UNDERSTANDING THE IMPACTS OF METAL POLLUTION ON BIOTIC COMMUNITIES IN TWO HIGH ELEVATION TRIBUTARIES

A Thesis

by

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ABSTRACT

UNDERSTANDING THE IMPACTS OF METAL POLLUTION ON BIOTIC COMMUNITIES IN TWO HIGH ELEVATION TRIBUTARIES

(December 2007)

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Two high elevation tributaries in Watauga County, North Carolina were chosen to investigate the effects of iron (Fe), manganese (Mn), and zinc (Zn) pollution on biotic communities. The physical environment of both tributaries was altered by anthropogenic activity. A result of this disturbance was the presence of seeps heavily laden with an orange biofilm. Chemical analysis of the tributaries downstream of the seeps indicated elevated levels of Fe, Mn, and Zn in both water and sediment samples compared to an undisturbed reference tributary. Bacterial biofilm communities were described using 16S rRNA clone library analysis. Results indicated that the communities in metal- rich portions of the stream were not notably different from the undisturbed reference tributary with the exception of the presence of Gammaproteobacteria and Comamonadaceae bacteria only in the metal polluted locations. However, index analysis of benthic macroinvertebrate community structure yielded several significant differences between disturbed and reference sites.

Lower abundance and diversity of macroinvertebrate communities in metal polluted locations suggests that increased Fe, Mn, and Zn deposition limits colonization and promotes drift. However, bacterial community structure is largely unaffected. The lack of any commonly recognized metal oxidizing bacteria indicates that Fe, Mn, and Zn deposition in these tributaries may be a biologically independent process. Interestingly, the loss of scraping macroinvertebrate taxa such as Heptageniid mayflies, indicates that Fe, Mn, and Zn deposition negatively influences the diversity of benthic communities. The effect of metal deposition on macrophyte communities on which Heptageniids depend may be greater than the effect on bacterial communities and may therefore play a larger role in determining macroinvertebrate community structure.

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DEDICATION

For my grandparents Marvin and Jean Culpepper and Elmer and Alberta Pitchford, whose love will always be with me.

For two of my very best friends Larry and Janice Pitchford, my Father and Mother.

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Introduction

Anthropogenic disturbance in the Appalachian Mountains, including the processes of road building, mining, and urbanization, has become increasingly common due to the demand for, and limited supply of, usable land. Many areas in Watauga County, North Carolina, currently being developed for residential and commercial purposes are adjacent to small tributaries and wetlands, thus placing severe stress on these ecosystems. The term *disturbance*, as it applies to stream ecosystems, is defined by Resh *et al.* (1988) as "any relatively discrete event in time that is characterized by a frequency, intensity, and severity outside a predictable range, and that disrupts ecosystem, community, or population structure and changes resources or the physical environment." An initial investigation of a tributary in the New River watershed in Watauga County indicated that anthropogenic activity had altered the physical and biological components (Greco 2005) and that certain portions of the tributary meet the criteria of a "disturbed" stream, thus further research into the implications of these disturbances may help in prescribing proper treatment and future management systems.

An alteration in the physical habitat of a tributary elicits a bottom-up effect on the biological community, where abiotic factors (i.e. temperature, pH, iron concentrations) determine the diversity and distribution of biotic communities (Dodds 2002). For example, increasing the concentration of naturally occurring metals can result in shifts in biofilm communities from algae, protists, and diatoms to metal depositing bacteria (Sheldon & Skelly 1990; Sheldon & Wellnitz 1998; Emerson & Weiss 2004; Morin *et al.* 2007). Furthermore, chemical and biological metal deposition negatively impacts substratum quality upon which benthic macroinvertebrate communities depend, oftentimes eliciting species specific responses (Lemly & King 2000; Courtney & Clements 2002). Graded responses by benthic macroinvertebrates to metal concentrations allow insect community structure to act as an index of metal pollution in a given stream (Winner *et al.*1980; Nelson & Roline 1996; Schmidt *et al.* 2002; Rhea *et al.* 2004).

Metal Interactions

Metal cycling is a continuous process in aquatic environments and is controlled by a combination of abiotic and biotic factors that impact both the chemical form and the mobility and availability of metals. Iron, manganese, and zinc are considered minor elements in aquatic systems, and their influence is diminished compared with other nutrients. However the cycling of these metals has considerable impacts (Cole 1988; Wetzel 2001) particularly in areas affected by anthropogenic disturbance where an increase in the concentration of iron, manganese, and zinc has been noted (Sheldon & Skelly 1990; Sheldon & Wellnitz 1998; Emerson & Weiss 2004; Greco 2005; Morin *et al.* 2007). Below is a brief introduction to the major forms of iron, manganese, and zinc in aquatic environments, and an explanation of ways in which abiotic and biotic processes regulate their chemical form and movement in groundwater, streambeds, and streamwater.

Iron (Fe) is one of the most abundant elements on the earth's surface. It exists in natural environments in two states: oxidized Fe^{3+} (ferric) and reduced Fe^{2+} (ferrous). The cycling of Fe in aquatic environments involves continuous chemical and biological transformations from ferric to ferrous states (Wetzel 2001; Cole 1988; Madigan & Martinko 2006). Fe entering oxygenated waters from soil drainage and groundwater is generally in soluble ferrous form, but surface runoff can have higher concentrations of insoluble Fe^{3+} (Cole 1988; Wetzel 2001). Temperature, pH, and redox potential all have large influences on Fe cycling, however the conditions regulating bacterial metabolism also have an effect on the spatial and temporal variations in the physical chemistry of Fe (Wetzel 2001).

