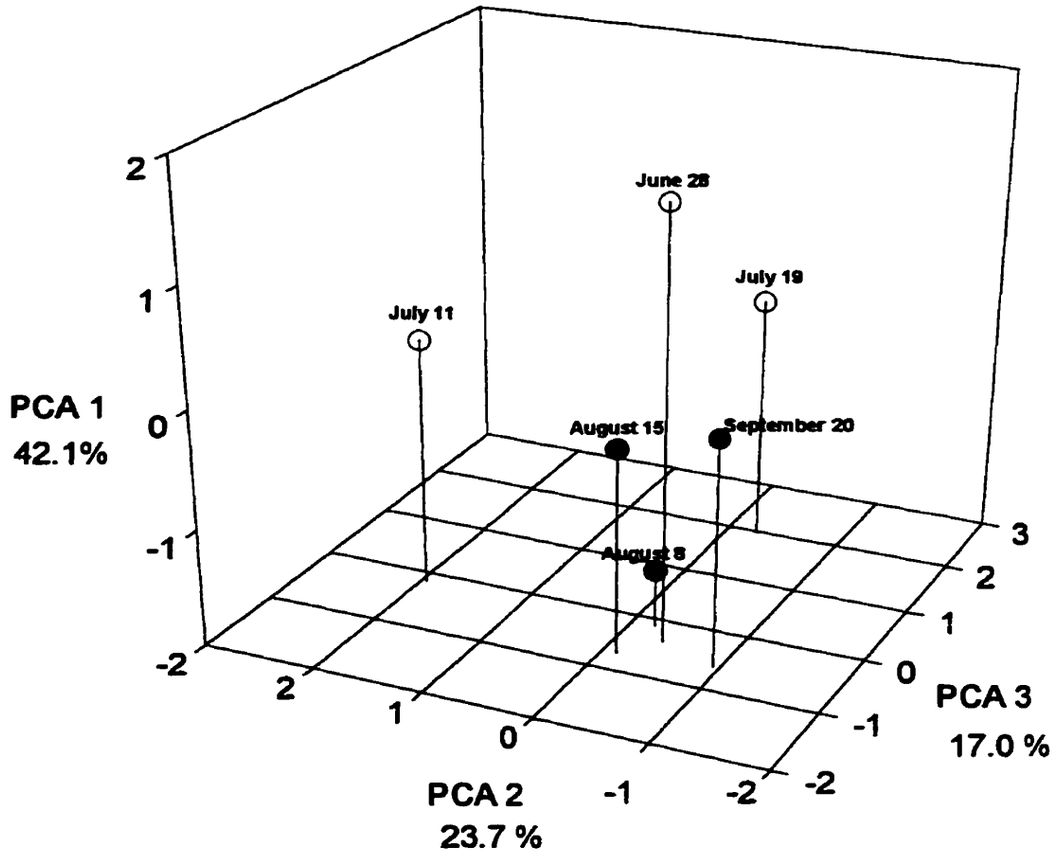


Fig. 3. **Results of principal components analysis (PCA) based on the crude sample with slope data. Early samples (June 28-July 19), indicated with clear symbols, and the late samples (August 8-September 20), indicated with solid symbols.**

