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Developing Methods for the Rapid Molecular Assessment of Aquatic Microbial Communities

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DEVELOPING METHODS FOR THE RAPID MOLECULAR ASSESSMENT OF AQUATIC MICROBIAL COMMUNITIES

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Molecular investigations of microbial community structure and dynamics involve costly and time-consuming methods. This approach is limiting when rapid assessment and detection of microbial organisms are needed. In aquatic environments, especially freshwater environments which may be used as a water source, rapid detection of pathogenic microbes is essential. Likewise, monitoring for the presence or absence of various functional genes can be used to indicate the type of microbial community present in the environment of interest. Therefore, the aim of this study was to develop and optimize methods necessary for the rapid assessment of freshwater microbial community structure and dynamics. Primers for several genes of interest (i.e. small subunit (SSU) rRNAs, *nifH*, *rbcL*, *stx I & II*, and primers targeting *Cryptosporidium parvum*, *Giardia lamblia*, *Entamoeba histolytica*, and *Listeria monocytogenes*) were either constructed *de novo* or synthesized from previously published sequences. These primers were used to establish optimal PCR amplification parameters and to create probes from type-strain cultures representing the twelve main divisions of bacteria (Actionmycetes, Aquificales, Low GC Gram positives, Cytophaga/ Flavobacteria/ Bacteroides [CFB], Cyanobacteria, Deinococcus, Green Non-Sulfur, Green Sulfur, Planctomyces, Proteobacteria [α, β, γ, and δ], Spirocheta, and Thermotogales), Archaea (kingdoms Crenarchaeota and Euryarchaeota), and Eukarya (Brown Algae, Green Algae, Red Algae, Diatoms, Dinoflagellates, Cryptomonas, Euglenozoa). Water samples from Mill Creek, in southern Wayne County (a fresh water environment) were collected seasonally. DNA extraction by sonication was optimized and used to collect total DNA from the water samples. Environmental DNA was labeled with ^{35}S using the random prime label method for hybridization to target genes immobilized on nylon membranes. This method is called Reverse Sample Gene Probing (RSGP). PCR amplification of specific gene targets was significantly improved for some template DNAs by including either a touchdown PCR method, titrating the Mg^{2+} concentration and/or diluting the DNA. However, some templates could not be amplified and were, therefore, eliminated from the study. Hybridization by RSGP was attempted twice, once using lower stringency conditions, and once using higher stringency conditions. In both cases the optimum conditions were not obtained and thus, it was concluded that the optimum parameters must lie somewhere between the parameters attempted. With further optimization and development, the application of RSGP can provide a rapid and inexpensive alternative to current methods used in microbial ecology studies of aquatic environments.

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PREFACE

Why Study Microbial Life?

Microbes are everywhere, literally, from the hottest thermal vents to the coldest reaches of Antarctica, to the most acidic and salty environments. In fact, most microbiologists will not venture to say, without some reservation, what the limits to microbial life are for fear that they too will succumb to the fate of their predecessors whom made such inferences only to be proven wrong. Microbes are an inherent, fundamental part of the global ecosystem.

It is not an overstatement to say that microbes are an essential component to all other life on earth and thus understanding the complexity and nature of these organisms is imperative. It is easy for the non-microbiologist to dismiss such statements as tyrannical rhetoric aimed at inflating the importance of the microbiology field. All scientists see their field of study as being the most important. Perhaps such beliefs have prevented most scientists, except the microbiologist, from seeing the truth. Louis Pasture realized the power of the microbe when he said "The microbes have the last word". In Pasteur's time only a small fraction of microbial life and diversity was understood. Today, microbial life has been more extensively studied. However, only a slightly larger fraction is still understood. .What scientists *do* know is that as microbes become more understood, Pasteur's statement is being proven true. For microbes will truly have the last word. In fact they had the first word. They were the first living organisms on earth and they will undoubtedly be the last organisms on the earth; for they are the ultimate survivors, existing in almost every environment.

Chapter 1

LITERATURE REVIEW

The Development of Methods for Microbial Biodiversity Determination

Diversification of life began approximately 3.5 billion years ago when cells developed a genome and gained the ability to replicate their genetic material. With the development of a mechanism for information storage, came the possibility of mutation and selection; and through this life gained the ability to adapted and survive in an ever-changing environment. This led to the speciation and diversification of today (Stiling, 1996). These early organisms were adapted to the earth's primordial conditions, and through time, changed the environment to make it inhabitable for eukaryotic cells, which further led to the evolution of plants and animals (Staley, 2002).

The importance of studying biodiversity was realized in the late 1950's when a series of publications by R.H. MacArthur (1955; 1957) and G.E. Hutchinson (1959) described diversity as a means to measure processes such as resource partitioning, competition , succession, and community productivity and stability (Morris et al., 2002). However, these studies were limited to plants and animals – the macroscopic world.

It was not until the late 1960's that microbiologists began studying biodiversity and related it to the functions and structures of microbial communities (Hairston et al., 1968; Swift, 1974). However, for several reasons, these studies proved to be much more difficult than diversity studies involving macroscopic organisms. First, there was a lack of existing methods to correctly classify and establish phylogenetic relationships between microbes. At the time, diversity studies were limited to morphologic and physiologic determinations. These methods worked well among

macroscopic organisms however; the simplicity of microbial cell morphology (Woese, 2002), and vast physiologic diversities (van Niel, 1946) rendered these studies misleading and non-informative.

Second, most studies were cultivation-based. Bacteria cultivated from the environmental do not reflect the true biodiversity of the sample (Jones, 1977). Although this may not have been clearly understood at the time, most microbes from the environment are not cultivable using standard techniques (Amman et al., 1995). Thus, studies based on cultivation were incomplete.

Third, existing technology limited the study of microbial communities *in situ*. Understanding microbial diversity requires the ability to examine the microbial life in its natural environment, for the environment and the microbial community exists in an intimate, dynamical nature, constantly redefining and changing each other (Hurst, 1991; Morgan and Winstanley, 1996). Without taking this into account, a true representation of the biodiversity can not be obtained. It was the mid-1970's before methods were developed and implemented which allowed microbial diversity to be more accurately investigated (Woese, 2002).

Revitalized interest in microbial ecology and diversity began when molecular methods, which were more commonly used by evolutionists, were applied to determine microbial relationships. Two new methods were indispensable to the field. The Sanger method, which allowed nucleic acids to be sequenced (Sanger et al., 1975; Sanger et al., 1977), and comparative analysis of molecular sequences, first developed by Zuckerandl and Pauling, (1965). These techniques facilitated higher level classification of bacteria and ultimately freed microbial ecologists from constraints of cultivation by allowing them to study nucleic acids, which could be isolated directly from the environment.

With the molecular tools in place, the focus was on determining phylogenetic relationships between microbes. Many molecular sequences were compared, however, the ribosomal Ribonucleic Acids (rRNAs) proved to be most reliable for phylogenetic studies (Sogin et al., 1972; Fox et al., 1977). Woese (1987) found the small subunit (SSU) rRNAs were relatively easy to sequence and highly reliable for sequence comparison. These molecules were later used to establish a Universal Phylogenetic Tree of Life (Figure 1.1) consisting of three main branches termed domains: Bacteria, Archaea, and Eucarya (Woese et al., 1990).

With the foundations of microbial phylogeny in place, microbial ecologist began to focus on microbial diversity and community structure. Stahl et al. (1985) characterized the microbial community of a hot spring in Yellowstone National Park. This was the first study to used culture-independent methods to characterize a microbial community. Later studies (e.g., DeLong et al., 1989; Risatti et al., 1994) used oligonucleotide probes to detect microbial community structure at numerous taxonomic levels (e.g., species, genus, and family). These methods led the way for many techniques that are now used to study of microbial ecology, some of which will be reviewed in greater detail below.

Methods for Studying Biodiversity

The Purpose of this method review is to compare and contrast current and past methods used to assess microbial diversity and community structure. A brief description of theory and technique followed by a discussion of advantages and limitations is presented for several methods. Therefore, only the most commonly used methods will be discussed here.

Cultivation Based Methods

The classical method for studying microbial diversity is by cultivation of microbial cells on a growth medium followed by counting the number of colonies that are present on the medium after a defined incubation period. Cells can then be isolated and identified by colony appearance, cell morphology, and biochemical properties. Estimated species diversity can be calculated by using traditional diversity models such as the Shannon-Weaver index (Shannon and Weaver, 1963). Early diversity studies (Sieburth 1979; Gunderson et al., 1972) reported that most marine environmental habitats yielded low species diversity and cell numbers. However, direct microscopic counts showed the same environmental samples contained much higher numbers (Jannasch and Jones 1959; Ferguson et al., 1984). Staley and Konopka (1985) termed this phenomena the "Great Plate Count Anomaly" and reported that as little as 1% of the total organisms in oligotrophic environments were being cultivated using the viable plate count method. Studies using culture-independent methods (molecular based studies) have proven this estimate to be accurate not only for marine environments, but for other environments as well (DeLong, 1992; Giovannoni et al., 1990; Fuhrman et al., 1993).

The cause of this phenomenon is still not clearly understood, however, studies have shown that it may be due to a combination of several factors. Some cells enter a viable but not cultivable state when exposed to oligotrophic conditions and/or low temperatures and transition from these conditions to a nutrient rich, culture medium, may inhibit growth. Although these cells may not be actively reproducing and metabolizing, they do still synthesize proteins and take up substrates (Roszak and Colwell, 1987) and where found to utilize significant amounts of nutrients in aquatic environments (Giovannoni et al., 1990). Therefore, these cells are considered to be a major influence in the environmental. Other influences may include the absence of growth factors such

as cytokines, chemical communication *via* methods such as quorum-sensing, or other unknown substances produced by other microbes and macroscopic organisms (Guan et al., 2000; Mukamalova et al., 1998; Breznak, 2002). Although new methods such as producing dilution cultures with sterile sea water have shown some improvements (Schut et al., 1993; Button et al., 1993), biodiversity studies based solely on cultivation methods are rarely used today.

Culture-Independent Methods

Direct Microscopic Counts

The oldest culture-independent method to survey microbial communities is by microscopic examination. Traditionally these examinations are done by using a light microscope. Several staining procedures, such as Gram staining, are used to facilitate cell detection and identification. In theory, counts could be preformed directly on environmental samples, thereby eliminating the need for cultivation and providing a more accurate cell estimation than by cultivation based methods. However, there are many disadvantages to this method. As stated earlier, microbial cell identification by morphology alone can provide misleading results since many microbes have similar morphologies making it difficult to differentiate them taxonomically. Additionally, misidentification of cells from aquatic environments is common, since these environments may contain microbes with nondistinct and/ or variable morphologies, especially cells found in oligotrophic environments (Sieburth, 1979).

Francisco et al. (1973) and Hobbie et al. (1977) used fluorescence stains and an epifluoroescenct microscope to facilitate the direct counting of bacterial cells from filter-concentrated aquatic environmental samples. By using a fluorescent based method, it was easier to detect and count the cells. The use of flurochrome stains such as DAPI, which binds to cellular

DNA, allowed for the direct enumeration of bacteria coupled with the use of flow cytometry making the procedure more automated by eliminating the need for sample filtration and hand counting (Kepner and Pratt, 1994).

The advances in direct microscopic counting methods improved the ability of counting microbial cells and thereby, allowed for a more accurate estimation of total microbial cells in aquatic environments. However, advances in these microscopic methods did little to contribute to the ability to understand microbial diversity by means of taxonomic differentiation; since there is no correlation between morphological diversity and genetical diversity among most microbes. Only until the implementation of molecular based methods such as lipid biomarker determination and nucleic acid differentiation was taxonomic differentiation and diversity estimation possible. The use and continued improvement of these methods has allowed the complexity and diversity of the microbial more accurately understood.

Lipid Biomarkers

All organisms contain lipids within their cells, and some lipids are characteristic of certain groups of organisms. It is possible to determine biomass and biodiversity based upon the analysis of these signature lipids (White, 1994). Phospholipid fatty acids (PLFAs) are common biomarkers which are used in this method. PLFAs are found in the cytoplasmic membrane of viable cells. Upon cell death, the molecules degrade rapidly to lipid diglycerides (White and Tucker, 1969). This property makes it possible to survey the diversity of cells only.

In this method, total PLFA are extracted from an environmental sample using organic solvents and analyzed by gas chromatography. These data can be analyzed in two ways: either as total PLFA patterns characteristic to the entire community, or on an individual PLFA pattern basis

to distinguish the presence of certain microbial groups. Findlay and Watling (1998) used lipid biomarker to determine patterns of seasonal variation in a marine benthic microbial community. They found PLFAs more characteristic to marine algae, were more abundant in colder months than in warmer months and was always more predominant than the PLFAs characteristic to diatoms, which also showed a similar seasonal trend. By using lipid biomarkers, Findlay and Watling (1998) were able to detect community dynamics within the marine environment on a seasonal basis and demonstrate how microbial communities can shift given certain environmental changes.

Lipid biomarkers are an effective way to measure environmental biomass and monitoring community changes among major microbial groups, there are certain disadvantages when using this method; as many microbes may contain overlapping PFLA patterns, so detection below a major group level is difficult (White et al., 1997). Because of this limitation, the use of nucleic acids, that can be more specific at virtually any taxonomic level, are mainly used. These methods have proven very useful for both taxonomic analysis and diversity studies. Several of the most commonly used methods are reviewed below.

Nucleic Acid Based Methods

Denaturant gradient gel electrophoresis

Denaturant gradient gel electrophoresis (DGGE) is a method that is based on the analytical separation of nearly identical fragments of DNA by electrophoresis. In microbial diversity studies, DGGE is used to analyze the Polymerase Chain Reaction (PCR) amplified gene fragments amplified from environmental samples (Muyzer et al., 1993). Fragment separation is based on changes in electrophoretic mobility of DNA fragments in a polyacrylamide gel containing a linearly increasing concentration of DNA denaturants (e.g., urea or formamide). As the DNA

fragments reaches the region of the gel containing sufficient denaturant, denaturation occurs in certain regions termed the melting domains. This slows DNA fragment migration. Sequence variation within each domain alters DNA melting behavior, and sequence variants of the amplification products migrate differently in the denaturing gradient (Lerman et al., 1984; Myers et al., 1987).

DGGE analysis of PCR amplified gene fragments provides a rapid method to estimate community complexity. However, only a specific banding pattern can be established. More specific community determinations (i.e., assigning populations t specific taxa) require additional PCR, cloning and sequencing steps (Muyzer et al., 1993).

Cloning

Pace et al. (1985) were the first to apply cloning methods to study microbial diversity and phylogeny. They used a method termed "Shotgun cloning" to study diversity among planktonic marine environments. In this method, total microbial DNA is isolated and fragmented by restriction enzymes. DNA fragments are ligated into lambda bacteriophage (or plasmid) vectors and transformed into *E. coli* to make a clone library. The library is then screened for the clones containing the gene fragments of interest using a gene-specific probe (Schmidt et al., 1991). The successful clones are then partially characterized to cluster identical clones and then the inserts are sequenced. These sequences are aligned to other sequences obtained from known organisms and the environmental sequences are classified by clustering based on sequence identity. In this manner, phylogenic relationships can be established based on sequence similarity and microbial diversity estimated.

The major advantage to this method of cloning is that no biases are introduced to interfere with the cloning of certain microbial groups and that libraries can be screen for several genes at the

same time (Embley and Stackebrandt, 1991). However, a limitation to this method is the amount of time required to screen the library, as only a small percentage of the clones with have the gene fragments of interest. Schmidt et al. (1991) found that only 0.02% to 0.30% of the total clones contained the rRNA genes, which are most commonly used for phylogenetic studies.

This cloning method was modified slightly by Ward et al. (1990) to retrieve and clone only selected genes from hot springs in Yellowstone National Park. 16S rRNA from the environment was extracted and reverse transcription was used to make complementary DNA (cDNA). The cDNA was then cloned into a vector.

The advantage of using rRNA as the initial template is that organisms which contain a large number of rRNA are more likely to be selected, and the amount of rRNA in a cell reflects the state of metabolic activity. Thus, viable cells will contain more rRNA. Therefore, this method is more likely to detected viable microbes than other cloning methods (Weller and Ward, 1989). The transcription reliability of the enzyme reverse transcriptase is a limitation; as it may not always produce accurate, full-length copies (Weller et al., 1991).

The simplest and most common method of cloning is by adding an initial nucleic acid amplification step by the polymerase chain reaction (PCR). PCR is performed on the extracted community DNA with primers that target the specific gene of interest. The primers can be designed to amplify at different taxonomic levels, from kingdom (DeLong, 1992; Britschgi and Giovannoni, 1991) to family (Giovannoni et al., 1990) to genus or species (Champliaud et al., 1998).

Adding PCR to the cloning method greatly increases cloning efficiency, as all of the successful clones contain the target gene. However, there are limitations to this method. PCR introduces the possibility of chimeric gene sequences (Giovannoni, 1991). Chimeric genes can

form in a mixed population sample when two partially amplified gene segments from different organisms combine to form a single amplicon. The product is a gene segment which is not representative of an actual organism. During subsequent PCR cycles, the gene segment can be amplified to significant numbers thus; during cloning procedures, a large number of clones may contain a DNA sequence representing a non-existent organism. It may be difficult to distinguish chimeric genes from actual genes (Giovannoni, 1991). Another limitation comes from primer-probe annealing affinity inequalities, in which some templates in a mixed population sample will have a higher annealing affinity for the primers than others. This results in biased amplification of some templates over others, and thus a true representation of the community is not achieved (Theron and Cleote, 2000).

Slot- or dot-blot hybridization

Blot hybridizations provide a rapid method for determining microbial diversity without the need for cloning or sequencing. In this method, DNAs are extracted from an environmental sample. These target nucleic acids are denatured and applied to a membrane either in a round (dot) or longitudinal (slot) formation, depending on vacuum chamber configuration (Kafatos et al., 1979). The nucleotides are fixed to the membrane by either heat or UV cross linking and either radiolabeled or fluorescently labeled DNA probes or either partial regions of a gene or the entire gene is hybridized to the target nucleic acids. Quantification can be achieved by comparing probes of specific taxa with universal probes, and the relative abundance of microbes can then be calculated by dividing the amount of taxon-specific probe hybridized by the amount of universal probe hybridized (Theron and Cloete, 2000). This technique allows for the detection of a gene in numerous environmental samples at the same time. However, probing with multiple probes simultaneously is limited and difficult (Wang and Wang, 1995).