Fe^{2+} is found in aquatic environments chemically bound to humic material from soil drainage, as dissolution from insoluble iron hydroxides and iron phosphates, or as deposited soluble ferrous sulfide (FeS_2). Submerged soils containing reduced Fe have a characteristic bluish-gray to greenish-gray color typical of submerged anoxic soils (Mitsch & Gosselink 1993). Fe^{2+} is soluble and is readily leached from soils and carried by groundwater to oxygen interfaces (Gambrell 1994). In environments where pH is neutral, Fe^{2+} entering oxic zones can be oxidized in several ways.

Free Fe^{2+} ions are considered very unstable and readily react chemically with O_2 when entering aerobic zones in the streambed and stream water. This process results in the formation of a flocculent ferric hydroxide, $Fe(OH)_3$, that precipitates automatically in oxygenated environments where pH values range from 7.5 to 7.7 (Cole 1988; Dodds 2002) (see equation 1: Madigan & Martinko 2006).

Equation 1: $$Fe^{2+} + \frac{1}{4} O_2 + 2\frac{1}{2} H_2O \rightarrow Fe(OH)_3 + 2H^+$$

The precipitate has a characteristic brown/orange color and often settles to the stream bottom. Precipitates may eventually reach anoxic regions of the stream bottom where they dissociate into hydroxide and free ferrous Fe ions (Mitsch & Gosselink 1993). Ferrous Fe can also readily be oxidized chemically at neutral pH by phosphate and become another immobile precipitate $FePO_4$. $FePO_4$ percipitate eventually settles into anoxic stream sediments and dissociates into phosphate and ferrous ions in a similar manner as ferric hydroxides (Dodds 2002). In some cases ferric oxides adsorb to algae or dead particles where they are assimilated into living systems, although the mechanisms by which this occurs is unclear (Wetzel 2001).

Biological oxidation of Fe occurs at the oxic/anoxic interface at groundwater inputs and in areas where anoxic stream sediments enter oxic zones in the stream bottom. There are several genera of chemoautotrophic Fe oxidizing bacteria including *Gallionella*, *Leptothrix,* and *Ochrobium* (Wetzel 2001; Mitsch & Gosselink 1993). These bacteria are able to oxidize Fe before it reacts with oxygen chemically thereby gaining necessary energy for growth. The niche for these bacterial types is in areas with a neutral pH where the redox gradient is very steep. In these habitats they are able to effectively compete with oxygen for reduced forms of Fe (Wetzel 2001). Very little energy is obtained from this reaction, so these bacteria must oxidize large amounts to meet their metabolic requirements (Madigan & Martinko 2006).

Fe entering stream water via soil drainage and anoxic sediments can be held in solution by humics and organic compounds that form ionic bonds with Fe, thereby preventing precipitation and allowing Fe to become soluble and move downstream in stream water (Cole 1988; Dodds 2002). Iron bicarbonate ($Fe(HCO_3)_2$ is an example of compound that is oxidized by species of aquatic heterotrophic bacteria from the genera *Cladothrix, Leptothrix,* and *Siderocapsa. Siderocapsa* growth typically coincides with high amounts of rainfall that introduce Fe humates into stream water from soil runoff. *Leptothrix* and *Cladothrix* growth occurs primarily in oxygenated regions of the water column where they deposit Fe oxides on their sheaths when oxidizing organic material like $Fe(HCO_3)_2$ for energy. Resultantly, insoluble Fe oxides are produced and precipitate from the water column. A typical Fe humate oxidation reaction is shown in equation 2 (Wetzel 2001)

Equation 2: $4Fe(HCO_3)_2 + O_2 + 6H_2O \rightarrow 4Fe(OH)_3 + 4\ H_2CO_3 + 4CO_2 + 58\ \text{kcal}$

In acidic environments Fe^{2+} is stable and is available to chemosynthetic bacteria in the presence of oxygen (Mitsch & Gosslink 1993). Common types of these bacteria include *Acidithiobacillus ferroxidans* and *Leptospirillum ferroxidans.* Each thrives in acidic environments in the presence of large amounts of reduced Fe (Madigan & Martinko 2006). Acid mine drainages (AMDs) have just the right conditions for this type of bacterial growth. The presence of chemosynthetic bacteria in AMDs has been shown to increase Fe^{2+} oxidation by a factor of 10^6 (Mitsch & Gosselink 1993). Recovery zones in streams affected by AMDs are visible where masses of flocculent ferric hydroxides, often referred to as "yellow dog", has formed. These regions mark recovery from AMDs because pH and oxygen levels have risen and allowed spontaneous Fe oxidation to occur (Cole 1988).

Fe^{3+} is reduced both chemically and biologically in anaerobic environments like groundwater. Abiotic reduction occurs when $Fe(OH)_3$ and $FePO_4$ precipitates are dissociated, as mentioned previously. Dissociation occurs when immobilized Fe oxide precipitates settle to anaerobic humic layers of the soil (Dodds 2002).

Both chemolithotrophic and chemoorganotrophic bacteria also play a significant role in Fe^{3+} reduction by using it as an electron acceptor in anaerobic respiration. Often this process is coupled to the oxidation of organic substances. *Geobacter metallireducens* is a well known Fe reducer that couples the reduction with the oxidation of acetate (see equation 3: Madigan & Martinko 2006).

Equation 3: $$\text{Acetate}^- + 8\ Fe^{3+} + 4\ H_2O \rightarrow 2\ HCO^{3-} + 8\ Fe^{2+} + 9\ H^+$$

The bacterium *Desulfovibrio* indirectly facilitates Fe reduction. A metabolic by-product of these bacteria is H_2SO_4 which promotes Fe reduction and solubilization.

Free Fe^{2+} ions can form salts, complexes with ammonia, and complexes with sulfides (iron pyrite-FeS) in anaerobic zones, or can be leached from soil by groundwater and begin migration to aerobic environments to be oxidized (Mitsch & Gosselink 1993). The complete Fe cycle in freshwater ecosystems is provided in Figure 1.