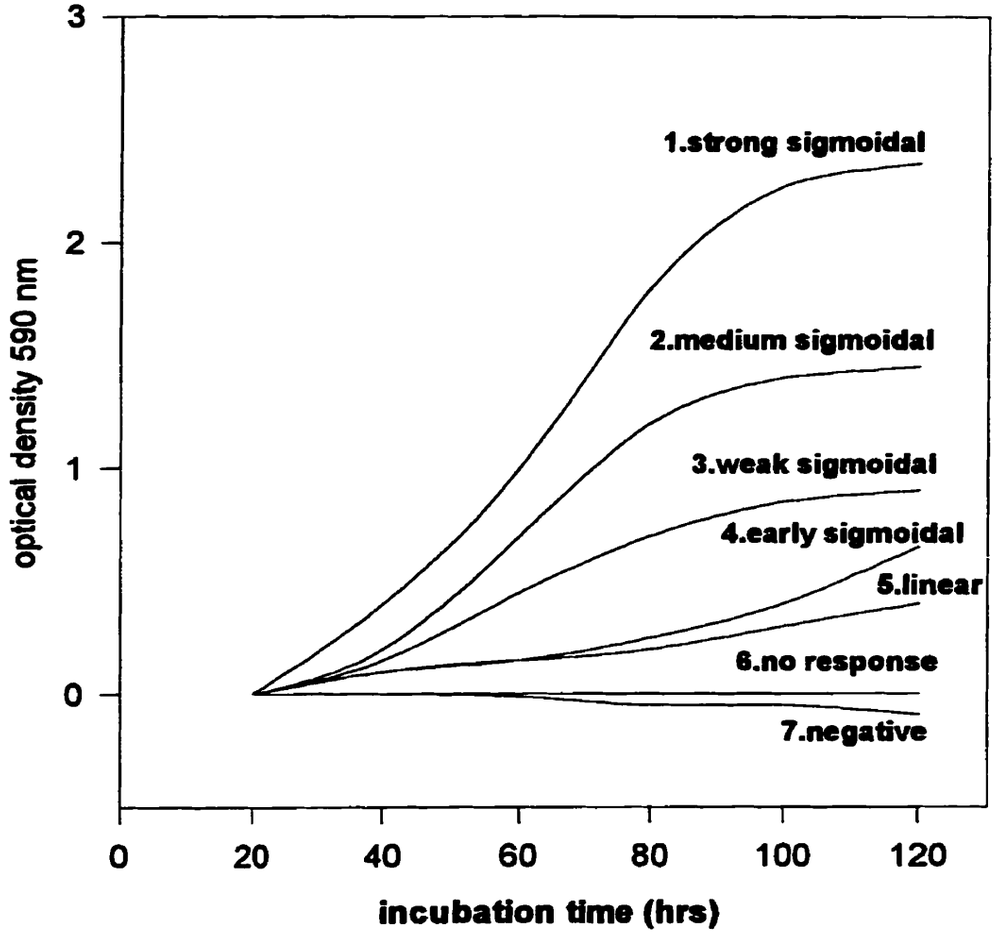


Fig. 4. Hypothetical substrate response curves.