Reverse Sample Gene Probing

Reverse Sample Gene Probing (RSGP) is a method similar to slot- or dot blot hybridizations. However, the probe and target relationship is reversed. The genomic DNA from various reference organisms or target genes is denatured and immobilized on a membrane support. DNA extracted from the environment is then labeled and hybridized to the reference DNA (Stahl, 1997).

This method was developed by Voordouw et al. (1993, 1996) to analyze the diversity of sulfate-reducing bacteria (SRB) in oil fields, and has since been mostly limited to this area, although, this method could be applied to numerous molecular microbial ecological studies. It gives more rapid results than cloning with multiple samples, more specific microbial detection than DGGE and lipid analysis, and it has the potential for a wider range of detection than standard slot-blots. Moreover, this method can be used to assess a wide range of environments, including both soil and aquatic.

Whole cell *in situ* hybridization

Whole cell *in situ* hybridization combines microscopic techniques with nucleic acid hybridization, thereby allowing for the detection of single, whole cells, while preserving cell morphology. Giovannoni et al. (1988) demonstrated that fixed, whole cells were permeable to short, radioactively labeled probes and that, under the right hybridization conditions, these probes would hybridize to intracellular nucleic acids. This provided *in situ* cell detection at various phylogenetic levels determined by probe specificity. DeLong et al. (1989) used fluorescently labeled probes instead of radioactively labeled probes, and the technique was termed FISH (fluorescent *in situ* hybridization). The fluorescent technique yielded a much higher resolution and

facilitated detection methods by use of epifluorescence microcopy, flow cytometry or confocal scanning laser microscopy (Amann et al., 1990a; Kenzaka et al., 1998; Caldwell et al., 1992).

There are several advantages to this method. It incorporates both morphology identification and abundance in one procedure. Also, the need for nucleic acid extraction, purification, and amplification is eliminated, thereby removing steps which may bias the results. Likewise, there are several limitations to *in situ* hybridization. Low cell numbers and low numbers of target molecules can produce weak fluorescence intensity (Amann et al., 1995). Probe permeability and probe hybridization may also be affected by cell growth state. Kenzaka et al., (1998) found that as much as 68% of all cells counted from a eutrophic site by staining procedures could be detected using FISH and a universal probe; however, only 39% of all cells from a oligotrophic site hybridized to the same probe. This study suggests that FISH may not be an ideal method for studies in oligotrophic environments.

Molecular Systematics

Molecular Chronometers

Cellular macromolecules can be used as evolutionary chronometers. That is, they can be used to measure evolutionary changes, and infer evolutionary distances between organisms. These relationships are established by measuring the number of differences in nucleotide or amino acid sequences of homologous molecules, assuming that the numbers of sequence differences are proportional to the number of stable mutational changes fixed in the DNA encoding the molecule in organisms that diverged from a common ancestor (Madigan et al., 1997; Pace, 1997). Thus, organisms whose homologous molecules exhibit close similarity are likely to have diverged from a

common ancestor more recently than those who have fewer sequences in common. Based on the evolutionary distance between organisms, a phylogenetic tree is constructed (Logan, 1994).

A molecule must posses several properties to be a molecular chronometer. It must: (1) have universal distribution across the taxa of interest, (2) have functional homology in each organism (3) posses the ability to be aligned for comparison, (4) have a mutation rate that is proportional to evolutionary distance, (5) be of adequate size, and (6) not be influenced by lateral gene transfer (Madigan, 1997; Stahl, 1997).

A limited number of molecules are used as molecular chronometers, including cytochromes, elongation factors and ATPases (Goodfellow and O'Donnell, 1993); however, the most commonly used molecules are the rRNAs. Prokaryotes contain three types of rRNA molecules: 5S (approximately 120 nucleotides), 16S (approximately 1500 nucleotides), and 23S (approximately 2900 nucleotides). The homologous rRNA molecules in eukaryotes are slightly larger and are termed the 5.8S (approximately 156 nucleotides), 18S (approximately 1900 nucleotides), and 28S (approximately 4700 nucleotides) respectively.

Of these rRNAs, the 16S and the 18S, (the small subunit [SSUs] rRNAs) have proven to be the most useful as chronometers because they are much easier to sequence entirely than the 23S or 28S molecules, and provide significantly more phylogenetic information than the 5S or 5.8S rRNAs. The SSUs are ideal for phylogenetic work because they contain both conserved and variable regions. Conserved regions are more slowly evolving sections that facilitate alignments and are ideal for phylogenetic comparisons at higher taxonomic levels, e.g., domain and kingdom. The most variable regions are more rapidly evolving and are useful for lower taxonomic determinations, e.g., genus and species.

The Universal Tree of Life

Woese et al. (1990) described all life on earth as belonging to one of three Domains: Bacteria, Archaea or Eucarya. Each domain includes several kingdoms. Based upon sequence analysis of 16S and 18S rRNA of a wide range of prokaryotic and eukaryotic organisms, a universal phylogenetic tree of life was constructed (Figure 1.1).

The Bacterial Domain

Woese (1987) initially described The Bacteria as containing 12 major groups based on 16S rRNA analysis, however, Hugenholtz et al. (1998) reported that at least 24 additional major groups exist, most of which have no cultured representatives. This study was limited to the 12 original groups proposed by Woese and therefore, only those groups will be discussed here.

Proteobacteria. The Proteobacterial kingdom is comprised of five subclasses (alpha, beta, gamma, delta and epsilon) and represents the largest, most physiologically diverse group of all currently known bacteria (Madigan et al., 1997; Logan, 1994). All genera within this kingdom are Gram-negative (i.e., containing several layers of lipoproteins, lipopolysaccharieds, and phosolipids, but only a single layer of peptidoyglycan in their cell walls). It includes representatives that are: phototrophic (anoxygenic and oxygenic), non-phototrophic, pathogenic, sulfur oxidizing, and sulfur and sulfate reducing among others.

Gram-positive bacteria. This kingdom is distinguished from the proteobacteria by its cell wall characteristics (containing a thick layer of peptidoglycan), and can be divided into subdivisions based on DNA base composition, those species containing a high molar percentage (> 50%) of G-C pairs (high GC) and those species with low molar percentage)(< 50%)of G-C pairs (low GC). The high GC subdivision (Actinobacteria) consists primarily of species that are

pleomorphic or exhibit branching growth. They are aerobic and common in soil. The low GC subdivision contains genera that are aerobic, anaerobic and some endospore-formers and these organisms live in a variety of habitats from soil to human skin (Woese, 1987, Logan, 1994).

Cyanobacteria. A large morphologic and physiologic diversity exists within this kingdom. However, all members are oxygenic phototrophs that utilize chlorophyll *a* as a photosynthetic pigment. Cyanobacteria are important contributors to carbon and nitrogen cycling, especially in aquatic environments (Graham and Wilcox, 2000). This kingdom includes the closest common ancestor of the chloroplast of green plants and green algae (Logan, 1994).

Green Sulfur Bacteria. This kingdom consists of anoxygenic phototrophs that gain energy by oxidizing reduced sulfur compounds under anaerobic conditions. Species in this group are usually found in anoxic regions of aquatic environments were sufficient light is available for growth (Woese, 1987). Some planktonic species possess gas vesicles which allow them to move in response to changes in light and H_2S levels (Lansing et al., 1999).

Spirochetes. This kingdom is comprised of helical and coiled bacteria with one or more polar flagella that wrap around the cells inside the Gram-negative-like outer membrane. Movement is facilitated by the flagella and by cellular flexing and rotation. The habitat range of these organisms is vast, ranging from fresh and marine waters, to soils and sediments, to parasitic relationships with insects, molluscs and mammals (Woese, 1987; Logan, 1994). The best known member of the kingdom is *Treponema pallidum*, the causative agent of syphilis.

Cytophaga-Flexibacter-Bacteriods (CFB). This kingdom contains a mixture of strict anaerobes and strict aerobes. Gliding bacteria are found within the genera *Cytophaga* and *Flexibacter* (Madigan et al., 1997). Members of *Bacteroides* can be found in the oral cavity and intestinal tract of humans and other animals where they benefit the host by degrading cellulose and

other complex carbohydrates. *Cytophaga* species also degrade complex carbohydrates and are commonly found in soil and aquatic environments were they contribute significantly to cellulose decomposition.

Deinococcus-Thermus. The Deinococci are unique in that are highly resistant to radiation and desiccation. These organisms have an unusual ability to repair damaged DNA even when it has been fragmented (Madigan et al., 1997). *Deinococcus radiodurans* can withstand 50-100 times more ionizing radiation than most other bacteria (Daly et al., 1994). *Thermus* species are thermophilic, hot spring dwellers and are of scientific interest because they produce heat stable enzymes (Madigan et al., 1997). For example, A DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) is commonly used in PCR reactions.

Green Non-Sulfur Bacteria. This kingdom contains only three genera *Chloroflexus*, *Heliothrix* and *Herpetosiphon*. It includes phototrophic bacteria that do not oxidize sulfide to obtain energy. *Chloroflexus* species are thermophilic, photoorganotrophs or chemoorganotrophs depending on environmental conditions, and can be seen forming orange-reddish mats in neutral to alkaline hot springs (Lansing et al., 1999). *Heliothrix* are photosynthetic and are commonly found in lakes where sufficient light reaches the anoxic zone (Woese, 1987; Logan, 1994). *Herpetosiphon* are not photosynthetic and are common in soil (Lansing et al., 1999).

Planctomyces. Microbes within this kingdom are mostly aquatic and reproduce by budding. They are unusual among bacteria in that they lack peptidoglycan in their cell walls (Madigan et al., 1997). These bacteria are common in aquatic habitats, and contain flagella and stalk structures that facilitate cell attachment to surfaces (Schmidt and Star, 1989).

Thermotogales. Species within this kingdom have been isolated from hyperthermophilic marine sediments and geothermally heated soils. These organisms have unique cell wall

structures, in that they lack peptidoglycan and contain large amounts of proteins, and cells are enclosed in a loose sheath (toga-like) envelope. Metabolically, *Thermotogales* are fermentative chemoorganotrophs (Madigan et al., 1997; Logan, 1994).

Aquificales. This is the earliest branching bacterial kingdom from the common ancestor of the Bacterial domain. These microbes are hyperthermophilic, chemolithotrophs that generate energy by oxidizing hydrogen or sulfur compounds. There are only nine known genera in this group and all are physiologically similar (Madigan et al., 1997). *Aquifex* species are the most thermophilic of all known bacteria. They have optimum growth temperatures up to 95°C (Staley, 2002).

The Archaeal Domain

The Archaea are often characterized by growth under extreme conditions, although some types are abundant in non-extreme habitats. They have unusual metabolic characteristics and cell structures. These prokaryotic microbes lack peptidoglycan in their cell walls and have unique ether-linked lipids (unlike Bacteria and Eukarya which have ester-linked lipids). The Archaeal domain includes microbes that are hyperthermophiles, methanogens (produce methane gas from CO_2 reduction), extreme halophiles and acidophiles. The domain is comprised of three kingdoms: Crenarchaeota, Euryarchaeota and Korarchaeota (Staley, 2002).

Crenarchaeota. This kingdom contains mostly hyperthermophiles found in geothermally heated soils and waters containing sulfur or hydrogen sulfide (Fuhrman et al., 1992). These microbes are mostly obligate anaerobes and have an optimum growth temperature above 80^oC. New species are regularly discovered in this kingdom as more extreme environments are explored (Logan, 1994).

Euryarchaeota. This kingdom contains methanogens and halophiles. The methanogens are strict anaerobes obtaining energy by oxidizing hydrogen or simple organic compounds. They use the electrons generated to reduce carbon dioxide to methane. These organisms have been found in aquatic sediments, the intestinal tracts and rumens of animals, sewage sludges and oil field soils (Madigan et al., 1997). Halophiles can live in salt lakes, soda lakes, or heavily salted foods in which the sodium chloride concentration is greater than 8% (Logan, 1994).

Korarchaeota. This kingdom is comprised of microbes that have only been identified from 16S rRNA sequences obtained from terrestrial hot springs and marine waters. Their function in the environments is unknown, and there are no culture representatives for this kingdom (Madigan et al., 1997).

The Eukaryal Domain

The Eukaryal domain includes plants and animals, as well as several kingdoms of microorganisms. The microbial kingdoms include microscopic algae, protists and microscopic fungi However, only the algae and protists were included in this study. The diversity of eukaryotic microbes is vast and not well understood (Sogin, 1996). In general, they have 80S ribosomes, a nuclear membrane and intracellular organelles, e.g., mitochondria and chloroplasts and; based on 18S rRNA sequences, they appear to be more closely related to Archaea, than Bacteria (Staley, 2002).

Algae. Eukaryotic algae make up a large fraction of primary producers in aquatic environments (Graham and Wilcox, 2000; Ford, 1993). These organisms can be found in virtually any aquatic environment from cold arctic oceans to temperate waters. Microscopic algae can vary in organization from unicellular to multicellular to filamentous clusters. All algae contain plastids containing chlorophyll, however, some groups contain several accessory pigments that may mask

the chlorophyll, and therefore, give the cell a color other than green (Graham and Wilcox, 2000; Sorokin, 1999). Generally, algae are grouped by pigment color. This study included brown algae, red algae, green algae, and diatoms.

Protozoa. Protozoans (protists) are unicellular eukaryotic microbes that lack cell walls, and are motile by the use of a flagella, cilia, pseudopodia, or are non-motile. This kingdom is comprised of several separate lineages, but is usually combined together because of the common characteristics described above. Protists are found in a variety of freshwater and marine environments, soils, and as parasites in or on other organisms (Madigan et al, 1997). Most protists are primarily heterotrophic, although some genera, e.g., *Euglena,* contain chloroplasts and can exist as phototrophs (Graham and Wilcox, 2000). Aquatic protists are important components in the food web, for they act as predators on smaller eukaryotes and prokaryotes, and prey for larger invertebrates and vertebrates (Sorokin, 1999). This study was limited to the study of the following protozoans: ciliates, cryptomonads, dinoflagellates and euglenids.

Pathogen Detection in Aquatic Environments

Many waterborne pathogens are propagated through the intestinal tracts of humans and animals. They include commonly bacteria, protozoans and viruses. These organisms cause gastrointestinal illnesses and in severe cases, can cause death. Waterborne contamination occurs through fecal pollution of the water either from natural or anthropogenically-introduced sources. Several factors determine whether transmission will occur: (1) the concentration of pathogens in the water, (2) the infectious dose of organisms, and (3) the amount of exposure to the contaminated water (Moe, 1997).

Several methods are used for the detection of waterborne pathogens. However, most of these methods are time consuming, as they involve cultivation and/or microscopic identification. Identification by molecular methods such as oligonucleotide probing or PCR is becoming more popular, since it gives rapid results, allows for the detection of low cell numbers, and the detection of numerous pathogens simultaneously (Kaucner and Stinear, 1998). Additionally, probes or primers can be constructed that detect only pathogenic species or strains, something that may not be possible using other, non-molecular, methods.

Escherichia coli

Most strains of *Escherichia coli* are not pathogenic and exist in commensalistic or synergistic interactions the intestines of humans and warm blooded animals. However, some strains are pathogenic, causing severe gastrointestinal illnesses. The most severe infections come from the enterohemorrhagic strains. Among these, *E. coli* O157:H7 is the most well known. This strain not only adheres to and invades host tissue, but produces toxins that kill host cells. Host cell death leads to hemorrhagic colitis and possibly to hemorrhagic uremia and even death (Madigan, et al., 1997). These toxins are termed shiga-like toxins (STX) and are encoded by the *stx*I or *stx*II genes. Molecular probing for these genes allows pathogenic strains of *E. coli* to be distinguished from nonpathogenic strains, assuming that only the pathogenic strains will contain the toxin genes. The nucleic acid sequence for these genes contains some highly variable regions, but a few conserved regions are present and thus, it is possible to construct PCR primers which will amplify portions of both genes (Read et al., 1992). Primers amplifying conserved regions or these genes were used in this study.

Giardia lamblia

Giardia lamblia is a flagellated protozoan that is commonly found in freshwater streams and rivers. The organism exists in the environment in cyst form and germinates upon entering the intestinal tract of a warm-blooded animal. The differences between the pathogenic species *G. lamblia* and the non-pathogenic *G. muris* are difficult to distinguish, even at a molecular level, as many genes are too similar (Kaucner ad Stinear, 1998). However, the giardin (*grn*) gene, which encodes for a cytoskeleton component, does have some variability between the two species. This makes it an ideal gene for primer and probe development (Kaucner ad Stinear, 1998). Primers amplifying this region of the *G. lamblia* genome were used in this study.

Cryptosporidium parvum

Cryptosporidium parvum is also a protozoan parasite in vertebrates with a life cycle similar to *G. lamblia*, in that it exists as an oocyst while in the environment and germinates in the intestines of the host animal. Only those who are immunocompromised are at risk of serious, life-threatening symptoms. Healthy individuals experience only mild symptoms. Of the eight species of *Cryptosporidium*, only *C. parvum* is pathogenic to humans. Thus, it is important to be able to differentiate between species present in aquatic habitats. Primers (CpR1f and CpR1r) amplifying regions of the repetitive oocyst gene have been shown to differentiate between several species (but not all) of *Cryptosporidium* (Champliaud et al., 1998). These primers were used in this study.

Entamoeba histolytica

Entamoeba histolytica is also a protozoan that produces a cyst that germinates within the epithelial lining of the intestine. Symptoms include abdominal cramping and severe bloody diarrhea. An infection of this type is often called amoebic dysentery. EH1 and EH2 (Hauge et al., 1998) primers targeting 18S rRNA were used in this study as a means to detect this organisms.

Study Objectives

The purpose of this study was to generate a gene grid for hybridization purposes that included specific microbial gene probes at known locations. Total environmental DNA could then be isolated, radioactively labeled and hybridized to the grid. The pattern of positive hybridizations could then be used to determine what taxa of microbes were present in the original water sample. The addition of gene probes for important functional genes and human pathogens was indented to reveal important information about the health and safety of the water. This work lays the foundation for the environmental DNA grid by identifying specific probes and determining the appropriate conditions for probe preparation.

Chapter 2

METHODS

Study Area

Microbial studies were conducted on a one-mile section of Mill Creek located within the future Tolsia Highway (US 52) wetland mitigation site in Wayne County, West Virginia. The mitigation area encompasses an approximate 40-acre floodplain section of Mill Creek that is characterized by a mixture of open fields, cropland and bottomland hardwood forest. Although the area was previously used for cattle grazing and hay cultivation, the site is currently relatively isolated from human impact (WVDOH, 1995), except as noted below.