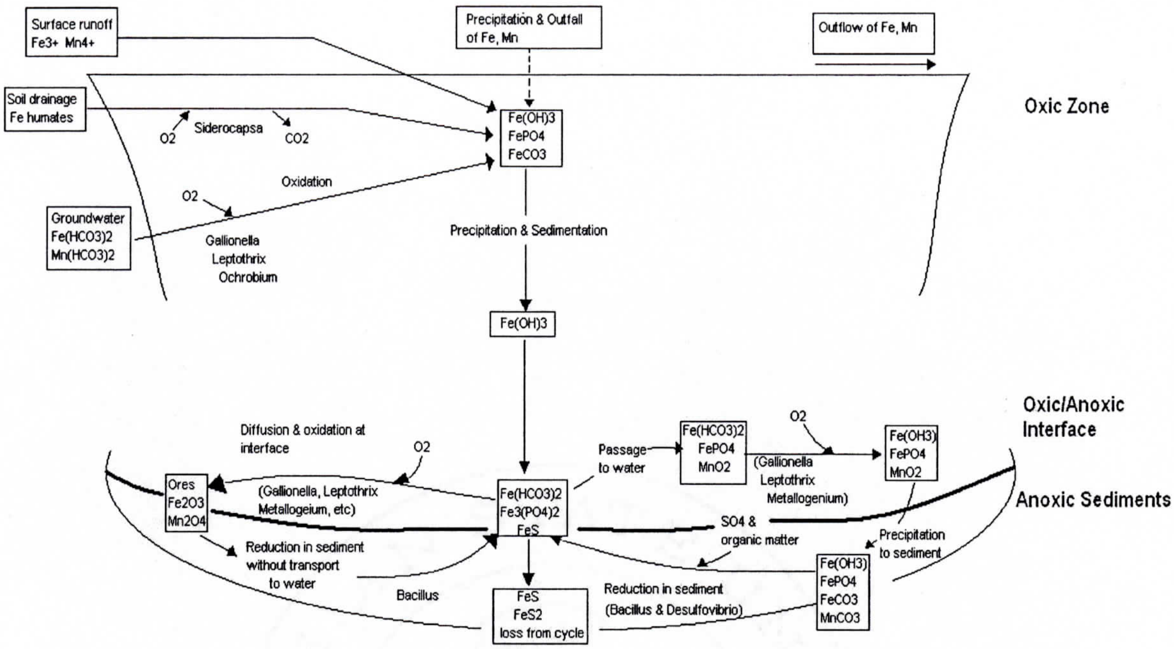

Figure 1. The Fe and Mn cycle in freshwater ecosystems. Adapted from Wetzel (2001).

Manganese (Mn) is a ubiquitous element in aquatic environments. It is the fifth most abundant metal on the Earth's surface (Gounot 1994), and plays a particularly important role in redox reactions in photosynthesizing organisms like algae (Dodds 2002). It has four valance states; the most stable of which are soluble Mn^{2+} (reduced) and less soluble Mn^{4+} (oxidized) (Cole 1988; Madigan & Martinko 2006). Mn in aquatic environments is most commonly observed as reduced soluble Mn^{2+}, soluble chelated Mn complexed with organic material, or in very stable particulate oxidized form bound with oxygen (MnO_2) or carbon ($MnCO_3$) (Ponnamperuma 1972; Cole 1988; Wetzel 2001). It has been established that microbes play a large role in Mn cycling either directly by using Mn as electron acceptors/donors, or indirectly by producing by-products that oxidize or reduce Mn (Nealson *et al.* 1988).

Soluble Mn in reduced form migrates from groundwater and submerged soils by mass flow or diffusion to oxygen interfaces in the streambed and commonly adsorbs to $Fe(OH)_3$ and MnO_2 and is oxidized, producing immobile Mn-rich nodules (Ponnamperuma 1972). This process is thermodynamically favored at neutral pH and high oxygen levels, however, the process occurs very slowly because the activation energy required for spontaneous oxidation is high (Gounat 1994).

At the oxic/anoxic interface of streambeds some species of sheathed aquatic bacteria have the ability to oxidize Mn^{2+}. Even aquatic environments with very low nutrient concentrations like artic lakes still support growth of Mn oxidizing bacteria (Gounat 1994). Common aquatic bacterial genera capable of Mn oxidation include: *Leptothrix, Hyphomicrobium*, and *Sphaerotilus* (Madigan & Martinko 2006). Mn oxidation is exergonic and there is some evidence that it is an energy yielding process for these bacteria (Madigan & Martinko 2006). It has been hypothesized that Mn^{2+} oxidation is coupled to the electron transport chain in the generation of a proton motive force in the cell wall of bacteria (see equation 4: Madigan and Martinko 2006).

Equation 4: $$Mn^{2+} + \frac{1}{2} O_2 + H_2O \rightarrow MnO_2 + 2\,H^+ \qquad \Delta G^{0'} = -68\ kJ$$

The major transformation of immobile Mn oxides in groundwater and submerged soils is reduction of immobile precipitated Mn^{4+} oxides to mobile Mn^{2+} (see equation 5: Wetzel 2001).

Equation 5: $$Mn^{4+} + 2e^{-} \rightarrow Mn^{2+}$$

Mn is considered somewhat more soluble than Fe in oxidized form; therefore Mn reduction occurs slightly before Fe on the redox scale (Mitsch & Gosselink 1993; Wetzel 2001). Reduction as part of a geochemical process can be coupled to sulfide production in groundwater and submerged soils with excess Mn oxides (Burdige & Nealson 1986). Although bacteria are also responsible for Mn reduction in these environments, Bratina *et al.* (1995) found that Mn^{4+} is reduced primarily by sulfide in anaerobic lake sediments.