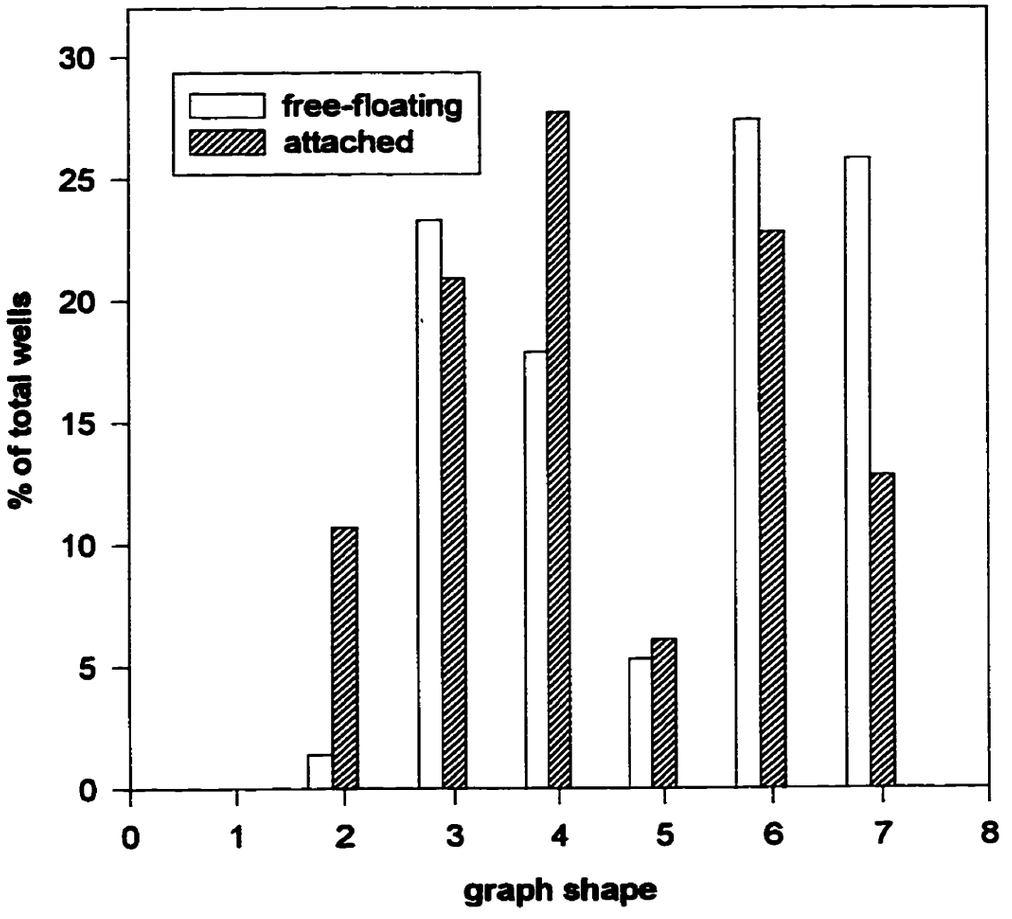


Fig. 5. Histogram showing the percentage distribution of graph shapes for the free floating and attached fractions.

Fig. 6. Plots of the mean corrected optical density (COD) for the early samples (June 28-July 19). Error bars represent 95% confidence intervals.