The mitigation site has been subdivided into five cells (termed A-E). Sections of the stream within each cell will be dammed to serve as the water source for the wetland. Water samples were taken from the section of the stream that was near the approximate middle of each cell (Figs. 2.1 and 2.2).

Site A. Collection site A (38º 04' 50" N, 82º 32' 29" W) was in a riffle section of the stream and was approximately 9 cm deep at normal flow. The water in this area contained an unusual red precipitate, red slimes and oily rock coating. Microscopic examination revealed that the precipitate and slimes contained microorganisms characteristic of unpolluted anoxic waters containing iron (Emersion and Revsbech, 1994). It was speculated that the iron was seeping from a sandstone cliff near the stream's edge. The stream bed was composed mostly of rock.

Site B. Collection site B (38º 04' 56" N, 82º 32' 29" W) was in a riffle section of the stream and was approximately 6 cm deep on average. Stream flow was impeded by the presence of vegetation during the spring and summer. The streambed was composed of fine silt.

Site C. Collection site C (38º 05' 05" N, 82º 32' 31" W) was in a riffle section of the stream and was approximately 15 cm deep on average. The streambed was composed of mostly rock and some fine sediment.

Site D. Collection site D (38º 05' 11" N, 82º 32' 25" W) was in a riffle section of the stream and was approximately 5 cm deep on average. The streambed was composed of mostly rock and some fine sediment. This area was close to an illegal trash-dumping site and numerous tires, rusting debris and trash were present in the stream during the sampling period.

Site E. Collection site E (38º 05' 15" N, 82º 32' 28" W) was in a pool section of the stream approximately 2 m deep on average. This area contained less foliage cover and thus received more sunlight than other sites. The area also contained several fallen trees in the stream, which served as a habitat for aquatic macrovertebrates (i.e., turtles and fish).

Environmental Sample Processing

Sample collection

Water samples (> 500 ml) were collected in sterilized 1 L Nalgene Polycarbonate bottles at each site (A-E) at least once per month from July 2001 through June 2002 (Table 2.1). Only surface water was collected and sediment was avoided as much as possible. Water samples were placed on ice until filtered at Marshall University (approximately one hour later), or were immediately filtered on-site. Samples were filtered using 250 ml pre-sterilized, disposable filter units containing a nitrocellulose membrane filter (47 mm diameter, 0.2-µm pore size) (Nalgene) until filter was clogged (approximately 250-500 ml depending on sample turbidity). Filters were stored in 50 mm petri dishes at -20ºC until further processed.

Samples collected on 5 dates were further processed. These sample dates were chosen by graphing the mean daily temperature (National Weather Service, www.nws.gov) for the duration of sample collection and establishing a weather trendline for daily mean temperatures (Fig. 2.3). Sampling dates closest to the trendline representing the high, moderate and low temperatures (Table 2.1) where chosen.

Optimization of DNA Extraction by Sonication

All glassware used during sonication was thoroughly cleaned by overnight submersion in a sulfuric acid bath, followed by rinsing in distilled water and baking at 250ºC. Glassware was then inverted for 1 hour in an oven and then covered and stored upright overnight or until used. All instruments that were in contact with samples (including sonicator probe), were immersed in 1 N HCl, rinsed with sterile water, immersed in 10 N NaOH, rinsed again in sterile water and then stored in 95% ethanol. Cleaning was repeated before each sample was sonicated. All samples were kept on ice throughout the sonication procedure to inhibit nuclease activity and avoid heat denaturation of nucleic acids during sonication.

Membrane filters (seven) carrying environmental sample retentates were placed in previously sterilized beakers containing 9.0 ml of lysis buffer (Appendix A) per filter and sonicated for a range of 0-120 seconds at 20 second intervals (one filter per each sonication time) using a Tekmar 600 Ultrasonicator with a 10.2 mm diameter probe, while maintaining a 15 power monitor reading (approximately 90 Watts). During sonication, the probe was immersed at least 3 cm into the liquid and centered over the filter to reduce occurrence of sample foaming. All sample vessels were placed in a cold ethanol bath continuously bubbled by dry ice to reduce temperature elevation. The sonicated samples (including all filter particles) were transferred to 25-ml polypropylene screw-cap centrifuge tubes. Probe and beaker walls were rinsed with 1 ml of sterile

1× SSC, which was added to the centrifuge tubes. Tubes were immediately frozen (−70°C) until further use.

This procedure was repeated again, sonicating in 24 ml of lysis buffer over a time range of 80-180 seconds. The process was repeated for a third time using 35 ml lysis buffer over a time range of 60-220 seconds. Sonicated samples were frozen (−70ºC) until further use for DNA concentration determinations.

Frozen sonicated samples were thawed in a 50°C water bath and divided into 15 ml aliquots in glass 25-ml Corex ® II centrifuge tubes. The crude lysates were extracted with an equal volume (15 ml) of phenol: chloroform: isoamyl alcohol (Appendix A). Tubes were centrifuged at 7,000 x g for 10 minutes and the aqueous phases were transferred to 30 ml polycarbonate centrifuge tubes. Partially purified DNAs were ethanol precipitated by the addition of 1/10 volume of 7.5 M ammonium acetate, and an equal volume of cold isopropanol, and stored overnight at −20°C. DNA was pelleted by centrifugation at 16,000 × g for 30 minutes at room temperature. The liquid was discarded and pellets were washed with 70% ethanol, centrifuged again for 15 minutes, decanted, and dried. Pellets were resuspened in 100 µl of TE buffer pH 8.0 (Sambrook et al., 1989).

DNA was quantified using a TD-360 Fluorometer (Turner Designs, CA) containing a 360 nanometer wavelength excitation filter and a 460 nanometer wavelength emission filter, a Fluorescent DNA Quantification Kit (Bio-Rad, Hercules, CA) and 1 cm^2 methacrylate fluorescence cuvettes. A standard curve was generated (Fig. 2.4) and quantifications were carried out according to the manufacture's instruction for a 0.1 µg/ml Hoechst 33258 dye solution and low range DNA (10-500 ng/ml) detection.

Primer Design

All primers used in this study were either constructed *de novo*, taken from previous literature, or adapted from previously described primers. Primers taken from previous literature were checked by aligning (ClustalX 1.8, Thompson et al., 1994) all gene sequences available (or one representative sequence from each genus available) from the National Center for Biotechnology Information (NCBI) GenBank database for the gene of interest with the primers. Any sequence discrepancies were eliminated by adapting the primer sequence to the aligned sequences. Primers constructed for this study were done so by searching for conserved regions within the aligned sequences and avoiding areas that would require numerous degeneracies within the primers. Primers were synthesized at the Marshall University DNA Core Facility, Huntington, WV. See Table 2.2 for primers used in this study.

Microbial Group Detection

The 16S rRNA gene was chosen for the microbial taxon amplification portion of this study because of the abundance of sequences available for this gene. Upon alignment, the Universal primer pair 530f and 907r (Lane, 1991) proved to be most useful for this study because the primers amplify a 341 base pair hypervariable region flanked by two highly conserved regions (primer locations). Primers were adapted for some groups to reduce degenerate pairing and increase specificity.

The consensus sequence for Crenarchaeota revealed sequence discrepancies between the 530f primer and the template at the third priming position. Therefore, 530f-cren (see Table 2.2 for primer sequence) was designed and synthesized. Consensus sequences for both the Euryarchaeota and Crenarchaeota showed a thymine deletion at primer position 13, as well as, an adenine

substitution for guanine at primer position 16 on 907r. To avoid potential problems, primer 907r-eury/cren was made (Table 2.2).

The epsilon-Proteobacterial group showed that the consensus sequence at the 907r priming location contained one substitution; the sixth primer position contained a thymine instead of adenine (Appendix B). Primer 907r-epsilon (Table 2.2) was synthesized to amplify epsilon-Proteobacterial sequences.

The euglena group had a cytosine substitution at the tenth priming position of primer 530f. Primer 530f-euglena (Table 2.2) was constructed to amplify euglenoid sequences.

Detection of Functional Genes and Microbes of Health Concern

Primers used for the detection of functional genes and pathogenic microbes were designed as stated above; however, genes segments which were highly conserved for a particular function, toxin, or protein (depending on gene of interest) were chosen and Genbank sequences for the corresponding genes were retrieved and aligned. The most conserved regions that would yield the largest gene fragments were chosen. For primers used in the detection of functional genes and pathogens, in most cases, few or no adequate sequences were available on GenBank for alignments. Therefore, primers were checked for priming specificity by performing an NCBI blast. All primers sequences returned no matches (in the case of genes with no sequences available), or resulted in matches only to the target organisms. A sufficient number of full length sequences were found for the large subunit of Rubisco, therefore, these primers were aligned to check for accuracy. Alignments revealed that the *rbc*L gene was highly conserved among both eukaryotic and prokaryotic organisms, with only a few variable regions. The primer pair rub1 and

rub2 (See Table 2.2 for primer sequence) was found to amplify a 395 bp conserved region of the *rbc*L gene.

Pure Culture Processing

Harvesting Cell from Reference Cultures

Representative pure culture, reference strains from each phylogenetic group of interest were obtained from type culture collection (see Table 2.3). Cultures received on a solid medium were removed from the medium with a sterile swab and suspended in a 1.5 ml microcentrifuge tube containing 1 ml of sterile water by plunging the swab into the liquid several times until the suspension was visibly turbid. Freeze-dried cultures were resuspended in 1 ml of sterile water and 500 µl of the suspension was transferred to a sterile 1.5 ml microcentrifuge tube. The remaining culture was stored at −70ºC until further use. Liquid cell suspensions were pelleted by centrifugation at 20,000 × g for 10 minutes. The liquid was removed and the pellet resuspended in 100-500 µl (depending on pellet size) of TE buffer and stored at −20°C until further use.

DNA Isolation

DNA was isolated using either a DNeasy® Tissue Kit (Qiagen, Valencia, CA) or by Chelex extraction (de Lamballerie et al., 1992). DNeasy® isolations were carried out as specified by the manufacturer. For samples extracted by Chelex, 50 µl of a 5% Chelex suspension was added to 50 µl of cell suspension in a 1.5 ml microcentrifuge tube. Tubes were incubated in a dry bath at 56ºC for at least 1.5 hours for Gram-negative bacteria and eukaryotic cells, and overnight for Gram-positive bacteria. Following incubation, tubes were boiled for 8 minutes then

immediately centrifuged at 16,200 x g for 5 minutes. The supernatant was transferred to sterile 1.5 ml microcentrifuge tubes and stored at −20ºC.

DNA Amplification and Verification

DNA was amplified by the Polymerase Chain Reaction (PCR) using primers targeting a specific gene in the corresponding DNA template (Tables 2.2 and 2.3). Amplification mixtures were prepared using either a PCR Core Kit (Roche, Mannheim, Germany) as specified by the manufacturer or as given in Table 2.4. Amplifications were carried out in a Bio-Rad Gene Cycler™ with the following parameters: 1-5 minute hot start at 94°C (during which Taq Polymerase was added) followed by 30 cycles of denaturation (1 minute at 94°C), annealing (1.5 minute [temperature varies with primer pair, see Table 2.3]), and polymerization (2 minutes at 72°C), with a final cycle including an extended polymerization at 72°C for 10 minutes. After amplification was complete, amplicons were stored at −20°C. Amplifications were repeated either using fresh DNA template or previous amplicons until a total of > 2,500 ng of DNA was obtained for each organism.

PCR Optimization

Several optimization steps were used to amplify those templates that did not amplify under standard conditions (Fig. 2.5). Optimization included one or a combination of the following parameters: titration of Mg^{2+} concentration, titration of DNA concentration, and varying annealing temperatures using touchdown PCR.

Templates requiring Mg^{2+} titration were amplified as previously described, except reactions were set up with 0.5, 1.0, 1.25, 1.50, 1.75, 2.0, and 2.5 mM of $MgCl_2$. Amplification results were

analyzed by agarose gel electrophoresis. The Mg^{2+} concentration resulting in the most effective amplification was used for subsequent amplification of the corresponding template.

Templates requiring titration of DNA concentration were amplified as previously described except template concentrations were varied by adding 1, 10, and 20 µl of template DNA stock for each template in question. Amplification success was analyzed by agarose gel electrophoresis. The concentration yielding the best amplification was used for subsequent amplifications of that template.

Templates optimized by touchdown PCR were amplified as previously described except variable annealing temperatures were used. The annealing step contained several cycles, the temperature during the first cycle was 5°C above the estimated primer pair annealing temperature. During each following cycle, the temperature was reduced by 1°C (for 1.5 min) until the temperature had reached 5°C below the estimated annealing temperature.

Following the touchdown cycles, 30 rounds of standard amplification were done as previously described. For some templates, PCR optimization was unsuccessful and no amplicons could be obtained. For these instances, the reference organism (and the corresponding group or gene) was eliminated from the study.

Preparative Agarose Gel Electrophoresis of Amplicons

Amplicons were purified by agarose gel electrophoresis as follows. Amplicons of the same gene fragment were pooled (total volume ≤ 800 µl) and 1/10 volume of 10× loading dye (Appendix A) was added. The samples were transferred to a 1.2% low melting point agarose gel containing ethidium bromide (Appendix A) made with a 12 × 0.5 cm sample well. A 1 kilobase DNA ladder (Promega, Madsion, WI) was used as a standard to determine fragment length. DNA fragments

were resolved by electrophoresis using TAE buffer (Appendix A) and a Bio-Rad Sub-Cell® GT with Bio-Rad Power Pack Junior power supply at 100 V. Gels were viewed using an Ultra-Violet Products Transilluminator at 302 nanometers and photographed using Polaroid-Documentation Camera containing a Tiffen® 40.5 mm deep yellow filter with Polaroid type 667, ISO 3000 black and white film.

Agarose Gel Extraction Method Comparison

DNA bands of the correct size were excised from the agarose gel using a sterile razor blade, placed into pre-weighed sterile microcentrifuge tubes and extracted using the MiniElute™ Gel Extraction Kit (Qiagen) according manufacturer's directions for the. Alternatively, DNA was recovered by the freeze squeeze method. In the latter method, the gel fragments were completely melted at 65°C and 200 µl of phenol was added while the agarose was still molten. The tube was vortexed and incubated at 70°C for 5 min., then centrifuged for 15 min at full speed in an microcentrifuge tube. After centrifugation, the aqueous layer was removed and held on ice, and the residual agarose was saved. TE buffer (200 µl) was added to the agarose and phenol remaining in the tube and heated at 65°C for 4 min. The tube was centrifuged for 15 additional minutes. The aqueous phase was recovered and added to the previously recovered aqueous phase on ice. The total aqueous phase was extracted twice with equal volumes of phenol, ethanol precipitated as described above. Pellets were resuspended in 10 µl of sterile H_2O. Extracted DNA from both methods was resolved by electrophoresis in agarose gels and banding patterns were visually compared to determine which method had the greatest extraction efficiency. In most cases, the Qiagen Kit provided superior recovery, therefore, DNA extraction of reference cultures was carried out using the MiniElute™ Gel Extraction Kit and fluorometrically quantified as described above.

Reverse Sample Gene Probing

Membrane Preparation

Membranes with purified reference DNA (target DNA) were prepared as follows. Using a 24-well Hybri-Slot™ filtration manifold (Life Technologies). The manifold was thoroughly cleaned by submersion in 1.0 N NaOH, followed by rinsing in sterile water. Nylon membranes (Micron Separations Inc.) were first wetted in sterile water at room temperature and then soaked in 20× SSC (Appendix A) for 1 hour at room temperature. Two sheets of blotting paper (Whatman 3mm filter paper) per membrane were soaked in 20× SSC for 10 minutes. The blotting paper and membrane were placed into the filtration manifold and individual slots were filled with 800 µl of 10× SSC and the buffer was pulled through the membrane by applying a vacuum to the base of the manifold. The 10× SSC wash was repeated.

Target DNAs (100 ng/ 40µl) were denatured by the addition of 10 µl 1 N NaOH and incubated for 10 minutes. DNAs were then neutralized by placing samples on ice and adding equal volumes of cold 10× SSC, and then vacuum blotted immediately (See Fig. 2.6 for membrane configuration). Wells were rinsed with an additional 2 ml of 10× SSC per well. The membrane was dried under continuous vacuum for 5 minutes and then baked overnight at 37°C between 2 sheets of blotting paper. Membranes were stored at −20°C until used.

Probe DNA Labeling

Environmental DNAs (Probes) were radioactively labeled by random prime DNA labeling using [α-^{35}S]-Deoxycytidine-5- triphosphate (^{35}S-dCTP) (ICN, Irvine, CA) using a High Prime DNA Labeling Kit (Roche) as specified by the manufacturer for 25 ng of template DNA.

Slot-blot Hybridization Comparison

Membranes with bound target DNA were pre-hybridized in 50 ml Bellco AutoBlot® jars containing 4.6 ml (1 ml per cm^2 of membrane) of Blotto (Appendix A) and incubated overnight at 65°C in an AutoBlot® Micro Hybridization Oven (Bellco). Probes were added to the hybridization jar and incubated at 65°C for ($3\times C_o t_{1/2}$), as determined by the equation:

$$\text{Hours to } C_o t_{1/2} = (1/X) \times (Y/5) \times (Z/10)$$

Where X is equal to the weight of the probe in μg, Y is equal to the length of the probe in kilobases and Z is equal to the volume of the reaction in ml. Membranes were washed 6 times for 5 minutes each with wash reagent (Appendix A) at 65°C and then counts were checked using a Geiger-Mueller (GM) counter. Membranes were air dried and then exposed to Kodak Biomax MR Film to produce autoradiographic records.

To compare different slot-blot hybridization methods, several steps were modified (e.g., membrane filter material, hybridization time, and wash stringency). Hybond-N membranes (Amersham Pharmacia Biotech) were used as given by the manufacturer for slot-blot hybridization using the same DNAs given above. Hybridization was carried out as follows. Membranes were pre-wetted in water and then in pre-hybridization buffer (Appendix A). Membranes were placed in hybridization tubes and 10 ml of pre-hybridization buffer was added and pre-hybridized for 30 min

at 65°C in a hybridization oven. Labeled probes (as previously described) were added and allowed to hybridize overnight. Membranes were washed (1) briefly in 2× SSC, 0.1% SDS, (2) twice for 5 min each in 2× SSC, 0.1% SDS, (3) twice for 10 minutes each in 1× SSC, 0.1% SDS, (4) four times at 5 min each in 0.1× SSC, 0.1% SDS. All wash steps were conducted at 65°C using wash buffer solutions that were pre-warmed to the same temperature. X-ray film exposure was carried out as previously stated. Hybridization results were compared for both protocols by visually comparing autoradiographic records.