Mn reduction is accomplished directly and indirectly by microbes in oxygenated surface water, stream sediments, and anaerobic subsurface water (Gounot 1994). Indirect reduction occurs in oxic conditions of streamwater and streambeds when chelated Mn interacts with extracellular metabolites of microbes like hydrogen peroxide (Bratina *et al.* 1995) and nitrates (Gounat 1994) that act as Mn reductants. Fe^{3+} reducing bacteria are also indirect Mn reducers since Fe^{2+} is a Mn reductant as well (Gounat 1994).

Direct involvement of microbes in Mn reduction typically occurs in anoxic conditions of groundwater and submerged soils by chemoorganotrophic bacteria. Gounat (1994) showed that of 100 isolated bacterial strains in subsurface sediments associated with groundwater, 81 were responsible for Mn reduction. However, as microbial reduction persisted, the pH of anaerobic environments became slightly acidic facilitating indirect Mn reduction. As pH levels became so acidic that spontaneous reduction would not occur only 12 bacterial species still reduced Mn, indicating that these bacteria may rely on the process as part of the electron transport chain. Species of *Pseudomonas, Bacillus*, and *Acinetobacter* are a few examples of Mn reducers commonly found in acidic anaerobic environments (Gounat 1994; Bratina *et al.* 1995). The complete Mn cycle in freshwater ecosystems is provided in Figure 1.

In low concentrations, zinc (Zn) is present in rocks, soils, water, the atmosphere, and living organisms (Madigan & Martinko 2006). Like Fe and Mn, Zn is also a trace element required for cellular processes in many organisms. Rain carries 2.5 to 12 mg/m^3 of Zn, so it is not usually considered in short supply (Cole 1988). Zn transformations in aquatic environments are poorly understood, and most information about the cycling of Zn is inferred from other elements like Fe and Mn (Wetzel 2001).

Zn is found in aquatic environments in ionic form, chemically bound with organic material, adsorbed or precipitated on solids, or incorporated into crystalline structures (Wetzel 2001). In aerated surface water the overall amount of Zn is very small. In neutral pH stream water and streambeds soluble free Zn^{2+} ions may form stable complexes with organic material and remain mobile; therefore Zn solubility in groundwater and submerged soils will increase in environments where Mn and Fe reduction results in production of organic complexing agents (Gambrell 1994; Wetzel 2001). In soluble form Zn is readily assimilated by aquatic organisms, particularly in environments with elevated temperatures where it can become toxic to some fish species (Dodds 2002).

In oxygenated, neutral pH, aquatic environments the majority of Zn is adsorbed to particulate matter. In waters with low pH Zn solubility increases, and a small portion of adsorbed Zn may become mobile and available for assimilation in photosynthesizing organisms where it plays a role in hydrogen transfer or protein synthesis in heterotrophic organisms. If Zn is not readily assimilated, it may be co-precipitated in lake sediments as an immobile sulfide along with calcium carbonate ($CaCO_3$) and $Fe(OH)_3$. Therefore, Zn mobility is influenced most by pH of the aquatic environment (White & Driscoll 1987; Cole 1988; Wetzel 2001).

Zn is in immobile oxidized form when it forms complexes with hydroxides, sulfides, phosphates, or carbonates. It also becomes immobile if forming crystalline structures with salts or other Zn ions (Cole 1988; Wetzel 2001). In most streambed and groundwater environments, particularly those with acidic soils, these immobile forms of Zn are most commonly found. Overall, Zn mobility in groundwater and submerged soils is depressed when compared with other trace elements (Ponnamperuma 1972).

Inputs of Zn, like most other trace elements, are increasing in aquatic environments. Sources like combustion and industrial emissions are often the cause. In some cases acid rain can increase leaching of trace elements resulting in increased soluble concentrations.

Microbial Communities

Research describing microbial communities in freshwater tributaries and wetlands with elevated concentrations of Fe and Mn typically characterizes the microbial community using microscopic examination of either slides placed in stream

(Sheldon & Skelly 1990; Emerson & Weiss 2004) or microbial mat cores (Emerson & Revsbech 1994). Researchers have often determined that the presence of Fe deposits or Fe encrusted bacterial sheaths is indicative of the dominance of metal oxidizing bacteria from the genera *Leptothrix* and *Gallionella*. The recent emergence of molecular techniques including 16S rRNA gene library analysis and terminal restriction fragment length polymorphism (T-RFLP) analysis has aided in identifying dominant members of microbial communities in these systems (Chan *et al.* 2001; Stein *et al.* 2001; Bruneel *et al.* 2006). The following paragraphs provide a brief review of this research including descriptions of methodology and results.

Bruneel *et al.* (2006) examined the diversity of microorganisms in an iron-arsenic (As) acid mine drainage in Carnoules, France. Bacterial DNA was isolated from water samples collected in three sites downstream of Fe and As inputs from the Carnoules mining site in October 2002 and January 2003. Initially, T-RFLP analysis of sixty clones from each sampling site was conducted; and16S rRNA sequences were obtained for the most frequently occurring T-RFLPs. An average of 10 T-RFLPs was found at each site, indicating low bacterial diversity. Of the 31 sequences obtained, 80 % were either uncultured organisms, or organisms recently associated with acid mine drainage. Phylogenies of 16S rRNA gene sequences indicated that the closest known relatives of the majority of sequences were from the genera *Gallionella*, *Desulfobacterium,* and *Acidithiobacillus*.

Emerson & Weiss (2004) examined bacterial Fe oxidation in several sites within a Virginia spring-fed wetland with neutral pH over the course of a year and determined that Fe^{2+} concentrations of stream water ranged from 1.4 mg/L in fall, winter, and spring to 16.8 mg/L during summer months. Microscopic examination throughout the year described an abundance of Fe oxidizing *Leptothrix ochracea* sheath material as the principle component of Fe oxide flocs. A second research component examined a pH neutral thermal spring in Yellowstone National Park with Fe^{2+} concentrations averaging 5.6 mg/L, and discovered a bacterial community dominated by photosynthetic cyanobacteria. Comparing the results from the Virginia spring to the thermal spring in Yellowstone indicate that microbial communities can respond differently to increased Fe in pH neutral habitats.