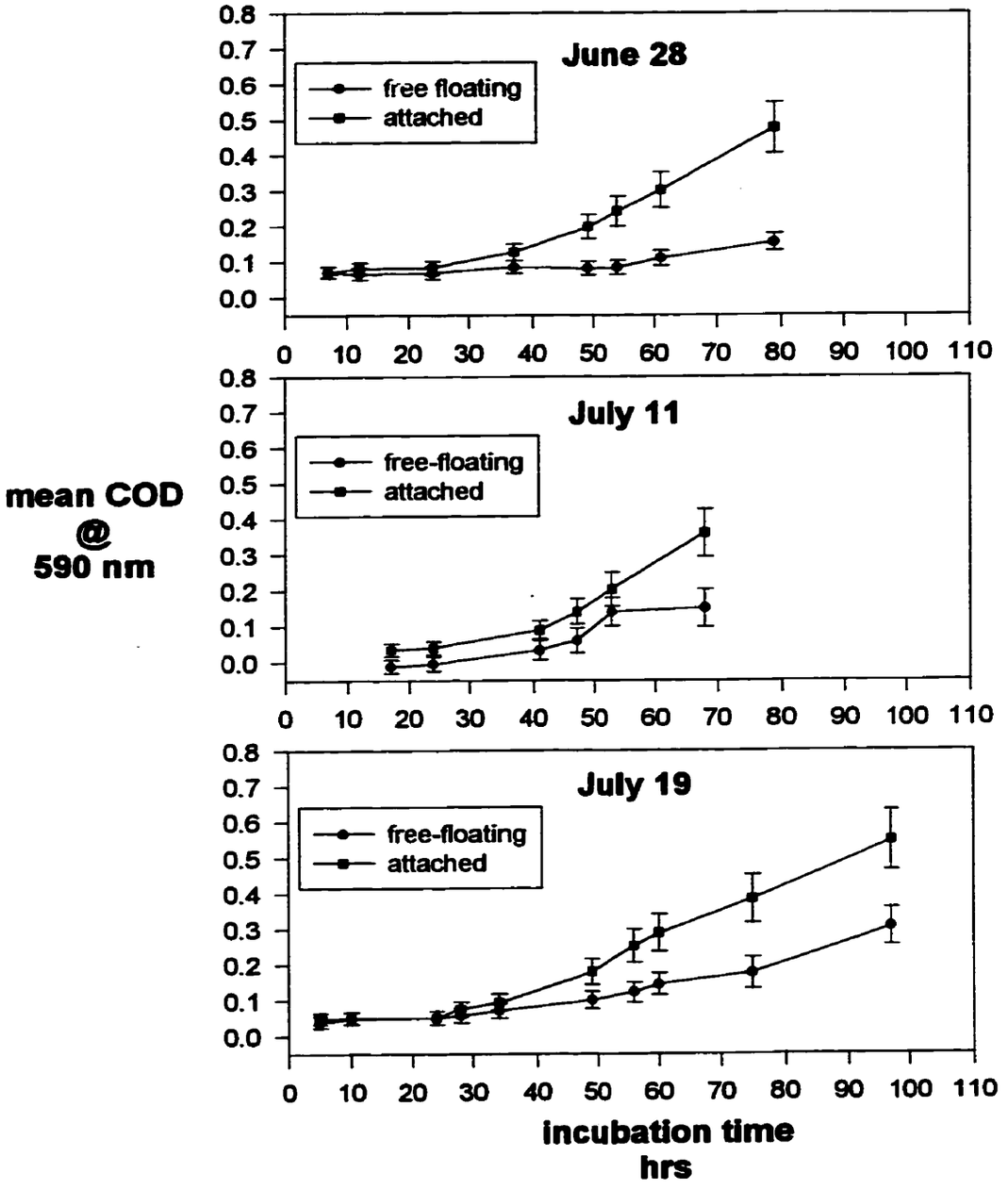
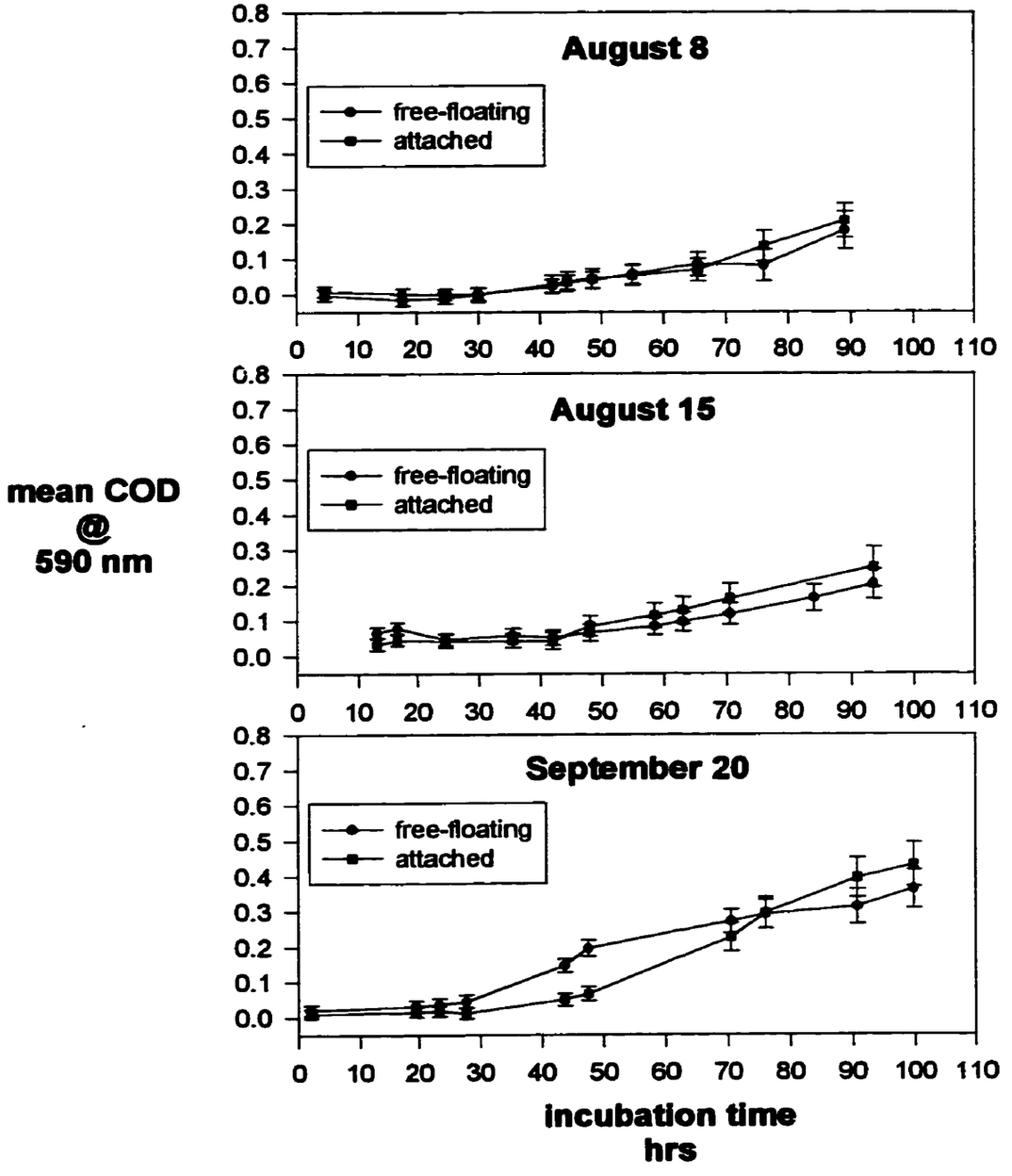

June 28
free floating
attached
July 11
free-floating
attached
July 19
free-floating
attached
mean COD
@
590 nm
incubation time
hrs

Fig. 7. Plots of the mean corrected optical density (COD) for the late samples (August 8-September 20). Error bars represent 95% confidence intervals.