Chapter 3

RESULTS

Optimization Results

Sonication Optimization. Sonication of membrane filters in 9, 24, and 35 ml of 1x SSC buffer showed similar extraction trends over a time range between 0 and 220 seconds (Fig. 3.1). Each volume showed an optimal sonication time where a maximum amount of DNA was extracted from the cell, and any further sonication beyond this time yielded less detectable DNA. For filters containing 9 ml of buffer, the optimum sonication time was 60 seconds. For filters sonicated in 24 ml and 35 ml of buffer, optimum times were 120 and 180, respectively. Sonication for 180 seconds in 30 ml of buffer was used for subsequent sonications as this volume was adequate to reduce the frequency of foaming (aeration) that occurred during the procedure.

Total DNA Extraction from Environmental Samples. DNA was extracted from filtered samples for the dates given in Table 2.1 and total DNA in each environmental sample was determined (Fig 3.2). The least amount of DNA was extracted on July 31, 2001. All sites yielded nearly the same quantity of DNA (approximately 3.0×10^2 ng /ml), except site E which contained considerably less (0.6×10^2 ng /ml). The five January 2002 samples resulted in the second lowest quantity of DNA. On this date, site E again had the lowest amount of DNA (1.3×10^2 ng /ml) and the remaining site had variable concentrations ranging from 5×10^2 to 3×10^2 ng /ml. October 2001 and April 2002 showed similar overall DNA concentrations as well as similar concentration trends among the individual collection sites (ranging from 2.0×10^2 to 9×10^2 ng /ml). June 2002 yielded the greatest concentration of DNA with site A have the highest (2.3×10^3 ng /ml) and site D having the lowest (1.1×10^3 ng/ml).

Primer Design. Sequence fidelities were checked for each primer pair (Table 2.2, Appendix B) used in this study, except for those genes which had few or no sequences available in GenBank. The universal primer pair 530f and 907r was found to amplify a fragment of approximately 341 bp for all groups of interest to this study. Primer and sequence alignments revealed that the annealing locations of these primers were highly conserved, however, the sequences between the priming locations was variable, thus making this primer ideal for producing a taxon –specific amplicon. Some groups did show degenerate primer annealing sites, so new primers were synthesized for those groups with numerous or destabilizing mismatches.

PCR optimization. Table 3.1 gives amplification parameters and optimization conditions for DNA templates that successfully amplified and were further used in this study. Table 3.2 gives attempted amplification parameters and optimization steps of templates eliminated from the study. Fig. 3.3A shows *Campylobacter fetus* amplicons titrated with Mg^{2+} concentrations between 0.5 and 2.5 mM. The gel reveals that 1.5 mM of magnesium worked the best and that concentration above that amount produced results below the detection limits of the gel (approximately 10 ng total of DNA). Fig. 3.3B shows *Actinomyces israelli* amplicons titrated with Mg^{2+} concentrations between 1 and 5 mM. For this template, 1 mM Mg^{2+} yielded the best amplification. Fig. 3.4 shows templates amplified by touchdown PCR that would not amplify under standard PCR conditions. Several bands are apparent on most of the amplified templates indicating some non-specific priming events. Note that some templates still did not amplify.

Gel Extraction Method Comparison. Agarose gel electrophoresis banding patterns of PCR amplicons revealed that gel extraction by both the MiniElute™ Gel Extraction Kit and the freeze squeeze methods yielded similar extraction efficiencies (Fig. 3.5).

Slot-Blot Hybridization Method Comparison. Hybridization using the MSI membrane, the Blotto blocking agent (Appendix A), and a low stringency wash, gave initial readings of approximately 4,000 cpm (count per minute) by a hand held radiation (GM) counter. Autoradiographic record revealed that background was extremely high (Fig. 3.6). It was difficult to distinguish back ground signal from hybridized DNA signal. Using a light table results were found as given in Table 3.3

Hybridization using the Hybond-N membrane, Denhardt's blocking agent and a high stringency wash, gave GM counts between 1,000 and 2,000 cpm. Autoradiographic records revealed that background levels were lower than previous hybridization, but still high (Fig. 3.7). However, results were inconclusive, suggesting that no signal due to hybridization was detectable.

Results of Method Development

This study involved determining methods for the rapid assessment of freshwater microbial community structure and dynamics with respect to temporal and spatial changes. The following are the optimum methods found for each step for the microbial assessment described above.

Sample collection. Water samples (>500 ml) should be collected in sterilized 1 L Nalgene Polycarbonate bottles. Only surface water should be collected in order to avoid sediment that may impede filtration steps. Immediately filter on-site using 250 ml pre-sterilized, disposable filter units containing a nitrocellulose membrane filter (47 mm diameter, 0.2-µm pore size) (Nalgene) until filter was clogged. Store filters in 50 mm petri dishes at -20ºC until further processed.

Sonication. All glassware used during sonication should be thoroughly cleaned by first, an overnight submersion in a sulfuric acid bath, followed by rinsing in distilled water and baking at

250ºC. Invert glassware for 1 hour in the oven, and then cover and store upright overnight or until used. All instruments that are in contact with samples (including sonicator probe), should be immersed in 1 N HCl, rinsed with sterile water, immersed in 10 N NaOH, rinsed again in sterile water and then stored in 95% ethanol. Repeat cleaning before each sample is sonicated. Keep all samples on ice throughout the sonication procedure to inhibit nuclease activity and avoid heat denaturation of nucleic acids during sonication.

Membrane filters carrying environmental sample retentates should be placed in previously sterilized beakers containing 35 ml of lysis buffer (Appendix A) per filter and sonicated for 180 s using a Tekmar 600 Ultrasonicator with a 10.2 mm diameter probe, while maintaining a 15 power monitor reading (approximately 90 Watts). During sonication, immerse the probe at least 3 cm into the liquid and center over the filter to reduce occurrence of sample foaming. Place all sample vessels in a cold ethanol bath continuously bubbled by dry ice to reduce temperature elevation. Transfer the sonicated samples (including all filter particles) to 25-ml polypropylene screw-cap centrifuge tubes. Probe and beaker walls should be carefully rinsed with 1 ml of sterile 1× SSC, and add the rinsing solution to the centrifuge tubes. Immediately freeze (−70°C) tubes until further use.

Thaw frozen sonicated samples in a 50°C water bath and divide them into 15 ml aliquots in glass 25-ml Corex ® II centrifuge tubes. Extract the crude lysates with equal volumes of phenol: chloroform: isoamyl alcohol (Appendix A). Centrifuge tubes at 7,000 x g for 10 minutes and transfer the aqueous phases to 30-ml polycarbonate centrifuge tubes. Ethanol precipitate partially purified DNAs by the addition of 1/10 volume of 7.5 M ammonium acetate, and an equal volume of cold isopropanol, and store overnight at −20°C. Pellet DNA by centrifugation at 16,000 × g for 30 minutes at room temperature. Discard the liquid and wash the pellets with 70% ethanol,

centrifuged again for 15 minutes, decant, and dry. Resuspend the pellets in 100µl of TE buffer pH 8.0 (Sambrook et al., 1989).

Quantify DNA using a TD-360 Fluorometer (Turner Designs) containing a 360 nanometer wavelength excitation filter and a 460 nanometer wavelength emission filter, a Fluorescent DNA Quantification Kit (Bio-Rad, Hercules, CA) and 1 cm^2 methacrylate fluorescence cuvettes. A standard curve should be generated (Fig. 2.4) according to the manufacture's instruction for a 0.1 µg/ml Hoechst 33258 dye solution and low range DNA (10-500 ng/ml) detection.

DNA Amplification. Amplify DNA by the Polymerase Chain Reaction (PCR) using primers targeting a specific gene in the corresponding DNA template (Tables 2.2 and 3.1). Amplifications mixtures should be prepared using either a PCR Core Kit (Roche, Mannheim, Germany) as specified by the manufacturer or as given in Table 2.4 using the parameters given in Table 3.1. Amplify DNA in a Bio-Rad Gene Cycler™ with the following parameters: 1-5 minute hot start at 94°C (during which Taq Polymerase was added) followed by 30 cycles of denaturation (1 minute at 94°C), annealing (1.5 minute [temperature varies with primer pair, see Table 2.3]), and polymerization (2 minutes at 72°C), with a final cycle including an extended polymerization at 72°C for 10 minutes. After amplification is complete, store amplicons at −20°C. Repeat amplification procedure using either fresh DNA template or previous amplicons until a total of > 2,500 ng of DNA is obtained for each organism.

Preparative gel analysis. Purify amplicons by agarose gel electrophoresis as follows. Pool amplicons of the same gene fragment (total volume ≤ 800 µl) and add 1/10 volume of 10× loading dye, and transfer the solution to a 1.2% low melting point agarose gel containing ethidium bromide (Appendix A) with a 12 × 0.5 cm well. Resolve DNA fragments by electrophoresis using

TAE buffer (Appendix A) and a Bio-Rad Sub-Cell® GT with Bio-Rad Power Pack Junior power supply at 100 V. View gels using a transilluminator at 302 nanometers and photographed using Polaroid-Documentation Camera containing a Tiffen® 40.5 mm deep yellow filter with Polaroid type 667, ISO 3000 black and white film.

DNA gel purification. Excise DNA gel fragments of the correct size from the agarose gel using a sterile razor blade, and place into pre-weighed sterile microcentrifuge tubes, and extracted using the MiniElute™ Gel Extraction Kit (Qiagen) according manufacturer's directions.

Reverse Sample Gene Probing. Prepare membranes with purified reference DNA (target DNA) as follows using a 24-well Hybri-Slot™ filtration manifold (Life Technologies). The manifold should be thoroughly cleaned by submersion in 1.0 N NaOH, followed by rinsing in sterile water. Wet nylon membranes (Micron Separations Inc.) first in sterile water at room temperature and then soaked them in 20× SSC (Appendix A) for 1 hour at room temperature. Two sheets of blotting paper (Whatman 3mm filter paper) per membrane should be soaked in 20× SSC for 10 minutes. Place the blotting paper and membrane into the filtration manifold and fill individual slots with 800 µl of 10× SSC. Pull the buffer through the membrane by applying a vacuum to the base of the manifold. Repeat the 10× SSC wash.

Denature target DNAs (100 ng/ 40µl) by the addition of 10 µl 1 N NaOH and incubated for 10 minutes. Neutralize DNAs by placing samples on ice and adding equal volumes of cold 10× SSC, and then vacuum blotted immediately (See Fig. 2.6 for membrane configuration). Rinse the wells with an additional 2 ml of 10× SSC per well. Dry the membrane under continuous vacuum for 5 minutes and then baked overnight at 37°C between 2 sheets of blotting paper. Store the membranes at −20°C until used.

Environmental DNAs (Probes) should be radioactively labeled by random prime DNA labeling using [α-^{35}S]-Deoxycytidine-5- triphosphate (^{35}S-dCTP) (ICN, Irvine, CA) using a High Prime DNA Labeling Kit (Roche) as specified by the manufacturer for 25 ng of template DNA.

Use Hybond-N membranes (Amersham Pharmacia Biotech) as given by the manufacturer for slot-blot hybridization using previously amplified DNA given above. Hybridization should be carried out as follows. Pre-wet membranes with bound target DNAs in water and then in pre-hybridization buffer (Appendix A). Place membranes in 50-ml Bellco AutoBlot® jars containing 10 ml of pre-hybridization buffer and pre-hybridized for 30 min at 65°C in an AutoBlot® Micro Hybridization Oven (Bellco). Add labeled probes (as previously described) and allowed to hybridize overnight. Wash membranes (1) briefly in 2× SSC, 0.1% SDS, (2) twice for 5 min each in 2× SSC, 0.1% SDS, (3) twice for 10 minutes each in 1× SSC, 0.1% SDS. Conduct all wash steps at 65°C using wash buffer solutions that are pre-warmed to the same temperature. Take radioactive counts using a GM counter. Air dry membranes and then exposed to Kodak Biomax MR Film to produce autoradiographic records.

Chapter 4

DISCUSSION

The purpose of this study was to develop a method for the rapid assessment of freshwater microbial community dynamics. These methods are intended to detect numerous prokaryotic and eukaryotic microbial groups, as well as functional genes and pathogens found in the environment. This study was done in hopes that the methods developed here will contribute to future studies on freshwater microbial ecology.

Different optimal sonication times using different volumes (Fig. 3.1) of buffer were most likely due to DNA degradation caused by heat generated at the tip of the sonicator probe. Samples containing less buffer were less likely to dissipate heat, and therefore, heat up more quickly, leading to DNA degradation. Also, foaming is more likely to occur, since the volume was smaller, causing aeration of the sample due to inadequate probe submersion. A larger volume of buffer reduces the heating and likelihood of aeration; however, increasing the volume too much may reduce the sonication efficacy and dilute the nucleic acids too much. Thus, it was imperative to find the correct volume and time to maximize extraction efficiency. Sonicating for 180 seconds in 35 ml of buffer solution was found to reduce both heating and foaming for samples in this study.

Extracted DNA data from the environmental samples only provided the total DNA in the environment, there was no differentiation between microbial and other DNA present in the sample. Seasonal trends could be seen among the October 2001, January 2002, April 2002, and June 2002 collection dates (Fig. 3.2). DNA concentrations were higher during summer months, possibly due to higher temperatures and longer daylight periods. These environmental conditions are usually more favorable for phototrophic growth, which in turn leads to increased heterotrophic growth.

October 2001 and April 2002 showed a decrease in DNA concentration from June 2002. This trend was expected as well, since the lower temperatures and reduced light periods associated with fall and spring can lead to a reduction of phototrophic activity and subsequently heterotrophic activity. As for the July 2001 date, it is unclear as to why DNA quantities were lowest. This date would be expected to yield DNA quantities similar to June 2002 since conditions were similar. Instead, this date yielded some of the lowest DNA yields among the sampling time frame. It is possible that results were compromised by DNA degradation due to nuclease activity, but repeated sampling is required to establish valid seasonal trends.

When DNA yield was compared on a site by site (spatial) basis, site E consistently yielded the lowest or a lower quantity than other sites during all collection dates. This site was on average 2 m deep at normal flow, much deeper than the other sampling sites. This factor could have led to the lower DNA quantities. However, collection of additional environmental parameters is needed to establish a correlation. The data from the remaining sites do not reveal a seasonal or spatial trend. It is likely that other parameters not measured (e.g., pH, dissolved oxygen, BOD, light intensity etc.) may contribute significantly to microbial growth in the creek and, therefore, effect DNA quantities extracted from collected water samples. However, the present data suggest that water depth is inversely correlated with microbial biomass in Mill Creek.

The total extracted DNA results are given only as preliminary data conducted to test method development for DNA extraction from environmental samples on membrane filters and to estimate the quantity of DNA obtained from filtered samples from the Mill Creek sites. These data are not conclusive and not intended to represent a complete study of the microbial temporal and spatial dynamics within Mill Creek. Some ambiguity could be eliminated in future studies by reducing the amount of time and steps used to process the samples (i.e., the immediate filtration,

sonication, and quantification of the sample). It is recommended that in future studies that sample preparation be done as soon as possible after collection.

For microbial group, functional gene, and pathogen detection, primers were designed to give gene fragments of greater than 100 bp, with ideal fragments being between 400-800 bp. This size was chosen to create probes large enough to allow for high stringency washes following the hybridization step, thereby, making detection more reliable. The primer pair 530f and 907r as reported by Lane (1991) contained some degenerate bases. These degeneracies were retained in universal primers for this study so the same primers could be used to amplify a wide range of microbial groups. However, when the primers were adapted to specific groups due to sequence discrepancies, degenerated based were remove from the primers and replaced with the correct consensus base (Table 2.2 and Appendix B).

Titrating Mg^{2+} concentration proved to be an important first step in troubleshooting PCR problems. Several templates that failed to amplify by other optimization steps, were successfully amplified after finding the correct Mg^{2+} concentration. Having the correct Mg^{2+} concentration is essential for good amplification, and the Mg^{2+} concentration that was appropriate for one template does not necessarily work for others. Therefore, it is recommended that the optimum Mg^{2+} concentration be determined for all target DNAs.

Touchdown PCR proved successful for some templates; however, lowering the annealing temperature usually produced numerous unwanted gene fragments due to nonspecific priming. Upon gel analysis, numerous bands could be seen for each amplicon, and in some cases, so many fragments we reproduced, that a smearing pattern was seen on the gel (Fig. 3.4). Because of this, touchdown PCR use was limited. In some instances, fragments of the correct size can be excised

and reamplified at higher annealing temperatures, however some templates yielded so many fragments that it was impossible to excise the correct fragment.

DNA extraction from agarose gels was performed using the MiniElute™ Gel Extraction Kit because the method was faster and easier than using the freeze squeeze method. Although both the freeze squeeze and the kit methods had similar extraction efficiencies, it was apparent that up to 50% of some DNA was lost in gel purification,. Fig. 3.5 shows a DNA sample before and after gel purification. The band intensity of DNA before gel extraction is much greater than the band intensity after gel extraction. The gel extraction step may have resulted in the largest loss of DNA. Finding ways to minimize DNA loss was an important issue in this study, as well as in other molecular studies. Therefore, finding alternative methods to eliminate the need for gel extraction would be advantageous.

Hybridization of DNA requires many optimization steps. The high background after hybridization and washing (Fig. 3.6) likely could have been reduced using a lower salt (higher stringency) wash buffer. Since even the negative controls showed some hybridization, it is difficult to determine how much signal indicated a true detection. However, the fact, that some slots produced a signal while other did not (Table 3.1), suggests that hybridization was successful The hybridization using the higher stringency wash and Denhardt's solution did show less background, but most of the probe signal was removed as well (Fig 3.7). The wash stringency was probably too high and the shorter hybridization time may have also contributed to the ambiguous results. The optimum hybridization parameters were not found in this study. It is speculated that using the Hybond-N membrane, the Denhardt's solution as a blocking agent, and using a moderately stringent wash procedure would yield optimal hybridization results.