An investigation of bacterial and archaeal populations in a Fe/Mn rich Green Bay, Wisconsin tributary was accomplished by constructing a 16S rRNA gene library. Two metal oxidizing groups related to *Leptothrix* and *Hyphomicrobium* were present along with the metal reducing groups related to *Magnetospirillum*. Of the 78 total clones 22% had gene sequences similar to organisms that either oxidize or reduce Fe or Mn. Archaeal populations were composed of a group of methanogens and a group of Crenarchaeota (Stein *et al.* 2001).

Chan *et al.* (2001) described the bacterial community of biofilm and in the water column of a lead (Pb)/Zn acid mine drainage in Mississippi. Scanning electron and transmission electron microscopy aided in detecting the presence of Fe oxide stalks characteristic of *Gallionella* and *Leptothrix*. 16S rRNA library analysis confirmed the presence of relatives of *Gallionella ferruginea,* but also indicated the presence of Actinobacteria, Acidobacteria, Bacteriodetes, Planctomyces. Sixty percent of the 186 sequences were from novel organisms, previously undescribed, and none of the sequences were relatives of *Leptothrix* (Chan *et al.* 2001).

Wellnitz & Sheldon (1995) observed diatom limitation in a neutral stream with elevated levels of Fe and Mn in Vermont. They initially hypothesized that diatom limitation resulted from confounding effects of high Fe and Mn concentrations and subsequent development of a ferromanganese-depositing bacterial bloom. Microscopic analysis indicated the major component of blooms to be the Fe depositing bacterium *Leptothrix orchracea*. An *in situ* experiment followed where soluble Mn was added to certain regions of the stream. Increasing the concentration of Mn led to further diatom displacement and Mn deposition. These results indicated that diatom limitation occurs due to increased Mn, and ferromanganese-depositing bacteria thrive in the absence of diatom competitors.

Emerson & Revsbech (1994) examined the bacterial community of an Fe seep and associated microbial mat in a small stream in Aarhus, Denmark using an acridine orange staining method and epiflourescent microscopy. A stone wall had been previously built into the stream and in several places water emanated from the wall onto microbial mats located in the stream bed below seeps. A correlation between bacterial type and flow rate became apparent. Sites with low flow rates (<2ml/s) consisted primarily of Fe encrusted sheaths of *Leptothrix ochracaea*. Cores taken from these portions of the mat showed that the first few millimeters contained vacated bacterial sheaths with only 7% of sheaths containing actual bacterial filaments. Corresponding Fe concentrations were 4 mg/L in shallow portions of the mat. Cell counts and Fe concentrations were higher in deeper portions of the mat. *Leptothrix ochracaea* filaments increased from 10^8 to 10^9 cells per cm^3 corresponding to Fe concentrations of 12 mg/L. Microscopic identification of bacterial stalks and associated oxides indicated the presence of small pockets of microaerobic bacteria from the genus *Gallionella* in areas close to water sources emanating from the wall. In high flow rate areas, unnamed unicellular bacteria and high concentrations of Fe oxide particulates dominated.

Sheldon & Skelly (1990) showed that the abundance of bacteria in an unnamed mountain brook in Virginia was from the genus *Leptothrix.* The presence of *Leptothrix* was significantly correlated with increased concentrations of Fe and Mn. Methods included monitoring Fe and Mn levels and the abundance and diversity of diatoms at eight sampling locations along a one kilometer stretch of a mountain stream. Microbial identifications were accomplished using microscopic examination of slides placed in stream for an extended period. In the sampling location with the highest levels of Fe and Mn, they noted a shift in the microbial community from diatoms to ferromanganous depositing bacterium *Leptothrix orchracea.* They discovered that a groundwater disturbance had occurred just upstream of this sampling location. Fe and Mn levels decreased at locations further downstream while diatom diversity increased indicating recovery of the biofilm community. At the last sampling location they noted that sixteen of the previous eighteen diatom species found upstream of the disturbance were present.

Benthic Macroinvertebrates

The use of benthic macroinvertebrate community analysis as a valid indicator of water/habitat quality is well established (Hauer & Lamberti 1996; Lemly & King 2000; Courtney & Clements 2002). Diversity and abundance of aquatic organisms, particularly macroinvertebrates, can indicate overall ecosystem health because they act as sensors of the quality of their habitat (Thorp & Covich 2001). The National Water Quality Assessment program of the United States Geological Survey (USGS) uses biomonitoring information on various spatial scales to better understand response and recovery of aquatic communities to disturbance. Biomonitoring data is also a useful tool for the Environmental Protection Agency (EPA) and various local water quality programs to evaluate compliance of industries and large scale agriculture to ordinances regulating point and non-point pollution. In addition to common regulatory purposes, the implementation of biomontoring programs has provided a more complete understanding of the physical, chemical, and biological relationships in aquatic habitats (Gurtz *et al.* 1994).

Species of the Ephemeropteran, Plecopteran, and Tricopteran (EPT) orders are generally classified as pollution-sensitive organisms. Their percentage in the macroinvertebrate community comprises the EPT score, a common index used to assess the ecological health of a given habitat. The EPT taxa are typically more sensitive to perturbations in water chemistry parameters such as nutrient inputs (Lemly & King 2000) and metal ion concentration (Courtney & Clements 2002; Greco 2005). The presence or absence of certain genera or species from these orders can indicate variations in pH, dissolved oxygen levels, nutrient concentrations, and metal ion concentrations. Families of EPT, while not as specific, also have predictable responses to variations in water chemistry. In some cases the abundance of more tolerant families skew general order level percent EPT index scores, thereby misrepresenting water quality in assessed streams (Bode *et al.* 1996). Family level identification of EPT taxa may be a good balance between general order identification and tedious species or genus level identification. This allows for pollution tolerant families within EPT to be taken into account when considering the overall health of the system. A combination of both EPT along with family level identification (described below) may be the most comprehensive way to assess a given stream. It is best to analyze a macroinvertebrate data set using a variety of indices to completely understand ecological health (Hauer & Lamberti 1996).