August 8
free-floating
attached
August 15
free-floating
attached
September 20
free-floating
attached
mean COD
@
590 nm
incubation time
hrs

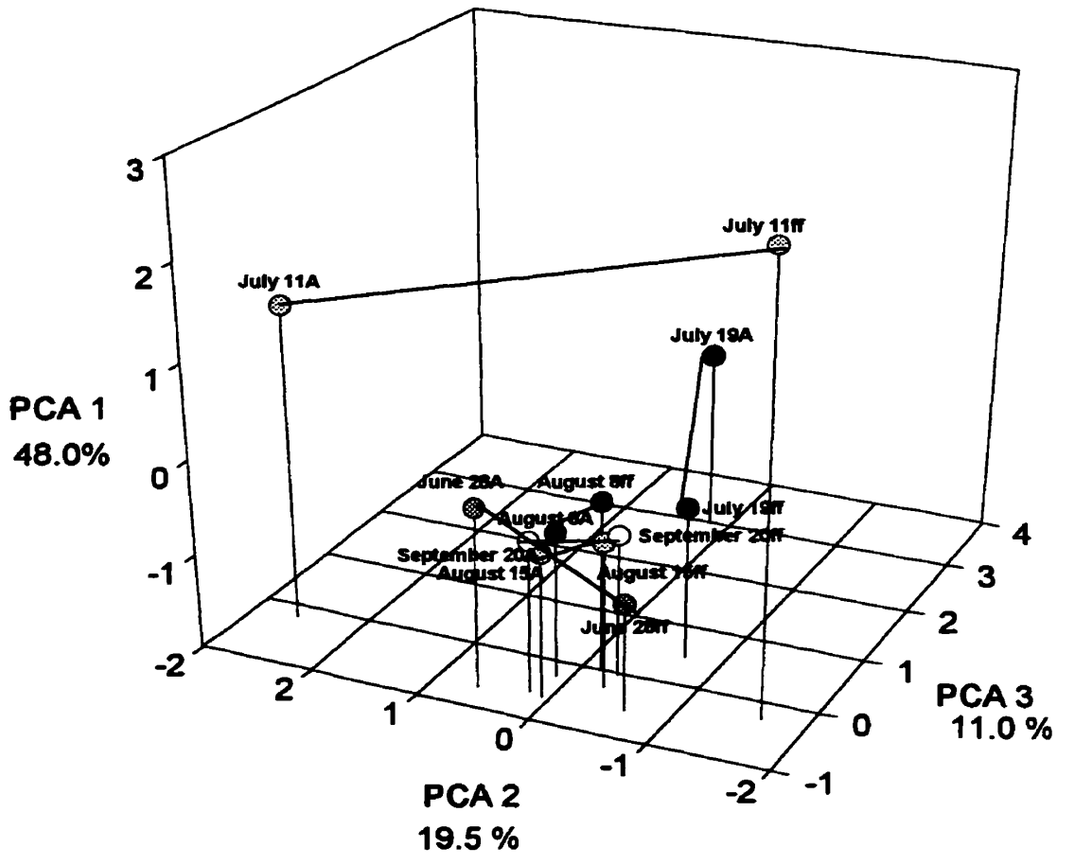


Fig. 8. Results of the principal components analysis (PCA) of the free floating and attached samples with slope data. The lines highlight the associated shifts. ff represents the free floating fraction. A represents the attached fraction.

Table 1. Comparison of early summer to late summer parameters. Figures in bold represent significant differences (Mann-Whitney U - Wilcoxon Rank Sum Test, $p \leq 0.05$).