CHAPTER 5

CONCLUSIONS

Initially the purpose of this study was to determine microbial community structure and dynamics of Mill Creek location within a future wetland mitigation site. However, method development proved to exhaust most of my time and budget, therefore, the optimization, troubleshooting, and protocol comparisons were reported. However, the commitment to the Appalachian Transportation Institute and the future Mill Creek mitigation site was not lost, in that methods developed in this study could be implemented in future studies conducted at the Mill Creek site as well as other similar sites and studies concerning wetland pre- and post-construction.

PCR optimization consumed the majority of the project, since numerous templates were used and optimization was required for most of them. The universal primers 530f and 907r proved to amplify most prokaryotic templates; however, those primers targeting functional genes and pathogenic microbes did not do as well. It was unfortunate that a large number of groups, organisms, and functional genes were eliminated from the study, for including these groups would give more detailed and accurate data on the community structure and dynamics.

With further development, the methods described in this study could give a rapid determination of microbial community structure and dynamics in any aquatic environment, not just freshwater. Based on results from this study, it is recommended that in future studies, steps to expedite sample processing be implemented, for it is believed that DNA loss (whether by nuclease degradation or physical loss during precipitation) is an important limiting factor. These steps for rapid sample processing include: sample filtration on-site, and sonicated, extracted, labeled, and hybridized immediately upon return to the lab, thereby eliminating most variables that compromise DNA yield. Membranes with target DNA could be mass produced and stored until needed.

In general, this study determined methods to assess the microbial community structure and dynamics of freshwater environments on a seasonal and temporal basis using more rapid techniques than previously descried in similar studies. Other methods such as cloning, DGGE, and lipid analysis may be time consuming and give less exhaustive results at lower taxonomic levels. Reverse Sample Gene Probing (Voordouw, 1993) is a rapid method for assessing microbial community structure at various taxonomic levels. However, applications of the technique are not commonly applied to freshwater environments. Therefore, this study developed methods which adapted the Reverse Sample Gene Probing method given by Voordouw (1993) for use in freshwater environments. These methods could easily be applied to future freshwater studies and with little adaptation to other environments as well.

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Figure 1.1. The Universal Tree of Life. Genetic distances are based upon comparison of 16S rRNA and 18S rRNA sequences, adapted from Woese et al., 1990.

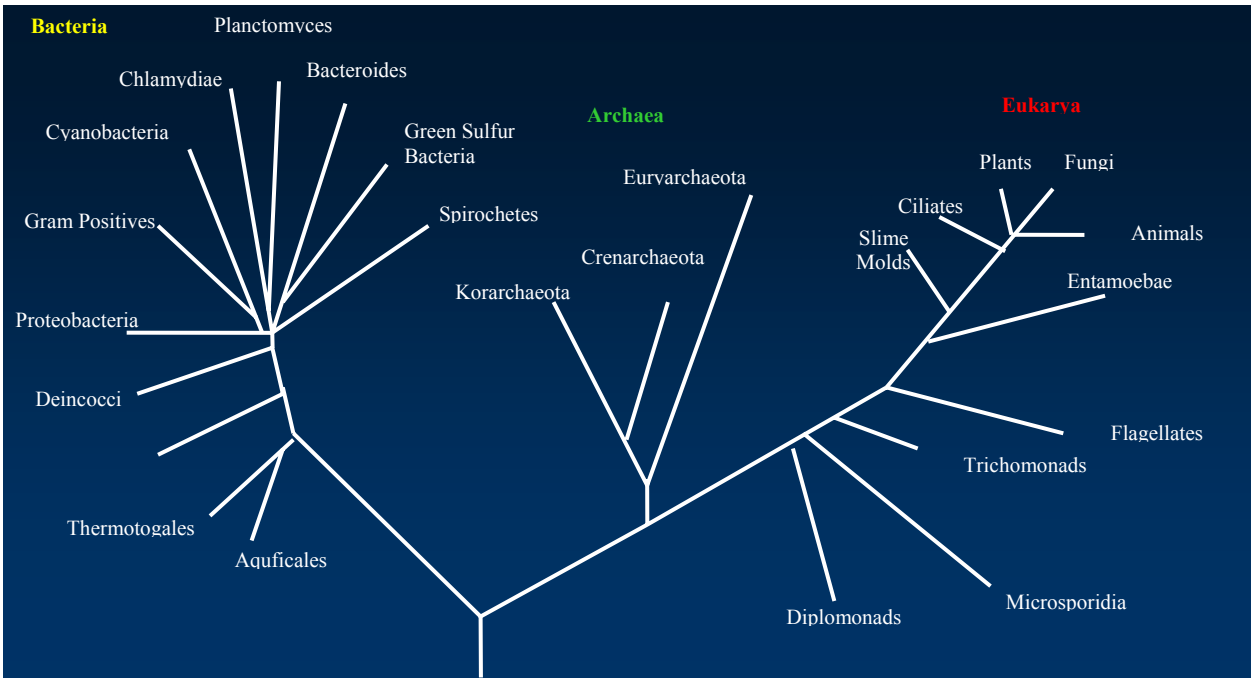


Figure 2.1. Map of Mill Creek Mitigation site showing cells A-E and collection sites. Geographical coordinates were as follows: site A, 38º 04' 50" N, 82º 32' 29" W; site B, 38º 04' 56" N, 82º 32' 29" W; site C, 38º 05' 05" N, 82º 32' 31" W; site D, 38º 05' 11" N, 82º 32' 25" W; site E, 38º 05' 15" N, 82º 32' 28" W.

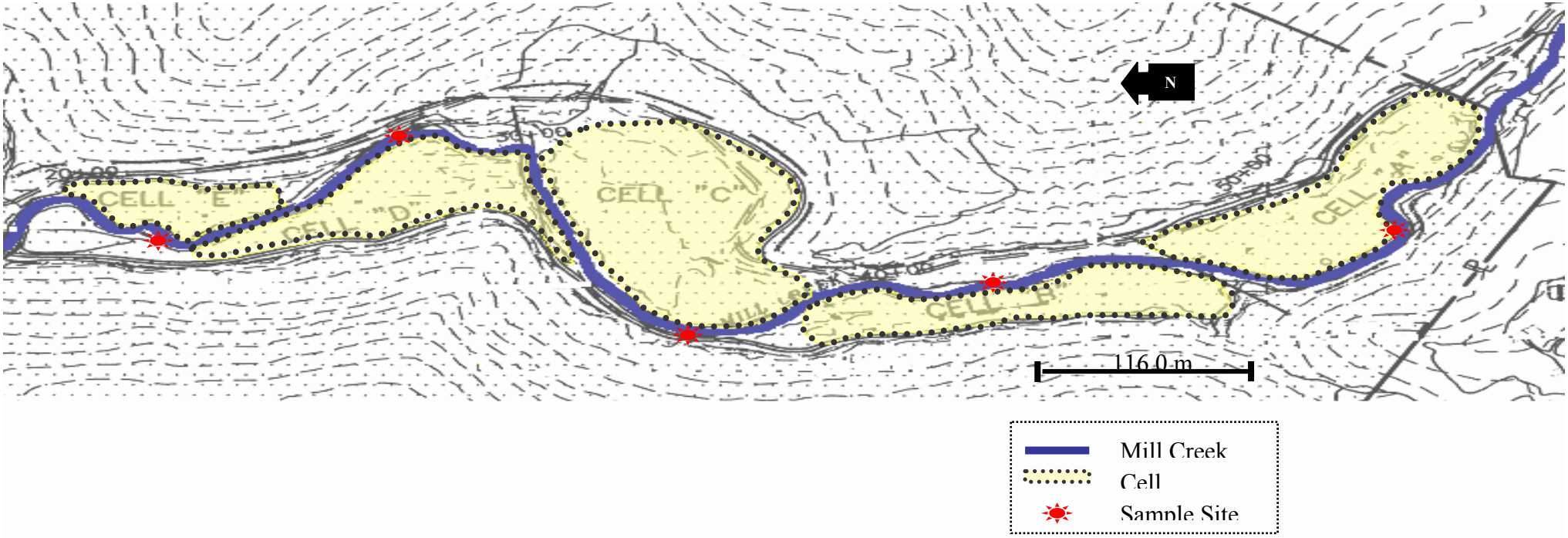


Figure 2.2. Water collection site for each cell A-E. Location of one-liter bottle indicates location of sample site. Photographs of cells A-D were taken on Sept. 20, 2001 and cell D was photographed on Nov. 15, 2001.

Cell A

Cell B

Cell C

Cell D

Cell E

Figure 2.3. Mean daily air temperature data for Huntington, WV during the period from June 2001- July 2002. This graph was used to determine which sampling dates would best represent seasonal high, low and moderate temperatures. ♦Represents mean daily temperature. ● Represents date closest to trend line. ▲ Represents collection date of samples that were further processed. Data obtained from The National Weather Service for Huntington, WV.

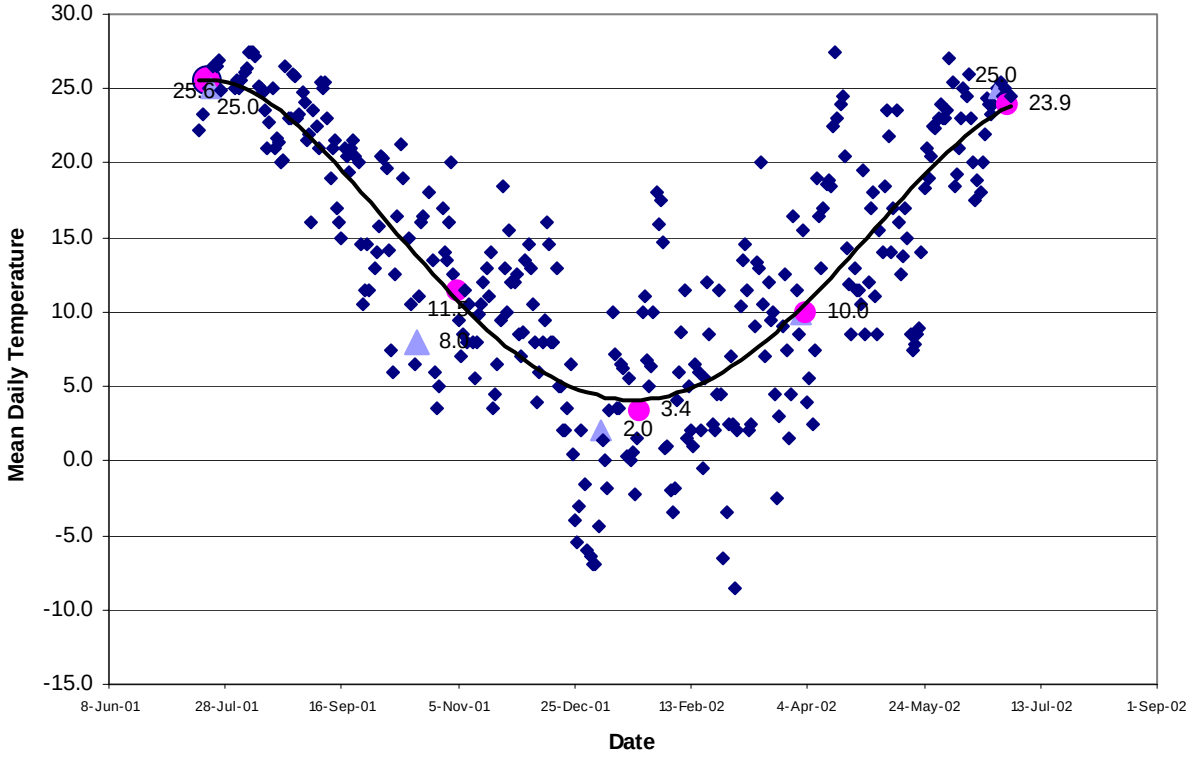


Figure 2.4. Standard fluorescence curve for Hoechst 33258 and a TD 360 Mini Fluorometer using known concentrations of calf thymus DNA. A standard curve was generated using stock DNA concentrations of 1000, 500, 250, 100, 50, 25, 10, and 1 ng/ml according to manufacture's direction for a Fluorometer DNA Quantification Kit (Bio-Rad, Hercules, CA)

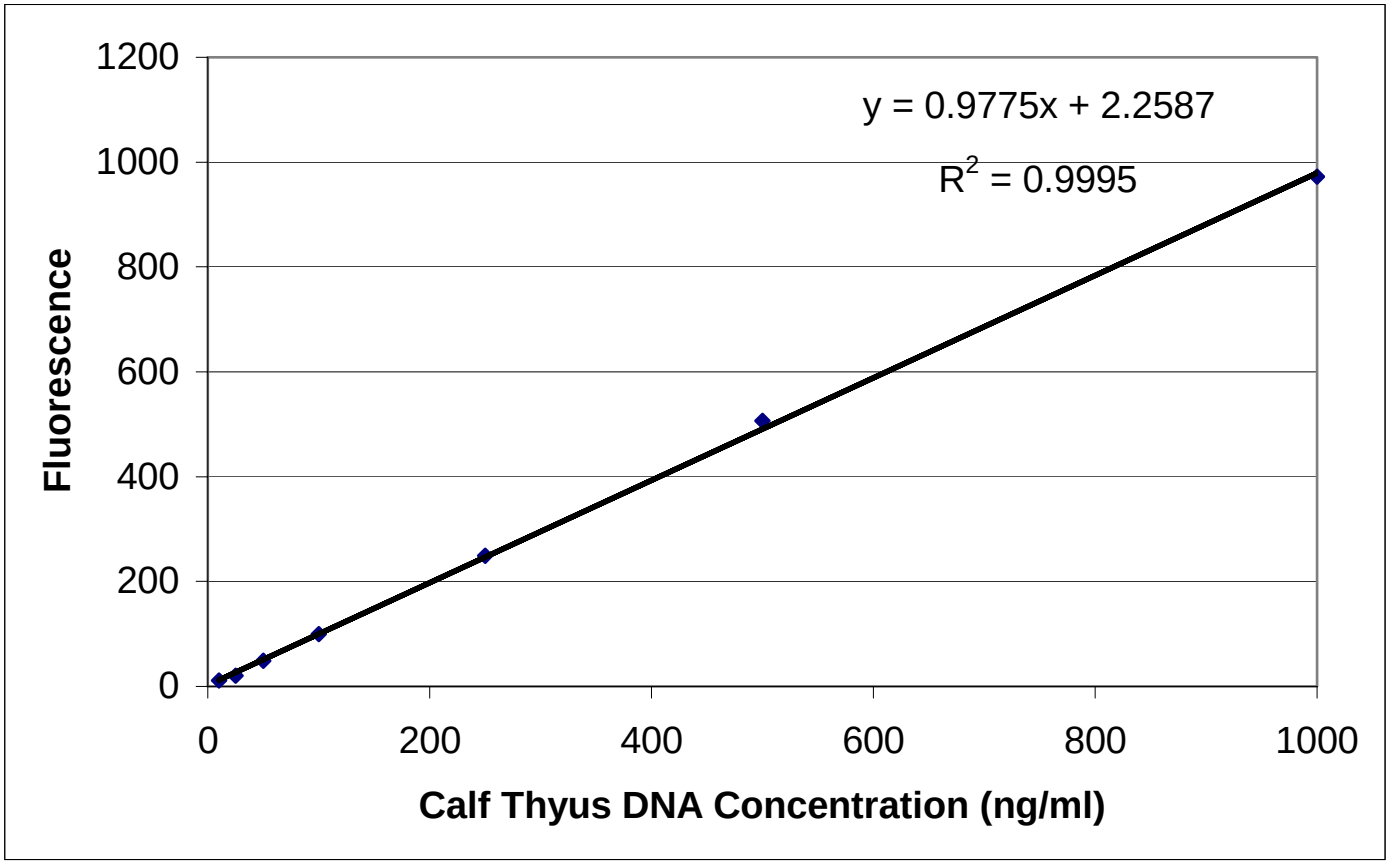


Figure 2.5. Flow chart of methods used in this study. ---- Represents steps that required optimization and — Represents steps that required no optimization.