The Family Biotic Index (FBI) is a reliable tool for assessing a stream with large numbers of facultative or tolerant EPT taxa. This index assigns tolerance scores, ranging from zero to ten, for all macroinvertebrate families. Tolerance scores were originally derived from a study of fifty-three Wisconsin streams with varying degrees of nutrient pollution. A total of 2,000 stream samples were used to compare the occurrence of each species and genera with levels of nutrient pollution in each sampling location. Family tolerance values represent a weighted average of tolerance values of individual species and genera within each family (Hilsenhoff 1988). While a FBI score of zero is assigned to a pollution sensitive Leuctrid stonefly (order Plecoptera), a more tolerant Hydropsychid is assigned a tolerance score of four. Tolerance scores (t) are multiplied by the number of individuals collected in that family (n), the products added, and this total is divided by the total number of organisms (N) to calculate the Family Biotic Index for the entire sample (see equation 6: Hilsenhoff 1988).

Equation 6: $$FBI = 1/N \sum n_i t_i$$

Distribution of FBI scores is given in Table 1 (Hauer & Lamberti 1996). This type of analysis appropriately addresses the problem of tolerant families within EPT taxa by allowing all taxa to contribute to the health rating of a habitat, and is a nice compromise between tedious genus or species level biotic indices and very general % EPT indices (Bode *et al.* 1996).

Table 1 . Water quality ratings based on FBI scores.

Family Biotic Index	Water Quality	Degree of organic pollution
0.00-3.75	Excellent	Unlikely
3.76-4.25	very good	possible slight pollution
4.26-5.00	Good	some probable pollution
5.01-5.75	Fair	fairly substantial pollution likely
5.76-6.50	fairly poor	substantial pollution likely
6.51-7.25	Poor	very substantial pollution likely
7.26-10.00	very poor	severe pollution likely

Benthic community structure is negatively affected in lotic ecosystems with elevated levels of metal ions. A study of an Ohio tributary showed that aquatic worms (family Oligochaeta) and chironmids (family Diptera) made up a large percentage (75% - 81%) of macroinvertebrate communities in metal polluted locations compared with a small portion (10%) of communities in unpolluted locations (Winner *et al.* 1980). Other studies monitoring tributaries and rivers in Colorado and Montana have shown that the abundance of Ephemeropteran, Plecopteran, and Tricopteran (EPT) taxa decreases in Zn polluted habitats compared to unpolluted (Rhea *et al.* 2004), and that members of the family Heptageniidae disappear altogether (Courtney & Clements 2002; Clements *et al.* 2000). Similar research in the Arkansas River, Colorado indicated an inverse relationship between dissolved metal concentration (Zn, copper (Cu) and cadmium (Cd)) and macroinvertebrate diversity and abundance. An observed absence of metal sensitive species in regions of the river where Zn, Cu, and Cd were all present indicate a possible synergistic effect when comparing responses of macroinvertebrates to stream regions where Zn and Cd, or Zn alone was present (Clements 2004).

Other research has shown similar losses of EPT taxa in habitats with elevated concentrations of Fe (Nelson & Roline 1996; Schmidt *et al.* 2002; Turchey-Dooley & Wallace 2002) and Mn (Dills & Rogers 1974). However, monitoring research in Fe and Mn habitats is limited. Further research is necessary to understand the "bottom up effect" of elevated levels of Fe and Mn on macroinvertebrate communities.

Research Objectives

The purpose of this thesis is to addresses physical disturbance as it relates to Fe, Mn, and Zn pollution, concurrent biofilm production, and alterations in macroinvertebrate community composition by answering the following questions:

1- Are the concentrations of Fe, Mn, and Zn in the water and the sediment of two disturbed mountain streams different than those of an undisturbed reference stream?

2- Are the bacterial biofilm communities of two disturbed mountain streams different than those of an undisturbed reference stream?

3- Are the macroinvetebrate communities of two disturbed mountain streams different than those of an undisturbed reference stream?

Answering these questions may help to provide further insight into understanding the mechanism by which physical habitat alterations impact biotic communities.

Materials and Methods

Site descriptions

Two second order streams in the New River watershed of Watauga County, North Carolina (Figure 2) were chosen for this study. Site choice was based on the presence of groundwater seeps and a distinct rust colored biofilm either around the seeps or covering the substrate on the stream bottom.