	28ppt (CFU/mL)	6ppt (CFU/mL)	AODC (cells/mL)	fecal coliform (cells/mL)	total coliform (cells/mL)	activity (cells/mL /hr)
ATTACHED FRACTION						
early summer						
mean	3.0E4	3.9E4	1.9E7	**2.0**	5.8	**3.5E4**
standard deviation	2.6E4	4.4E4	7.5E6	**0.43**	0.70	**3.4E4**
late summer						
mean	5.9E3	1.9E4	3.0E7	**0.57**	4.2	**4.1E3**
standard deviation	7.1E3	8.7E2	1.8E7	**0.40**	2.0	**4.3E3**
FREE FLOATING FRACTION						
early summer						
mean	9.7E2	1.1E3	1.5E6	0.08	0.70	1.2E3
standard deviation	9.9E2	1.3E3	5.6E5	0.05	0.40	2.0E3
late summer						
mean	3.4E2	3.5E2	3.2E6	0.10	0.82	1.2E3
standard deviation	2.7E2	2.4E2	1.6E6	0.08	0.21	2.2E2

Table 2. Comparison of attached and free floating parameters. Figures in bold represent significant differences (Mann-Whitney U - Wilcoxon Rank Sum Test, $p \leq 0.05$).

	28ppt (CFU/mL)	6ppt (CFU/mL)	AODC (cells/mL)	fecal coliform (cells/mL)	total coliform (cells/mL)	activity (cells/mL /hr)
EARLY SUMMER						
attached fraction						
mean	3.0E4	3.9E4	**1.9E7**	**2.0**	**5.8**	3.5E4
standard deviation	2.6E4	4.4E4	**7.5E6**	**0.43**	**0.70**	3.4E4
free floating fraction						
mean	9.7E2	1.1E3	**1.5E6**	**0.08**	**0.70**	1.2E3
standard deviation	9.9E2	1.3E3	**5.6E5**	**0.05**	**0.40**	2.0E3
LATE SUMMER						
attached fraction						
mean	**5.9E3**	**1.9E4**	**3.0E7**	**0.57**	**4.2**	4.1E3
standard deviation	**7.1E3**	**8.7E2**	**1.8E7**	**0.40**	**2.0**	4.3E3
free floating fraction						
mean	**3.4E2**	**3.5E2**	**3.2E6**	**0.10**	**0.82**	1.2E3
standard deviation	**2.7E2**	**2.4E2**	**1.6E6**	**0.08**	**0.21**	2.2E2

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GENERAL DISCUSSION

The application of a community approach for the study of a turbid estuary has shown that it can be used to evaluate the whole estuarine community as well as the attached and free floating portions within that community. The resultant shift in the functional potential of the attached fraction towards late summer, based on the slope of substrate response, graph shapes and COD has been shown to support the findings of others (30, 57, 59, 97), who have indicated that the attached and free floating fractions are similar with respect to uptake rates and specific growth rates within a season. The dissimilarity between seasons for attached and free floating bacteria is supported by Kirchman and Mitchell (65).

The balance between attached and free floating bacteria is in constant flux. The closeness of their metabolic potentials will be determined by the environmental conditions *in situ*. As Kirchman and Mitchell (65) have pointed out, differences in specific growth rates have been noted to occur with changes in the carbon source. The carbon source will ultimately determine the microbiota (11), therefore the sum of the physiological potentials will depend on the carbon source *in situ*.

Many studies which did not incorporate the Biolog system noted the importance of using diverse substrates when evaluating community responses (12, 19, 71). The Biolog system facilitates this.

Researchers have stated that the Biolog system exposes the community to a selective and enriching environment, the degree of which still remains unclear. Because of this, opponents have discredited the Biolog analysis of microbial communities. The selective enrichment which does occur excludes the possibility of the profile representing in situ abilities, as some have concluded (58, 117).

However, this does not proclaim the results to be invalid/invaluable. The results should be viewed as potentials (i.e. functional potentials or metabolic potentials) as stated

by Garland and Mills (40), Garland (38, 39)(personal communication, SUBMECO 1996), Winding (114) and Haack *et al* (52).

The end product of any community substrate response is based on the community environment from which it came. The resultant profile will depend on the community structure and function, predetermined by the carbon sources within the natural environment. Selective enrichment of community members will depend on the members present in the original environment. Therefore the profiles will represent individual community traits, thus, the Biolog system will be useful in terms of functional and structural studies.

Incorporating diverse methods of analysis and applying the functional and structural data to the taxonomic and genetic data would appear to be the most reliable method of community assessment.