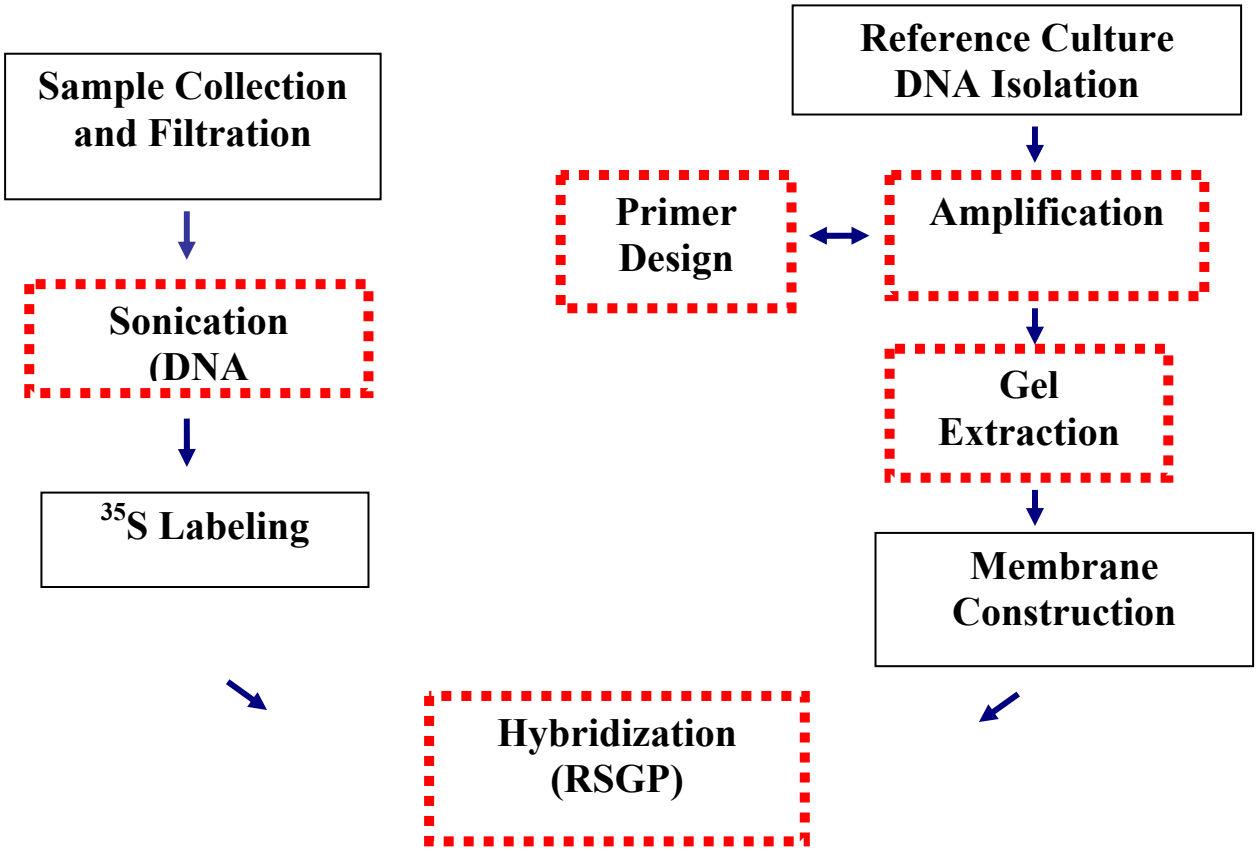
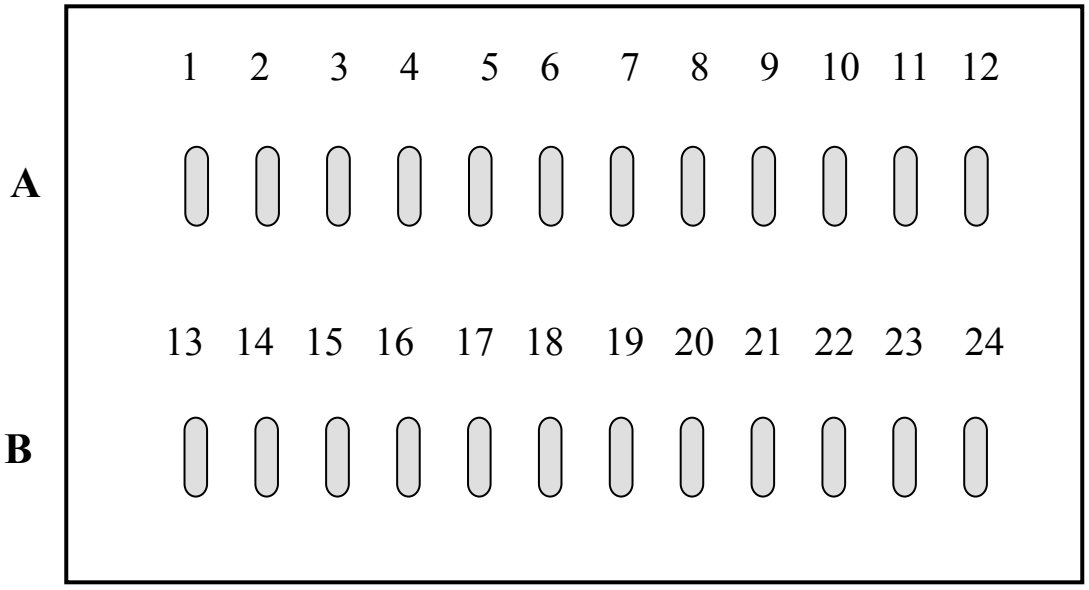


Figure 2.6. Function and slot orientation of DNA probes on hybridization membrane. * represents slots used for microbial group (phylogenetic) detection and † represents slots detecting functional genes.



Probe Orientation

A	B
1. Negative Control Salmon Sperm DNA	1 Positive Control (pUC 19) 1ng/µl
2. Euryarchaeota*	2 Green Non-sulfur*
3. Actinomyces*	3 Green Sulfur*
4. α-Proteobacteria*	4 Planctomyces*
5. Low G+C gram positive*	5 Spirochet*
6. CFB*	6 Thermotogales*
7. β-Proteobacteria*	7 Shiga Tox I†
8. Cyanobacteria*	8 Shiga Tox II†
9. Deinococcus*	9 *Entamoeba hystolytica*†
10. δ-Proteobacteria*	10 *Listeria moncytogenes*†
11. ε-Proteobacteria*	11 Nitrogenase†
12. γ-Proteobacteria*	12 Positive Control (pUC 19) 10ng/µl

Figure 3.1. Effects of sonication time and buffer volume on extracted DNA yield of filtered water samples. Samples were collected from the Ohio River at Huntington, WV in order to have a representative fresh water environment sample. Samples were collected at different times, therefore, filters do not contain equal biomass, and thus only trends were compared.

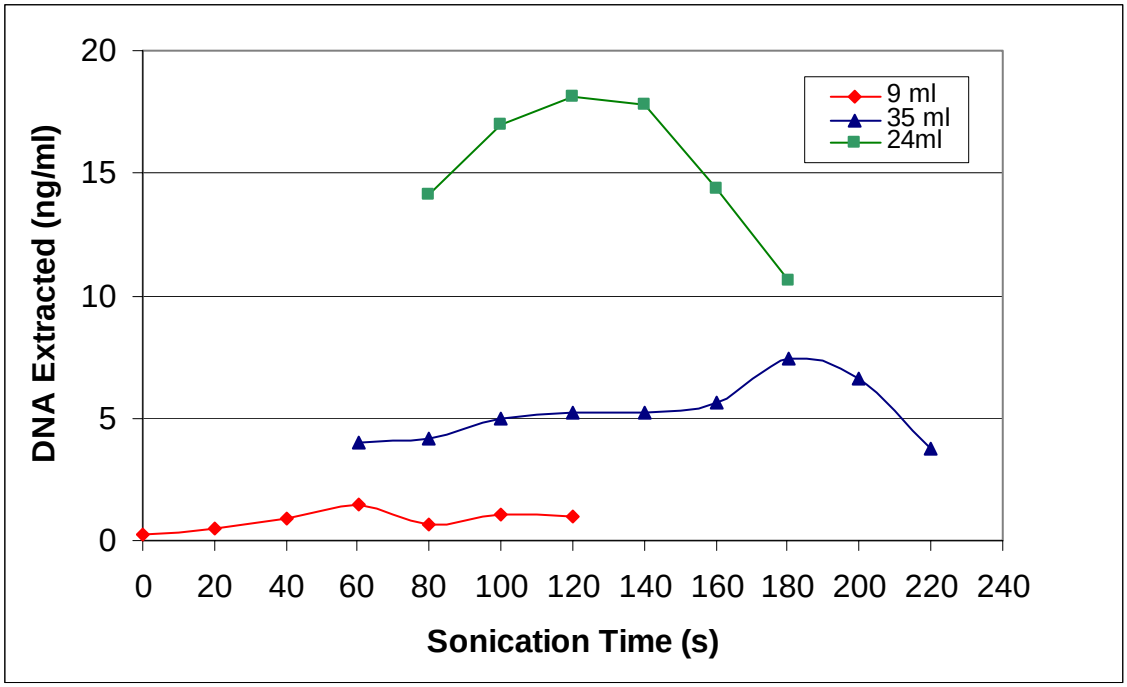


Figure 3.2. Total DNA extracted from filtered water samples collected from 2001-2002 at Mill Creek sites A-E. Values report the amount of DNA extracted per ml of water filtered.

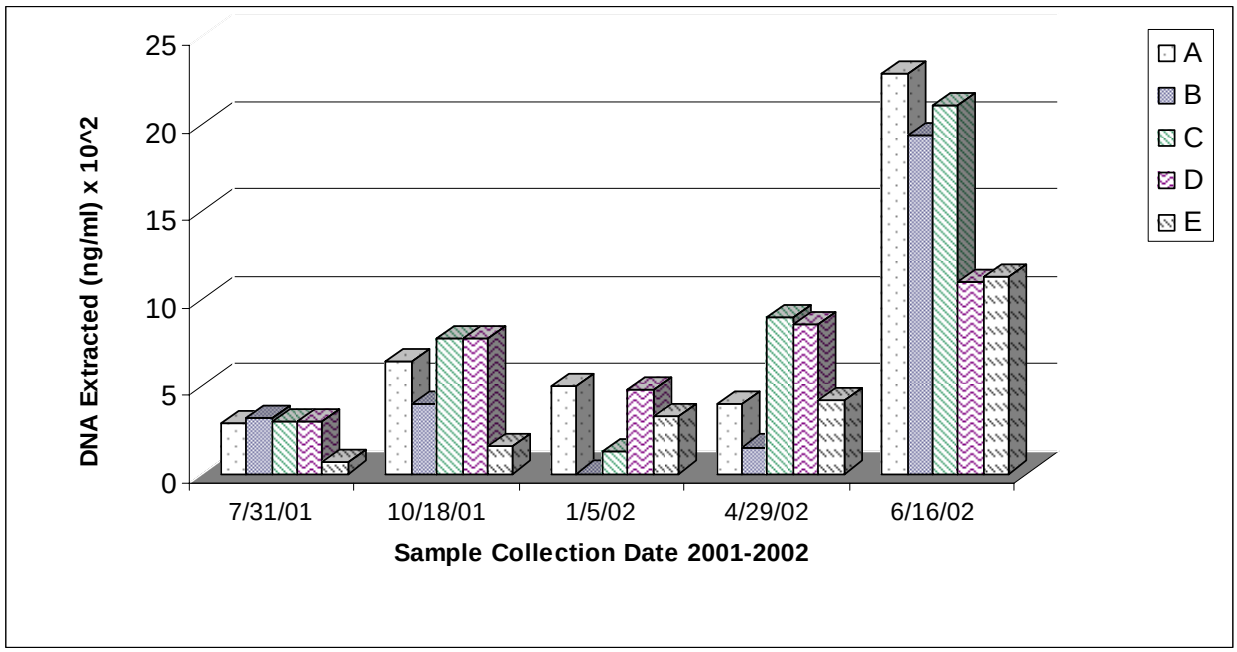


Figure 3.3. Results of Mg^{2+} concentration titration. (A) *Campylobacyer fetus* DNA amplified using: 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, and 5.0 mM Mg^{2+}as seen in lanes 2 through 8 respectively. (B) *Actinomyces israelli* DNA amplified using. 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, and 2.5 mM Mg^{2+} as seen in lanes 2 through 8 respectively.

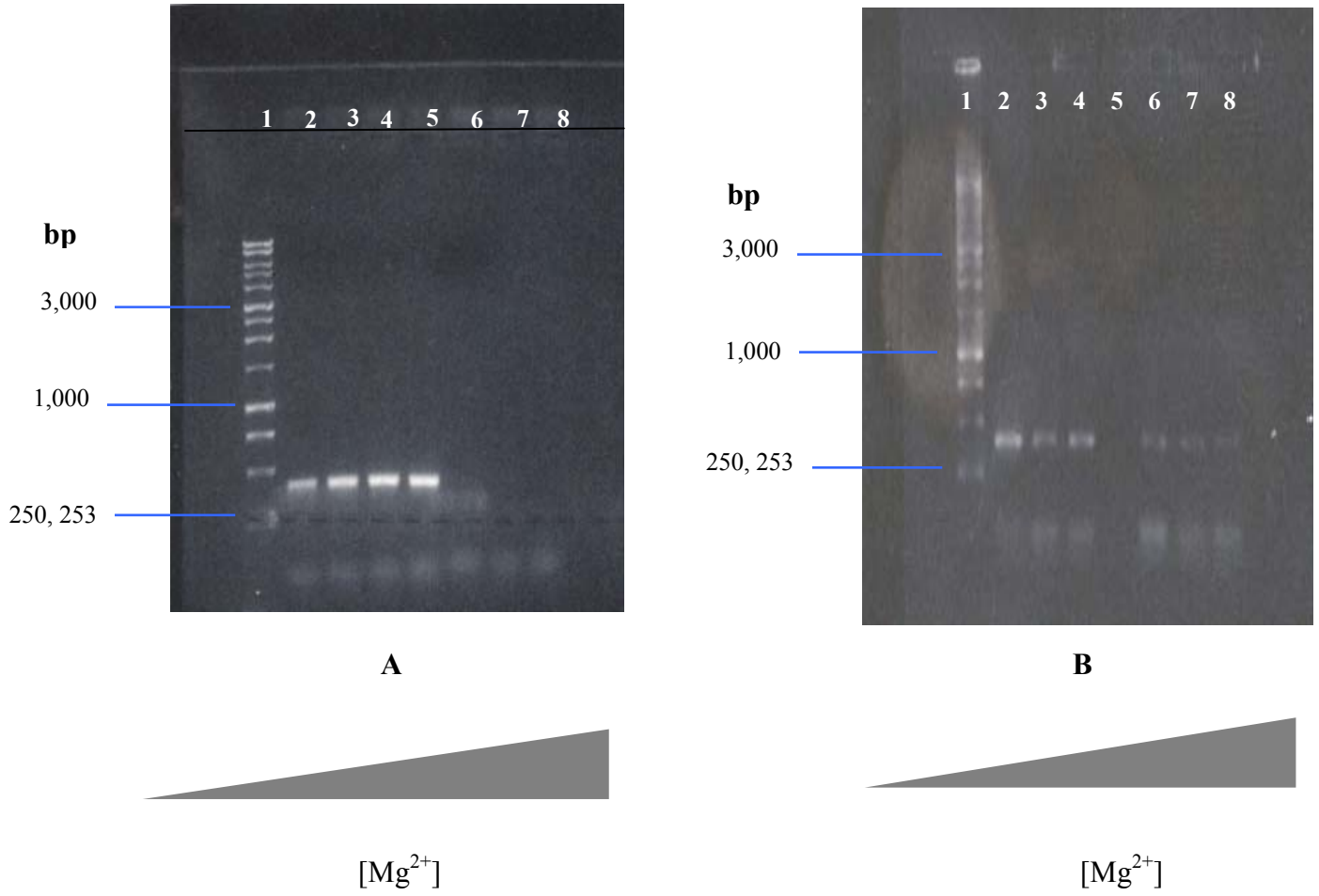


Figure 3.4. Various DNA templates amplified by touchdown PCR using 530f and 907r universal primers. Aliquots (5µl) of a 100 µl amplification reaction for each of the following organisms was resolved by electrophoresis: (2) *Archaeoglobus fulgidus*, (3*) Anabaena variabilis*, (4), *Sulfolobus solfataricus*, (5) *Deinococcus radiodurans*, (6) *Aquifex pyrophilus*, (7) *Actinomyces israelli*, (8) *Herpetosiphon geysericola*, (9) *Tetrahymena fugasoni*, (10) *Planctomyces maris*, (11*) Pyrocysitis lunula*, (12) *Ectocarpus variabilis*

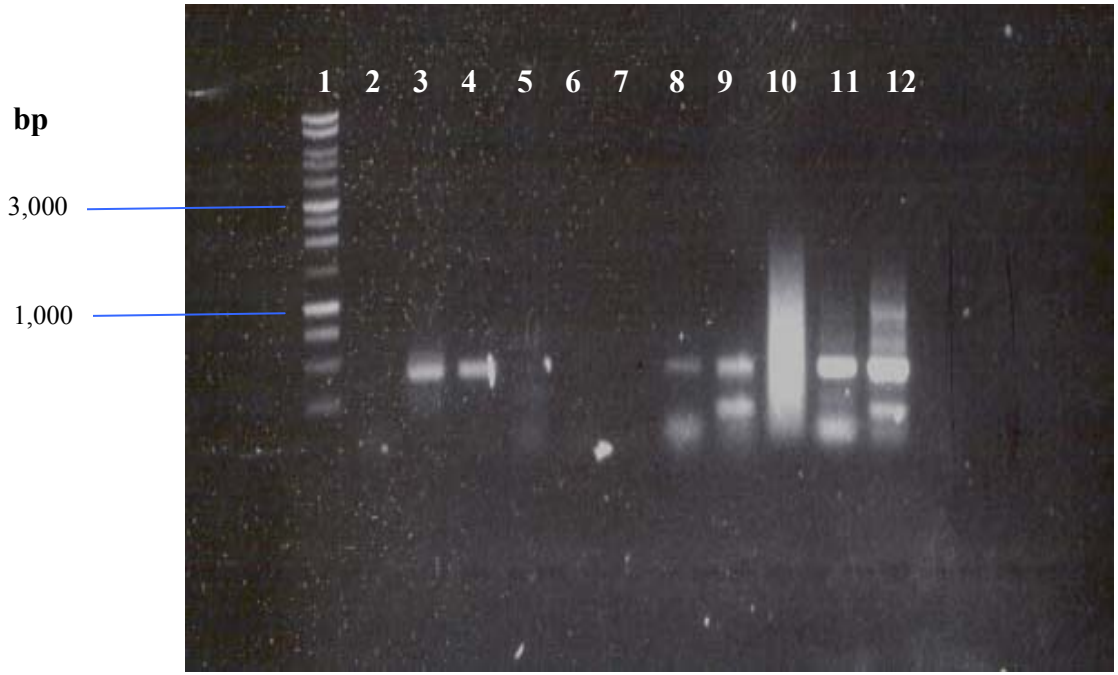
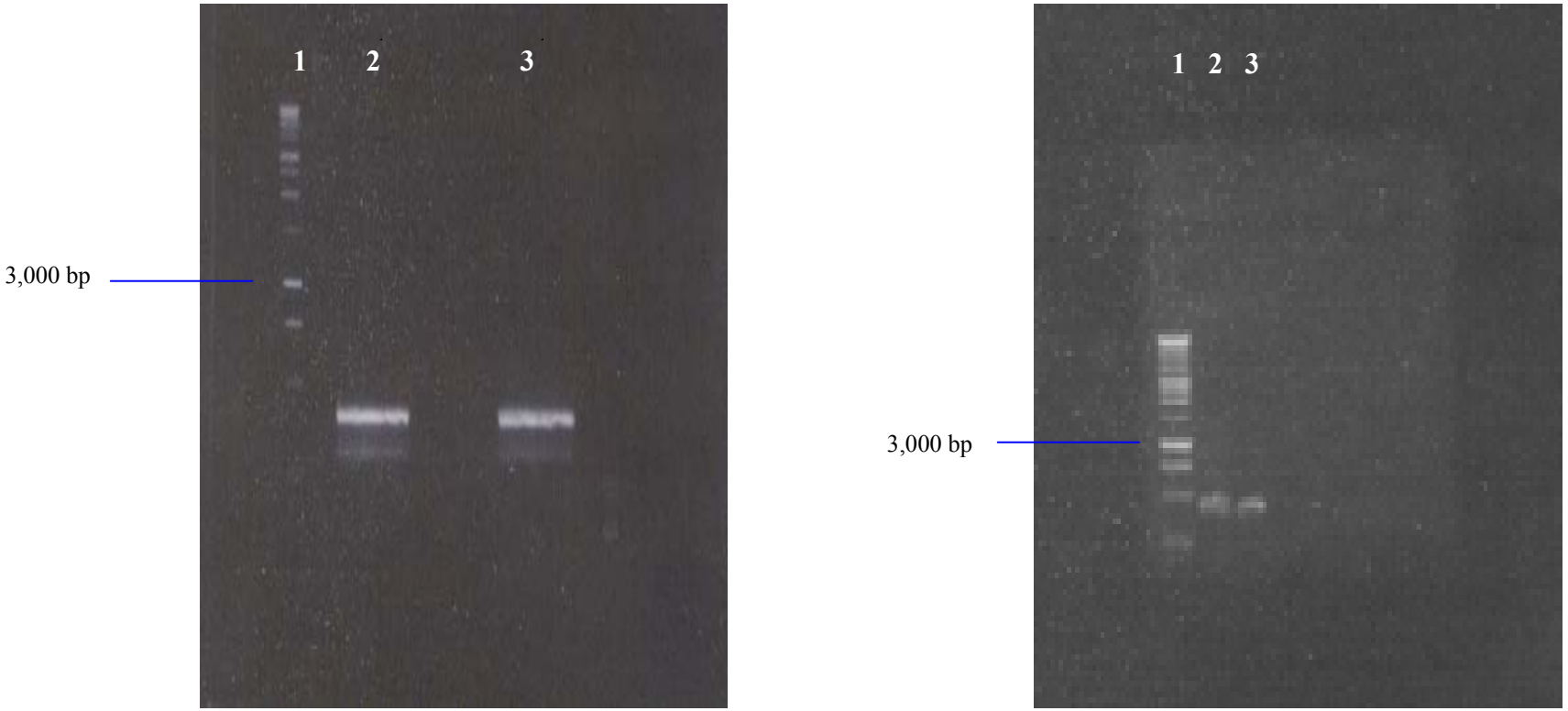


Figure 3.5. Comparison of agarose gel extraction methods. (A) *Anabaena variables* amplicons (50 µl in each lane) before gel extraction, (B) *Anabaena variables* amplicons (5µl in each lane) after extraction with lane two being extracted by Qiagen® Gel Extraction Kit and lane three extracted by the freeze squeeze method.