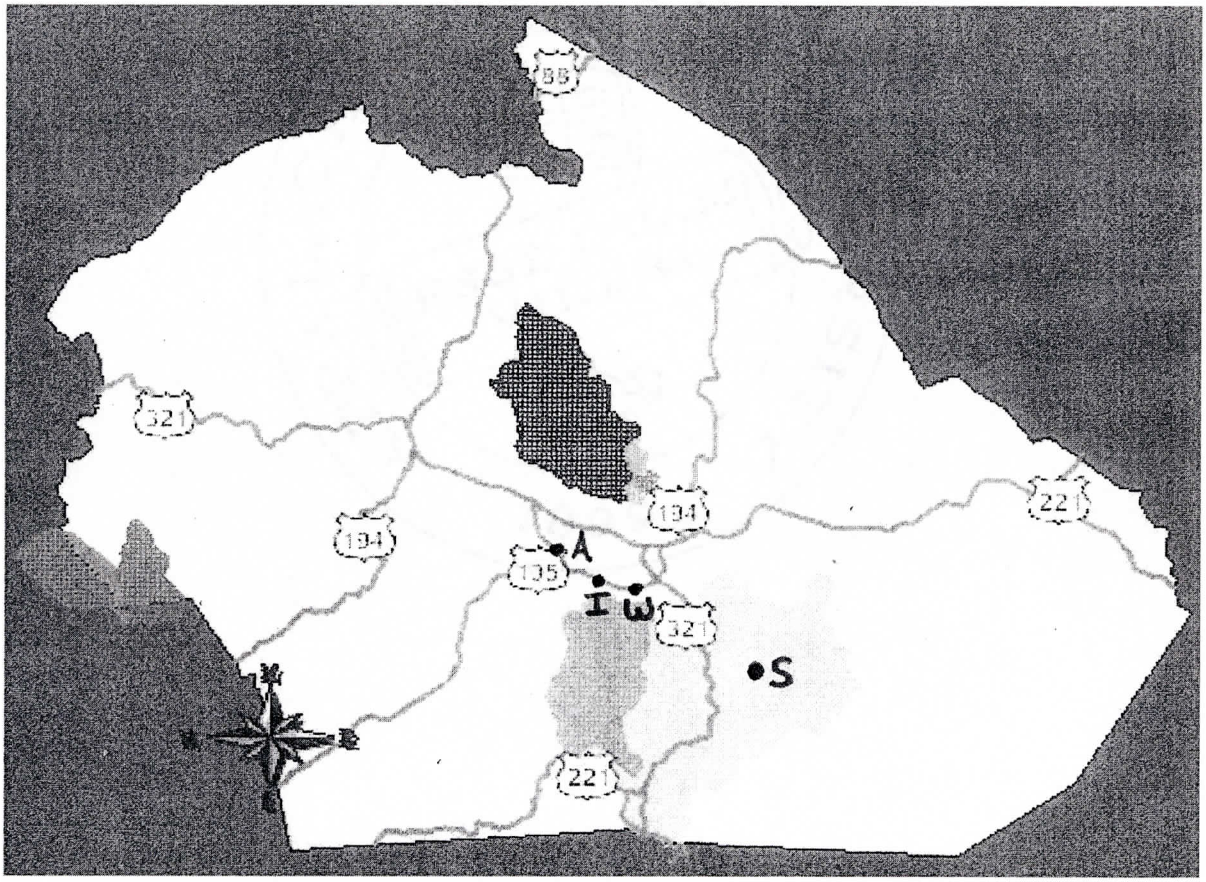

Figure 2. Sampling sites in Watauga County, NC. Sites include: Sorrento (S), Ingles (I), University Highlands (A), and Watauga High school (W).

The Sorrento (S) site is located in Sorrento Skies subdivision in Watauga County, NC. Figure 3 describes the placement of impoundments, seeps, and the four sampling locations located at the site.