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APPENDIX A

1. **Thymidine Incorporation:**

cells/mL/hr = ((dpm in sample / specific activity) * 4.5×10^{-13} * 2×10^{18} cells/mole)/ 20

2. **Acridine Orange Direct Counts:**

n = Yad / av = number of cells / mL
Y = mean number of cells / graticule
A = effective area of filtration
d = dilution factor
a = graticule area
v = volume of sample

3. **Coliform Counts:**

(number of colonies) * (dilution factor) / volume filtered = coliforms/mL

4. **Spiral Plating:**

CFU / mL = (number of colonies in area 1) + (number of colonies in area 2) / area constant

APPENDIX B

Logistic Function (logistz.fit)

```
*****************************************
This function curvefits the O.D. plots of the 96 Biolog wells.
The values of a, b, c and d, as well as the curvefit data are
 stored in columns 52 onward.
*****************************************
jsv4R
[Parameters] ;4 Parameter Logistic Function
          ;f=(a-d)/[1+(x/c)^b]+d, if b>0 it
          ;starts at a and falls to d.  If b<0
          ;it starts at d and rises to a.

;Modify these values for your data
a=2.0   ;asymptotic maximum
b=-5      ;slope parameter, b>0 gives slope<0
c=60     ;value at inflection point
d=.01      ;asymptotic minimum

[Variables]
x=col(1)     ;change to appropriate column
y=col(38)     ;change to appropriate column

[Equations]
f=(a-d)/(1+(x/c)^b)+d

fit f to y
;Five parameter logistic function
;Add parameter e to [Parameters]
                                   ;f=(a-d)/(1+(x/c)^b)^e+d
```

BOD Fit Transform

```
**************************************
This fit program calculates the L and K  factors
and curvefits the BOD plots.
**************************************
[Parameters]
L = 1000 ; set an estimate value
K = 1 ; set an estimate value
[Variables]
t = col(1)
bod = col(2)
[Equations]
f = l * (1-10**(-k*t)
fit f to bod
```

APPENDIX C

PLATES1.XFM Transform

```
;*****PARAMETER ESTIMATION FOR BIOLOG PLATES**********
;This transform is to be used on databases containing the two sets
;of Biolog plate data plus means, ranges and best fit estimates after
;using logistz.fit.  This will produce a database with 87 columns.
;The present transform continues and works out mu (the slope)
; and lambda (the lag phase) and tabulates it all for export to SPSS

;*****************Identification for SPSS*****************************
cell(90,1)=6          ;person identifier, change as necessary: Hélène=1,
                      ; Jacob=2, Scott=3, Shane=4, Joy=5, Trevor=6.
cell(90,2)=9          ;month identifier, change to reflect month of your
                      ;sample.
cell(90,3)=22          ;day identifier, change to reflect day of your sample.
cell(90,4)=1          ;plate row identifier, A=1, B=2, C=3, D=4, E=5, F=6
                      ;G=7, H=8.
cell(90,5)=1          ;details of your sample; specifically :-
                      ;for Hélène, summer=1, winter=2
                      ;for Jacob, HW/Hppt=1, HW/Lppt=2, LW/Hppt=3
                      ;LW/Lppt=4.
                      ;for Scott, crude=1, free-floating=2, attached=3
                      ;for Shane, always 1.
                      ;for Trevor, in situ=1, spikea=2, spikeb=3
cell(90,6)=1          ;invariate, the incrementer for the FOR loop.


;*********************Calculations************************************
for n=0 to 33 step 3 do
 a=cell(52+n,1)
 b=cell(52+n,2)
 c=cell(52+n,3)
 d=cell(52+n,4)
 mu=0.25*((d-a)*(b/c))
 lambda=c*exp(-2*(a+d)/((d-a)*b))
 person=cell(90,1)
 month=cell(90,2)
 day=cell(90,3)
 row=cell(90,4)
 detail=cell(90,5)
```

```
well=cell(90,6)
put person into cell(100,cell(90,6))
put month into cell(101,cell(90,6))
put day into cell(102,cell(90,6))
put row into cell(103,cell(90,6))
put well into cell(104,cell(90,6))
put detail into cell(105,cell(90,6))
put a into cell(106,cell(90,6))
put b into cell(107,cell(90,6))
put c into cell(108,cell(90,6))
put d into cell(111,cell(90,6))
put mu into cell(109,cell(90,6))
put lambda into cell(110,cell(90,6))
cell(90,6)=cell(90,6)+1
end for
```