A **B**

Figure 3.6. First hybridization attempt using Hybond-N membrane (Amersham Pharmacia Biotech). Blotto was used as a blocking agent for unwanted spurious DNA binding. The membranes with bound probes were subjected to an overnight pre-hybridization, followed by a 12 hr. hybridization time and washed with in 2 × SSC (low stringency washes). See Figure 2.6 for probe orientation.

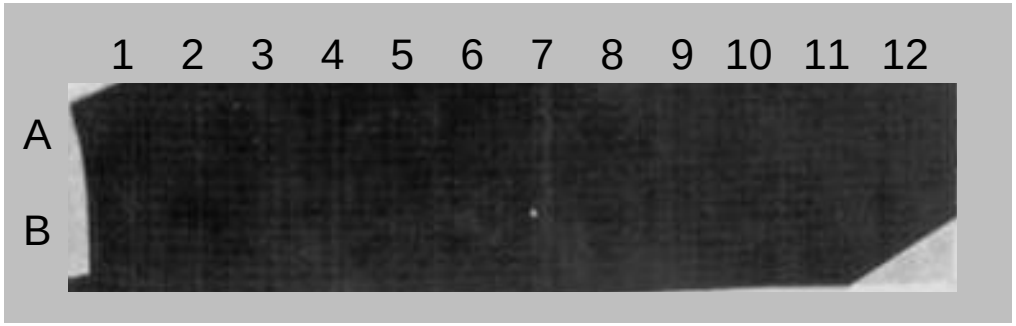


Figure 3.7. Hybridization using Hybond-N membranes with modified conditions. Denhardt's Solution was used as a blocking agent, followed by a 30 min. pre-hybridization period. An overnight hybridization period was added, as well as higher stringency washes in 2 ×, 1×, and 0.1× SSC. See Figure 2.6 for probe orientation.

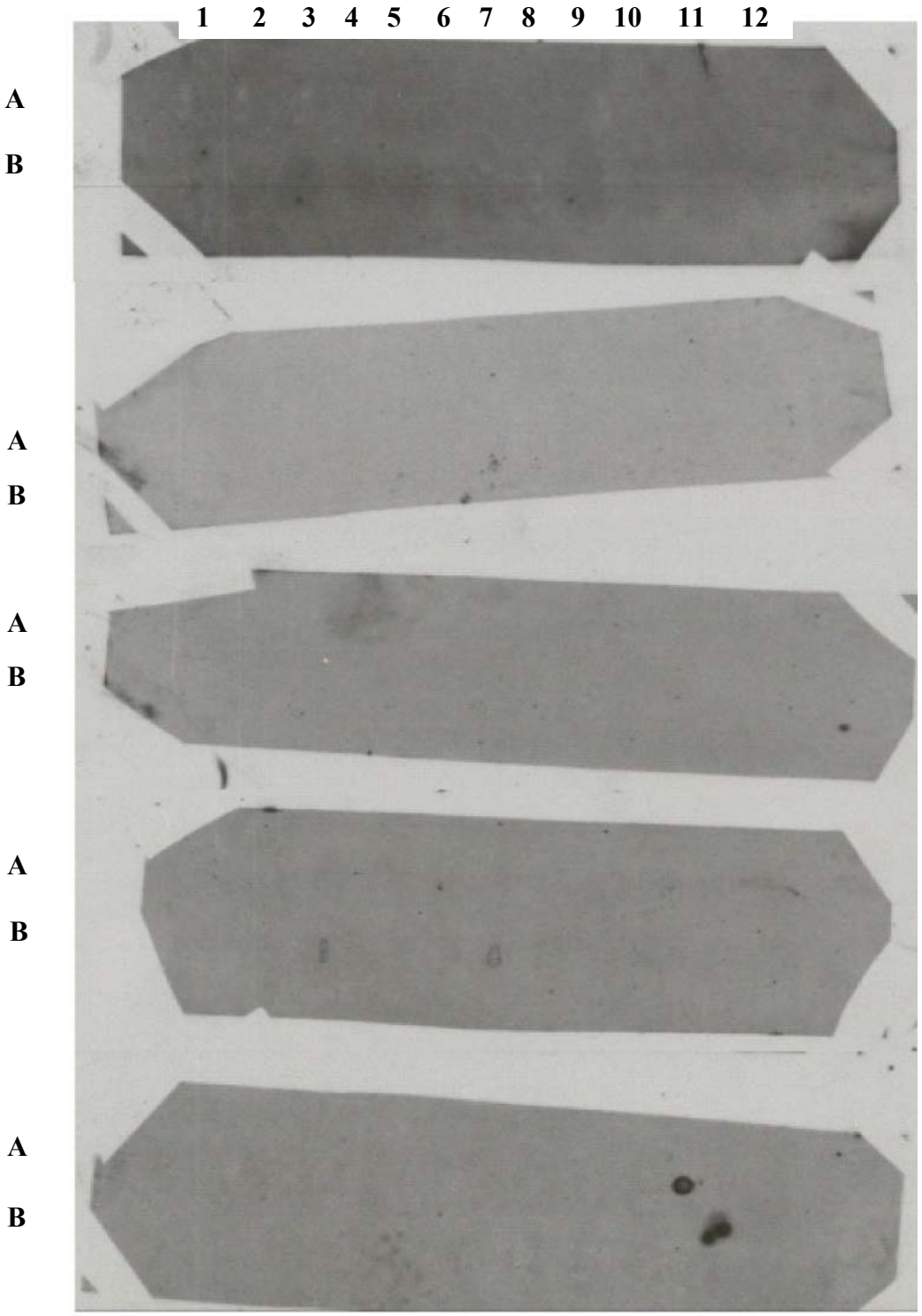


Table 2.1. Water Sample Collections (2001-2002). Rows correspond to the sample site and columns to sample date.

Volume filtered at cite (ml)	Date[a]																	
	7/7/01*	**8/9/01**	**8/22/01**	**9/20/01**	**10/5/01**	**10/18/01***	**11/15/01**	**12/18/01**	**1/5/02***	**2/11/02**	**2/25/02**	**3/29/02**	**4/15/02**	**4/29/02***	**5/15/02**	**5/31/02**	**6/16/02***	**729/03**
A[b]	290[c]	350	450	280	300	270	410	330	500	550	500	475	340	340	440	440	495	240
B	315	345	--	300	330	225	400	390	520	515	545	389	490	370	460	470	485	245
C	390	310	400	300	310	260	430	380	260	520	420	540	295	440	432	545	495	240
D	250	--	360	340	260	250	410	370	475	600	480	530	300	430	490	480	470	240
E	240	--	--	330	370	290	470	440	435	550	350	420	290	425	490	420	545	220

* Indicates that DNA was extracted as described in methods and materials from filters collected on this date.

[a] Columns designate date of sample collection.

[b] Rows correspond to sample location shown in Fig. 2.1 and 2.2.

[c] Tabular values indicate the volume of Mill Creek water filtered in ml.

Table 2.2. Primers Used In This Study.

Primer Name	Sequence (5'-3')	Target Gene[a]	Location (bp)[b]	Reference
530f	GTGCCAGCMGCCGCGG	16S rRNA	530	Lane, 1991
907r	CCGTCAATTCMTTTRAGTTT	16S rRNA	907	Lane, 1991
530f- cren	GTGTCAGCCGCCGCGG	16S rRNA	530	Adapted from Lane, 1991
907r-epsilon	CCGTCTATTCCTTTGAGT TT	16S rRNA	907	Adapted from Lane, 1991
907r-eury/cren	CCGTCAATTCMTTRAATTT	16S rRNA		Adapted from Lane, 1991
nifHf	TAYGGHAARGGNGGNATYGG	Nitrogenase	394	Lovell et al., 2000
nifHr	ACGATRTADATYTCYTSNGCYTT	Nitrogenase	800	This study
VTf	GARCRRAATAAKTTATATGT	*stx I & stx II*	495	Read et al., 1992
VTr	TGATGATGYCAATTCAGTAT	*stx I & stx* II	984	This study
hlyf	TCCGCCTGCAAGTCCTAAGA	*hly*	NA	Klien et al., 1997
hlyr	GCGCTTGCAACTGCTCTTTA	*hly*	NA	Klien et al., 1997
EH1	AATGGCCAATTCATTCAATG	18S rRNA	NA	Hague et al., 1998
EH2	TTTAGAAACAATGCTTCTCT	18S rRNA	NA	Hague et al., 1998

[a] *stx* I and *stx* II represent genes that encode for shiga toxin proteins found in some pathogenic *E. coli*; *hly* encodes the pore-forming cytolysin listeriolysin found in pathogenic *listeria monocytogenes.*
[b] Primer annealing locations based on *E. coli* numbering for 16S rRNA targets and

Table 2.3. Microbial Taxa Included In This Study And Their Representative Organisms. ATCC = American Type Culture Collection, Manassas, VA. DSM = German Collection of Microorganisms, Braunschweig, Germany. UTEX = University of Texas Culture Collection

Taxon Name	Type Strain	Strain ID Number
Crenarchaeota	*Archaeoglobus fulgidus*	DSM 4304
Euryarchaeota	*Sulfolobus solfataricuas*	DSM 1616
Actinomyces (High GC gram-positives)	*Actinomyces israelli*	ATCC 10049
Aquificales	*Aquifex pyrophilus*	DSM 6858
Low GC gram positives	*Enterococcus gallinarum*	ATCC 700425
Cytophaga/Flavobacteria/ Bacteriodes (CFB)	*Prophromanas ginivalis*	ATCC BAA-208D
Cyanobacteria	*Anabaena variabilis*	UTEX B 377
Deinococci	*Deinococcus radiodurans*	DSM 20539
Green Non-sulfur Bacteria	*Herpetosiphon geysericola*	UTEX 7119
Green Sulfur Bacteria	*Chlorobium tepidum*	DSM 245
Planctomycetes	*Planctomyces maris*	ATCC 29201
α-Proteobacteria	*Bartonella henselae*	ATCC 49882D
β-Proteobacteria	*Neisseria lactmaica*	ATCC 49142
γ-Proteobacteria	*Pseudomnas aeruginosa*	ATCC 9721
δ-Proteobacteria	*Desulfovibrio desulfuricans*	ATCC 7757
ε-Proteobacteria	*Campylobacter fetus*	ATCC 25936
Spirochetes	*Borrelia burgdorgeri*	ATCC 35210D
Thermotogales	*Thermotoga maritima*	DSM 3109
Brown Algae	*Ectocarpus variabilis*	UTEX LB 1636
Ciliphora	*Tetrahymena fugasoni*	ATCC 9357
Cryptomonads	*Cryptomonas ozolini*	UTEX LB 2194
Diatoms	*Frailaria shiloi*	
Dinoflagellates	*Pyrocysitis lunula*	UTEX LB 2166
Euglenozoa	*Phacus triqueter*	UTEX LB 2354
Green Algae	*Scenedesmus hystrix*	UTEX 2451
Red Algae	*Hilenbrandia rivularis*	UTEX LB 2622
Cryptosporidium parvum	--	
Entamoeba hystolytica	--	ATCC 50412
Listeria monocytogenes		ATCC 19115
Giardia lamblia		
Nitrogen Fixation	*Azotobacter chroococcum*	ATCC 9043
Rubisco	*Anabaena variabilis*	UTEX B 377
Shiga toxin I	*Escherichia coli*	ATCC 43889
Shiga toxin II	*Escherichia coli*	ATCC 43890

Table2.4. Standard Amplification Reaction Mixtures.

Reagent	**Volume**	**Concentration**
Sterile water	Variable	--
10× Taq Buffer	10 µl	1×
25 mM $MgSO_4$	Variable	Variable (see Table 3.1)
dNTPs [a]	2 µl	0.2mM each
Forward Primer	1 µl	1 µM
Reverse Primer	1 µl	1 µM
Template DNA	Variable	Variable
Taq DNA polymerase (Promega) [b]	0.5 µl	2.5 U/100 µl
Total	100 µl	

[a] deoxynucleotide triphosphates (GTP, ATP, CTP, TTP)
[b] Taq polymerase was added after reaction had reached a temperature of 94° C

Table 3.1. PCR Parameters For Templates That Successfully Amplified.

Group/Function/ Organism	Representative Organism	Primer Pair[a]	Annealing Temp (ºC)	Amplicon Size	[Mg^{2+}] mM	Amplification Method[b]
Euryarchaeota	*Sulfolobus solfataricuas*	530f cren & 907r eury/cren	38	376	2.0	*TD PCR
Actinomyces (High GC Gram-positives)	*Actinomyces israelli*	530f & 907r	42	377	1.0	PCR
Low GC Gram-positives	*Enterococcus gallinarum*	530f & 907r	42	377	2.0	PCR
Cytophaga/Flavobacteria /Bacteriodes (CFB)	*Prophromonas ginivalis*	530f & 907r	42	377	2.0	PCR
Cyanobacteria	*Anabaena variabilis*	530f & 907r	42	377	2.0	PCR
Deinococcus/ Thermus	*Deinococcus radiodurans*	530f & 907r	42	377	1.5	TD PCR
Green Non-sulfur	*Herpetosiphon geysericola*	530f & 907r	42	377	1	PCR
Green Sulfur	*Chlorobium tepidum*	530f & 907r	42	377	1.5	PCR
Planctomyces	*Planctomyces maris*	530f & 907r	42	377	1.75	PCR
α-Proteobacteria	*Bartonella henselae*	530f & 907r	42	377	2.0	PCR
α-Proteobacteria	*Neisseria lactmaica*	530f & 907r	42	377	2.0	PCR
γ-Proteobacteria	*Pseudomonas aeruginosa*	530f & 907r	42	377	2.0	PCR
Δ-Proteobacteria	*Desulfovibrio desulfuricans*	530f & 907r	42	377	1.0	TD PCR
ε-Proteobacteria	*Campylobacter fetus*	530f & 907r epsilon	46	377	1.5	TD PCR
Spirochetes	*Borrelia burgdorferi*	530f & 907r	42	377	2.0	PCR
Thermotogales	*Thermotoga maritima*	530f & 907r	42	377	2.0	PCR
Shiga toxin I	*E. coli*	VTf & VTr	42	489	2.0	PCR
Shiga toxin II	*E. coli*	VTf & VTr	42	489	2.0	PCR
Entamoeba hystolytica	--	EH1 & EH2	42	871	2.0	PCR
Listeria monocytogenes	--	hlyf & hlyr	50	713	1.0-1.75	PCR
Nitrogen Fixation	*Azotobacter chroococcum*	nifHf & nifHr	46	486	1.0-1.5	PCR

[a] primer pair sequence given in Table 2.2
[b] PCR = standard Polymerase Chain Reaction; TD PCR = touchdown PCR

Table 3.2. Amplification parameters tried for unsuccessful DNA templates.

Group/Function/ Organism	**Representative Organism**	**Primer Pair**	**[Mg^{2+}]** mM	**Amplification Method**
Crenarchaeota	*Archaeoglobus fulgidus*	530f-cren & 907r	Titrated*	PCR, TD PCR**
Aquificales	*Aquifex pyrophilus*	530f & 907r-aqui	Titrated	PCR, TD PCR
Brown Algae	*Ectocarpus variabilis*	530f & 907r	2	PCR
Ciliphora	*Tetrahymena fugasoni*	530f & 907r	2	PCR
Cryptomonas	*Cryptomonas ozolini*	530f & 907r	2	PCR
Diatoms	*Frailaria shiloi*	530f & 907r	2	PCR
Dinoflagellates	*Pyrocysitis lunula*	530f & 907r	2	PCR
Euglenozoa	*Phacus triqueter*	530f & 907r	2	PCR
Green Algae	*Scenedesmus dimorphus*	530f-euglena & 907r	2	PCR
Red Algae	*Hilenbrandia rivularis*	530f & 907r	2	PCR
Brown Algae	*Ectocarpus variabilis*	530f & 907r	2	PCR
Rubisco	*Anabaena variabilis*	Rbcf & Rbcr, Rub1 & Rub2	Titrated	PCR, TD PCR
Cryptosproidium parvum	--	Cpr1 & Cpr2	Titrated	PCR, TD PCR
Giardia lamblia	--	grdf & grdr	Titrated	PCR, TD PCR

* titrated templates were subjected to increasing Mg^{2+} concentrations of 0.5, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.5 mM during PCR. **TD PCR = touchdown PCR.

Table 3.3. DNA hybridization results for water sample June 16, 2002 site A. ^{35}S signals were visually determined using a light table. Intensities are given on a scale were + is the weakest signal present and +++++ is the strongest signal present.

Group (Row A)	^{35}S Signal Intensity	Group (Row B)	^{35}S Signal Intensity
1. Negative control	++	1. Negative control	++
2. Euryarchaeota	+	2. Green Non-sulfur	+
3. Actionomyces	++	3. Green Sulfur	+++
4. alpha-proteobacteria	+	4. Planctomyces	+
5. Low GC Gram pos.	+	5. Spirochet	++
6. CFB group	+	6. Thermotogales	+
7. beta-proteobacteria	+	7. Shiga tox I	++
8. Cyanobacteria	+	8. Shiga tox II	++
9. Deinococcus/Thermus	++	9. *Entamoeba histolytica*	+++
10. delta-proteobacteria	+++++	10. Listeria monocytogenes	+++
11. epsilon-proteobacteria	+++	11. Nitrogenase	+++
12. gamma-proteobacteria	++++	12. Negative control	++

Appendix A. Composition for reagents not commercially synthesized.

Item	Composition
Sterile water	16-18 megohm-cm purity, autoclave sterilized and/or filtered through 0.2 µm filter
Triton X-100	5% Triton X-100 in water (filter sterilized)
Phenol:chloroform:isoamyl Alcohol	Mixed to a ratio of 25:24:1 using buffer saturated Phenol
7.5 M Ammonium acetate	57.8 g of Ammonium acetate 100 ml sterile water
Tris-EDTA (TE) buffer	10 mM Tris 1 mM EDTA pH 8.0
5% Chelex	5 g chelex 100ml of water
1× Tris-acetate (TAE) buffer	0.04M Tris-acetate 0.001 M EDTA
1% Agarose gel	50 ml of 1× TAE buffer 0.1 g Agarose 2µl of Ethidium Bromide (10 mg/ml)
Lysis buffer	30 ml 1× SSC 40 µl 5 % Triton X-100
10 × Loading dye	30% Glycerol 0.25g Bromphenol blue/ 100 ml
20 × SSC	0.3 M Sodium citrate 3.0 M Sodium cholride

Blotto	0.25 g of dry milk to 100 ml of 6 × SSC and 1% SDS
Wash reagent	100 ml of 20 × SSC 5 ml of 20% SDS
Pre-hybridization buffer	5 × SSC 5 ×Denhardt's solution 5% SDS
100 ×Denhardt's solution	1 g Bovine Serum Albumin 1 g Ficoll 400 1 g Polyvinylpyrrolidone 50 ml sterile water

Appendix B: Alignments of NCBI database sequences to universal primers (530f and 907r) for bacterial groups used in this study.

Figure B.1. Aquifex sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Aquifex aeolicus	AJ309733	(Deckert et al., 1998)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGGCGG
Thermocrinis sp.	AJ278895	(Huber et al., 1998)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGGCGG
Hydrogenobacter acidophilus	D16296	(Shima et al., 1994)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGGCGG
Calderobacterium hydrogenophilum	Z30242	(Pitulle et al., 1994)	
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGGCGG

Figure B.2. Low GC sequence alignments.

Organism	Accession #	Reference	530. 907
			********** ********** ****
Megasphaera cerevisiae	L37040	(Doyle et al., 1995)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Zymophilus paucivorans	AF373025	(Schleifer et al., 1990)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Selenomonas flueggei	AF287803	(Paster et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Sporomusa sphaeroides	AJ279801	(Biebl et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Aerococcus sanguinicola	AJ276512	(Lawson et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Enterococcus sp.	AJ133478	(Bauer et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTKACGG
Streptococcus thermophilus	X68418	(Ludwig et al., 1992)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Lactobacillus sp. G22	AF308147	(Niamsup et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Gemella sp. oral strain C24KA	AY005051	(Paster et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Paenibacillus sp. P22-9	AJ297713	(Guinebretiere et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Pasteuria penetrans	AF375881	(Bekal et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thermoactinomyces intermedius	AF138734	(Yoon and Park, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bacillus sp.	U62623	(Cann et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Heliobacterium sulfidophilum	AF249678	(Briantseva et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Heliorestis baculata	AF249680	(Briantseva et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Butyrivibrio fibrisolvens	AF125217	(Diez-Gonzalez et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Natronincola histidinovorans	Y16716	(Zhilina et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Clostridium stercorarium	L09176	(Rainey et al., 1993)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Dehalobacterium formicoaceticum	X86690	(Magli, et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Desulfotomaculum sp. TPO	AY007190	(Plugge et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Natronoanaerobium aggerbacterium	AJ271452	(Jones et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Mycoplasma leopharyngis	U16760	(Brown et al., 1995)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.3. CFB sequence alignments.

Organism	Accession #	Reference	530. 907
			********** ** ********** ***
Chitinophaga pinensis	AF078775	(Sly et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Lewinella cohaerens	AF039292	(Sly et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Hymenobacter roseosalivarius	Y18834	(Hirsch et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Taxeobacter sp. Txc1	Y18837	(Hirsch et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Riemerella anatipestifer	U10877	(Ban, 1994)	GTGCCAGCAGCCGCGG.....AATCTCAAAGGAATTGGCGG
Flavobacterium xylanivorum	AF162266	(Humphry et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Polaribacter filamentus	U73726	(Gosink et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bacteroides uniformis	AB050110	(Miyamoto, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Prevotella disiens	L16483	(Paster et al., 1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Porphyromonas macacae	L16494	(Paster et al., 1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Rikenella microfusus	L16498	(Paster et al., 1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.4. Cyanobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Anabaena variabilis	AF247593	(Beltran and Neilan, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Anabaenopsis circularis	AF247595	(Beltran and Neilan, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Nodularia sp.	AJ224447	(Barker et al., 1999a)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Aphanizomenon sp.	AJ245457	(Barker et al., 1999b)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Chlorogloeopsis sp.	X68780	(Wilmotte et al., 1993)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Calothrix sp.	AJ133164	(Lyra et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Pleurocapsa sp	X78681	(Nelissen et al., 1995)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Prochloron sp	X63141	(Urbach et al., 1991)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Synechococcus sp.	AJ000716	(Nubel et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Cyanothece sp.	AF296872	(Turner et al, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Euhalothece sp.	AJ000713	(Nubel et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Dactylococcopsis sp.	AJ000711	(Nubel et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Halospirulina tapeticola	Y18791	(Nubel et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Spirulina sp.	Y18799	(Nubel et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Microcystis aeruginosa	D89031	(Kondo et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Stanieria cyanosphaera	AF132931	(Turner et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Gloeothece membranacea	X78680	(Nelissen et al., 1995)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Trichodesmium hildebrandtii	AF091322	(Janson et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Planktothrix sp. FP1	AF212922	(Pomati et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Prochlorococcus marinus subsp. pastoris	AF180967	(Rippka et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Oscillatoria sp.	AB003163	(Ishida et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Primer sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.5. Deinococcus/ Thermus sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Deinococcus geothermalis	AJ000002	(Vaisanen et al., 1998)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Deinococcus murrayi	Y13043	(Ferreira et al., 1997)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Deinococcus radiodurans	AF289089	(Brim et al., 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Deinococcus radiophilus	Y11333	(Rainey et al., 1997)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thermus aquaticus	X58340	(Weisburg et al., 1989)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thermus brockianus	Y18409	(Chung et al.,2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.6. Green Sulfur sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Clathrochloris sulfurica	X53184	(Witt et al., 1989)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Pelodictyon phaeoclathratiforme	Y08108	(Overmann, 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Chlorobium limicola	AB054671	(Imanaka et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Chloroherpeton thalassium	AF170103	(Stolzand and Buzzelli, 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.7. Green Nonsulfur sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Chloroflexus aurantiacus	M34116	(Oyaizu et al., 1987)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGNNNN
Dehalococcoides sp.	AF357918	Loeffler, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Heliothrix oregonensis	L04675	(Ward et al., 1992)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Herpetosiphon sp.	X86447	Bradford et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Oscillochloris sp.	AF149018	(Keppen et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thermomicrobium roseum	M34115	(Oyaizu et al., 1987)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.8. Alpha-Proteobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** ********* *********
Asticcacaulis biprosthecium	AJ007799	(Sly et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Sphingomonas sp	AB021492	(Yabuuch et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGACGG
Azospirillum sp	AF170353	(Coates et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Brevundimonas intermedia	AJ227786	(Abraham et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Caulobacter crescentus	M83799	(Stahl et al., 1992)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Phenylobacterium immobile	Y18216	(Eberspaecher and Ludwig, 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Mesorhizobium tianshanense	AF041447	(Wang et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Phyllobacterium rubiacearum	D12790	(Yanagi and Yamasato, 1993)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Rhizobium sp.	AF054930	(Yan et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Sinorhizobium morelense	AY024335	(Wang et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Agrobacterium albertimagni	AF316615	(Salmassi et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bartonella henselae	AJ223779	(Sander et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Ochrobactrum sp	AF229848	(Reiter and Sessitsch, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Blastochloris sulfoviridis	AJ012089	(Zengler et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bradyrhizobium japonicum	AF239844	(Chueire et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Hyphomicrobium W1-1B	U59505	(Fishbain et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Roseobacter sp. Och114	M59063	(Woese, 1991)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Oseovarius tolerans	Y11551	(Labrenz et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Paracoccus sp. KS2	U58016	(Jordan et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Rhodovulum robiginosum	Y15012	(Straub et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Rickettsia bellii	U11014	(Stothard et al., 1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Wolbachia sp.	AJ010275	(Bandi et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.9. Beta-Proteobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** *** **********
Neisseria weaveri	L10738	(Andersen et al., 1993)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Vitreoscilla stercoraria	L06174	(Dewhirst et al., 1992	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Dechlorisoma sp	AF170350	(Coates et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Propionibacter pelophilus	AF016690	(Meijer et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Ferribacterium limneticum	Y17060	(Cummings et al, 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Azoarcus sp. CC-11	AB033745	(Shinoda et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thauera terpenica	AJ005817	(Foss and Harder, 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Sterolibacterium denitrificans	AJ306683	(Tarlera, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Alcaligenes faecalis	AF155147	(Ansede et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bordetella avium	AF177666	(Kattar et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Taylorella asinigenitalis	AF067729	(Jang et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Brackiella oedipodis	AJ277742	(Willems et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Burkholderia sp. HY1	AF210314	(Kahng et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Oxalobacter formigenes	U49758	(Sharp et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Acidovorax avenae subsp. avenae	AB021421	(Anzai et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Aquaspirillum metamorphum	Y18618	(Schulze et al, 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Hydrogenophaga intermedia	AF019037	(Feigel and Knackmuss, 1993)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Tepidimonas ignava	AF177943	(Moreira et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Matsuebacter sp	AB024306	(Kawamukai, 1999)	GTGCCAGCAGCCGCGG.....AAATTCAAAGGAATTGACGG
Ralstonia sp	AF139729	(Schloemann and Seibert, 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Nitrosomonas europaea	AF037106	(Juretschko et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Thiobacillus sp	X97534	(Visser et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.10. Gamma-Proteobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Alteromonas sp	AB040466	(Kondo and Imai, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Acinetobacter sp	AJ275041	(De Baere, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Buchnera aphidicola	AJ296750	Martinez-Torres., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Escherichia coli	AB045731	(Akiko, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Haemophilus influenzae	AF224306	(Dewhirst et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Nitrosococcus oceani	AF338212	(Voytek, 1996)	GTGCCAGCAGCCGCGG.....GAACTCAAATGAATTGAGGG
Pseudomonas syringae	AB001439	(Sawada et al., 1997)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Serratia fonticola	AJ279002	Ashelford et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Vibrio sp	AF369642	(Burja, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAATGAATTGAGGG
Xanthomonas sp	AF335549	(Taylor et al., 2001)	GTGCCAGCAGCCGCGG.....GAACTCAAATGAATTGAGGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.11. Delta-Proteobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** ********** *********
Archangium gephyra s	M94273	(Shimkets. and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bdellovibrio stolpii	M34125	(Woese, 1990)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Bdellovibrio bacteriovorus	M59297	(Woese, 1991)	GTGCCAGGAGCCGCGG.....AAACTCAAAGAAATTGACGG
Chondromyces crocatus	M94275	(Shimkets and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Corallococcus exiguus	AJ233932	(Sproer et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Cystobacter fuscus	M94276	(Shimkets and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Desulfobacca acetoxidans	AF002671	(Oude Elferink et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Desulfococcus biacutus	AJ277887	(Swiderski, 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Desulfomicrobium norvegicum	AJ277897	(Swiderski, 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGAAATTGACGG
Desulfonema magnu	U45989	(Fukui et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Desulfovibrio sp	AJ251630	(Kulik, 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGAAATTGACGG
Geobacter grbicium	AF335183	(Achenbach. and Coates, 2001)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Malonomonas rubra	Y17712	(Ludwig., 1998)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Melittangium lichenicola	M94277	(Shimkets. and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Myxococcus xanthus	AJ233930	(Sproer et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Nannocystis exedens	AJ233947	(Sproer et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Nitrospina gracilis	L35504	(Weisburg et al., 1989)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Pelobacter sp	AJ271656	(Thamdrup et al., 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Polyangium cellulosum	M94282	(Shimkets and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Stigmatella aurantiaca	M94281	(Shimkets and Woese, 1992)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Syntrophobacter sp	X94911	(Zellner et al., 1996)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.12. Epsilon-Proteobacteria sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Alvinella pompejana	L35520	(Haddad et al., 1994)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Campylobacter jejuni	AL139076	(Parkhill et al., 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Dehalospirillum multivorans	X82931	(Scholz-Muramatsu et al., 1995)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Helicobacter sp	AF320621	(Dewhirst et al., 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Sulfuricurvum kujiense	AB053951	(Kodama and Watanabe, 2001)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Thiomicrospira sp	U46506	(Voordouw et al., 1996)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Campylobacter faecalis	AJ276874	(Kachler, 2000)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Flexispira rappini	AF034135	(Tee et al., 1998)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Arcobacter butzlerii	L14626	(Stanley et al., 1993)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Sulfurospirillum arcachonense	Y11561	Finster et al., 1997)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Uncultured epsilon proteobacterium	AF367496	(Corre et al., 2001)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.13. Planctomyces sequence alignments.

Organism	Accession #	Reference	530. 907
			******** ******* ********** *********
Gemmata obscuriglobus	X85248	(Fuerst et al., 1997)	GTGCCAGGAGCCGCGG.....AAACTCAAAGAAATAGACGG
Pirellula staleyi	M34126	(Woese and Oyalzu , 1990)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Uncultured *Planctomyces*	AF195423	(Kelly and Chistoserdov., 2001)	GTGCCAGGCGCCGCGG.....AAACTCAAAGGAATAGACGG
Isosphaera sp	X81960	(Ward et al., 1995)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Planctomyces brasiliensis	AJ231190	(Griepenburg et al., 1999)	GTGCCAGGAGCCGCGG.....AAACTCAAAGGAATAGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.14. High GC Gram positive (Actinomyces) sequence alignments.

Organism	Accession #	Reference	430. 907
			****** * ***** * **********
Actinomyces sp.	AF28774	(Paster et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Corynebacterium kroppenstedtii	Y10077	(Collins et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Agrococcus baldri	AJ309928	(Zlamala, et al., 2002)	GTGCCAGCWGCCGCKG.....AAACTCAAAGGAATTGACGG
Microbacterium sp.	AF287752	(Paster et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Subtercola sp.	AJ310412	(Behrendt et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Curtobacterium flaccumfaciens	AJ312209	(Behrendt et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Arthrobacter keyseri	AF256196	(Eaton, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Micrococcus luteus	AJ312751	(Wiese, 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Dermabacter sp. B46KS	AF287753	(Paster et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Georgenia sp	AJ308598	(Busse, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Pseudonocardia compacta	AJ252825	(Lee, 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Saccharothrix waywayandensis	AB020029	(Kinoshita et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Amycolatopsis rubidus	AF222022	(Huang et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Nocardia seriolae	AF254420	(Chen et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Rhodococcus sp.	X85242	(Soddell et al., 1995)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Tsukamurella columbiensis	AF272835	(Brown et al., 2000)	GTGCCARCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Mycobacterium holsaticum	AJ310467	(Richter et al., 2002)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Dietzia natronolimnaea	X92157	(Duckworth et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Clavisporangium rectum	AB062380	(Nakajima et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Streptomyces sp. SNG9	AF295602	(Mabrouk and Sabry, 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Propionibacterium avidum	AJ003055	(Dasen et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Slackia exigua	AF101240	(Wade et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Anaerobranca bogoriae	AF203703	(Prowe and Antranikian, 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Acholeplasma vituli	AF031479	(Angulo et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGACGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.15. Spirochet sequence alignments.

Organism	Accession #	Reference	530. . . . . .907 ********** ********** *********
Borrelia parkeri	AF307100	(Gage et al., 2000)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Borrelia burgdorferi	AJ224134	(Postic et al., 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Spirochaeta sp.	X97096	(Rheims et al., 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Treponema brennaborense	Y16568	(Schrank et al.,1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Leptonema illini	Z21632	(Hookey et al.,1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Leptospira biflexa	Z98591	(Hookey,1997)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Serpulina hyodysenteriae	U23036	(Jensen and Stanton, 1994)	GTGCCAGCAGCCGCGG.....AAACTCAAAGAAATTGAGGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG

Figure B.16 Thermotogales sequence alignments.

Organism	Accession #	Reference	530. 907
			********** **********
Caldotoga fontana	AJ237665	(Xu, 1999)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Fervidobacterium gondwanense	Z49117	(Andrews and Patel, 1996)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Thermotoga neapolitana	AJ401024	(Nesbo et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Thermotoga maritima	AJ401017	(Nesbo et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Thermopallium natronophilum	U37021	(Nesbo et al., 2001)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Petrotoga mobilis	Y15479	(Lien et al., 1998)	GTGCCAGCAGCCGCGG.....AAACTCAAAGGAATTGAGGG
Primer Sequence			GTGCCAGCMGCCGCGG.....AAACTYAAAKGAATTGACGG