MEAN.XFM Transform

```
;***MEANS AND RANGES FOR BIOLOGS****
;This transform averages two blocks of
;Biolog plate data.  The first block MUST be
;in columns 2 to 13, second block in
;columns 14 to 25.  Column 1 is reserved
;for time of sampling (hours).
;The means are placed in cols 27 to 38,
;ranges in cols 40 to 51.

;******* Data declaration***************
cell(26,1)=" "
cell(39,1)=" "
for n=2 to 13 do
 a=col(n)
 b=col(n+12)
 ;**********Calculations*******************
 f1(a,b)=(a+b)/2
 f2(a,b)=abs(a-b)
 mean=f1(a,b)
 range=f2(a,b)
 put mean into col(n+25)
 put range into col(n+38)
end for
```

PERCHAN.XFM Transform

```
**************************************************
This transform calculates the percent
change in each well over time from the
following formula:
((R-C/C) * 100%) - Background
ref = reference well
ctrl = control well
bckgrd = background color (column 98)
**************************************************
ctrl = col (2)
for n = 2 to 97 do
ref = col (n)
bckgrd = cell (98, n - 1)
f1 (ref,ctrl, bckgrd) = ((ref-ctrl)/ctrl) * 100 - bckgrd
result = f1 (ref,ctrl,bckgrd)
put result into col (97 + n)
end for
```

AWPC.XFM Transform

```
;*************************************************************
This transform calculates the AWPC (average well percent change)
for each time reading of a sample and places them in column 195.
;AWPC transform
;for the j loop change the 10 value to represent the total number of
; reading times.  6 reading times, enter a six.
;*************************************************************

for j = 1 to 10 do     ;Change '10' value to your number of time readings(rows)
   cell (190, 20) = 0.0
   for i = 100 to 194 do ;(cycles through columns)
     add = cell (190,20) ;(summation cell)
     temp = cell (i,j)
     total = temp + add ;(summing PC values)
     put total into cell (190,20) ;(storing sum of PC values)
   end for
    sum = cell (190,20)
    f1 (sum) = sum/95 ;(calculates the AWPC)
    AWPC = f1 (sum)
    put AWPC into cell (195,j)
end for
```

Parsing Macro (Quattro Pro)

This macro arranges the raw O.D. values from the Biolog plate reader so that each row in the spreadsheet consists of the 96 O.D. values for each time reading.

```
{DOWN}                {/ Row;Delete}
{/ Block;Move}                {DOWN}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{DOWN}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{DOWN}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{DOWN}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{DOWN}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
```

```
{END}
{DOWN}
{END}
{RIGHT}
{END}
{LEFT}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{DOWN}
{/ Block;Move}
{END}
{RIGHT}~
{END}
{UP}
{END}
{RIGHT 2}~
{/ Row;Delete}
{UP 5}~
{END}
{UP}
```

APPENDIX D

GNCOLOR.24

This file is a list of the background percentage correction factors for each well (96 in total); the percentage of the OD reading taken into account by the presence of substrate and tetrazolium dye.

row 1, column 1

	2.945	0.901	8.460	2.000
-0.000	5.679	1.556	2.766	0.982
8.597	1.107	1.268	6.283	1.767
7.680	1.201	1.808	-1.068	0.587
10.164	1.003	0.583	2.037	4.148
5.909	-0.813	2.914	0.815	9.203
-1.203	0.038	3.802	1.469	4.686
-1.988	4.584	1.388	1.699	5.382
-0.256	4.125	2.986	1.211	1.627
-3.699	-1.679	-0.036	3.459	-3.464
8.013	3.094	-3.450	4.036	3.202
-0.153	2.179	5.499	2.664	3.856
7.797	0.867	9.522	5.707	4.654
-4.961	5.938	-0.829	6.132	2.535
-0.318	0.292	6.762	-0.388	5.794
-2.067	3.714	-0.736	1.302	5.443
-0.876	11.971	4.133	5.434	9.723
4.886	7.282	-0.107	2.154	row 12 column
-2.040	-0.554	-0.031	5.139	8
-2.366	1.850	-4.145	4.308	