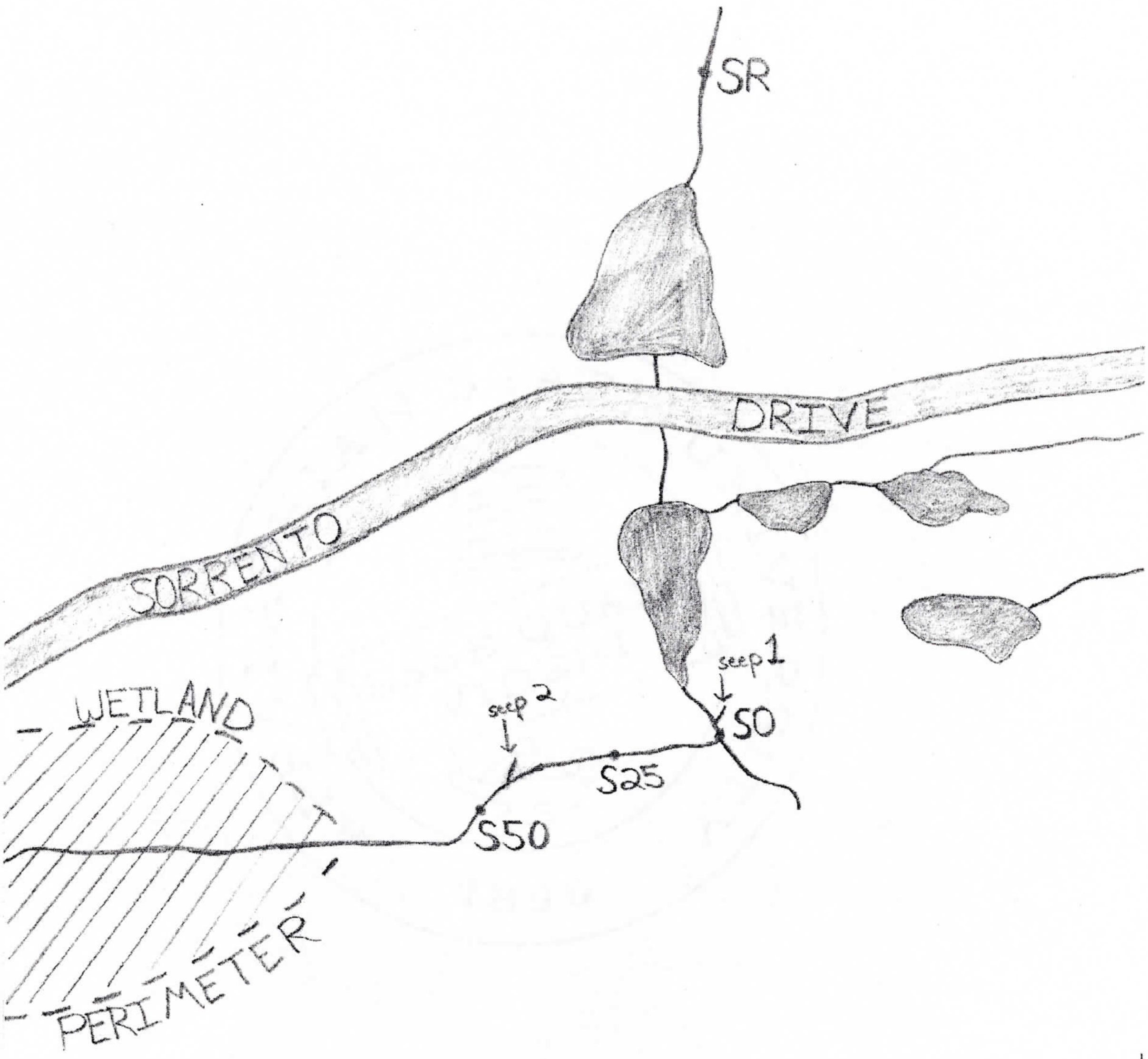

Figure 3. Map of Sorrento Site (S). This illustration displays sampling locations, and the location of seeps and retention ponds. The sampling locations are labeled SR (reference stream upstream of impoundments and rust colored seeps), S0 (first riffle downstream of impoundments and rust colored seeps), S25 (riffle approximately 25 meters downstream of S0, and S50 (riffle approximately 50 meters downstream of S0).

The second site, Ingles (I), is located behind a shopping center off of Highway 105 in Watauga County, NC. This stream emerges from under the parking lot from a galvanized six foot metal drain pipe. Once visible, the stream borders a small wetland for approximately fifty meters before returning underground via a second galvanized drain pipe. Figure 4 describes the placement of impoundments, seeps and sampling locations at this site.

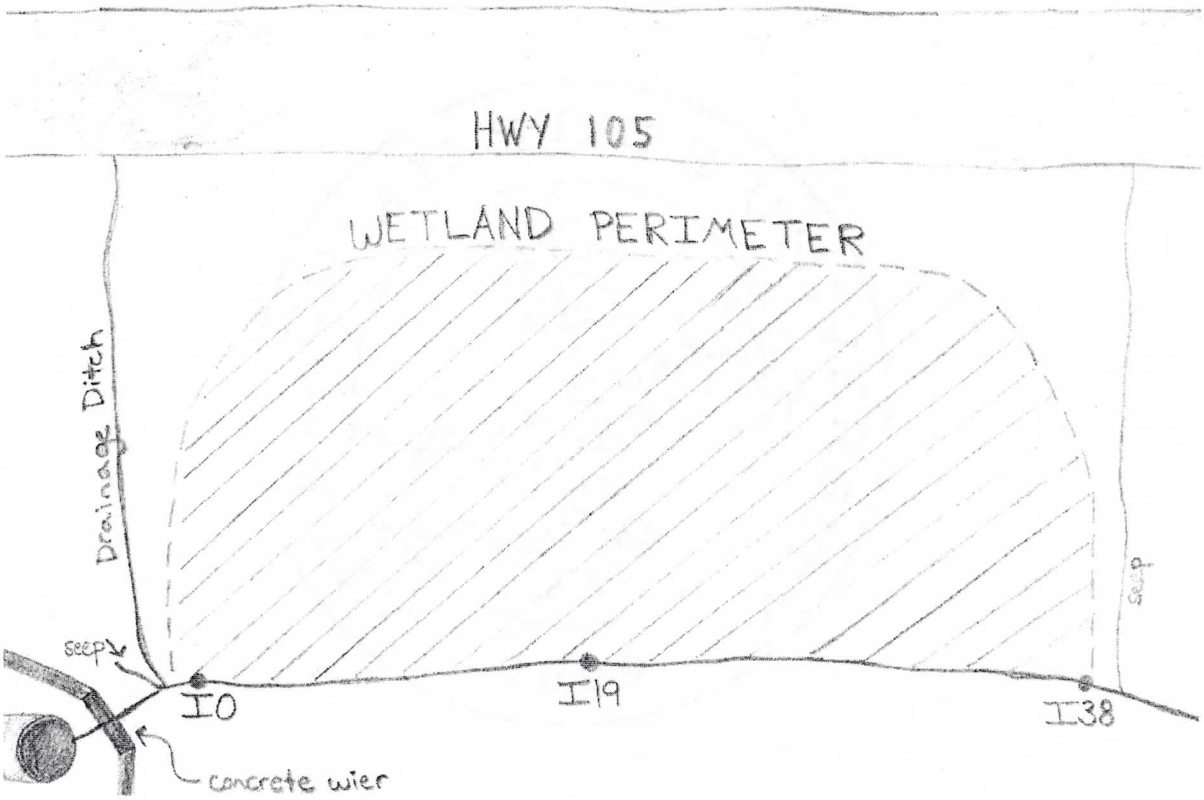

Figure 4. Map of the Ingles Site (I). Sampling locations, impoundments, and seeps are indicated. The sampling locations are labeled I0 (first riffle downstream emerges from drainage pipe), I19 (riffle approximately 19 meters downstream of I0), and I38 (riffle approximately 38 meters downstream of I0).

In addition to these sites, two other streams (Figure 2) were chosen for metal analysis: one at University Highlands (A) and another near Watauga High School (W). Site A is located on the Highway 105 bypass in Boone, NC and site W is located between the Watauga High School football field and Winkler's creek in Boone, NC. Each site contained a sampling location at the start of the stream, and also at twenty-five meters and fifty meters downstream (Figures 5 and 6). These sites are first order streams that have a rust colored biofilm covering the stream bottom. A first order reference stream (WR) is located at the W runs parallel approximately fifty meters away from W0. This stream was chosen as a reference because at the time of the project there was no presence of a rust colored biofilm.

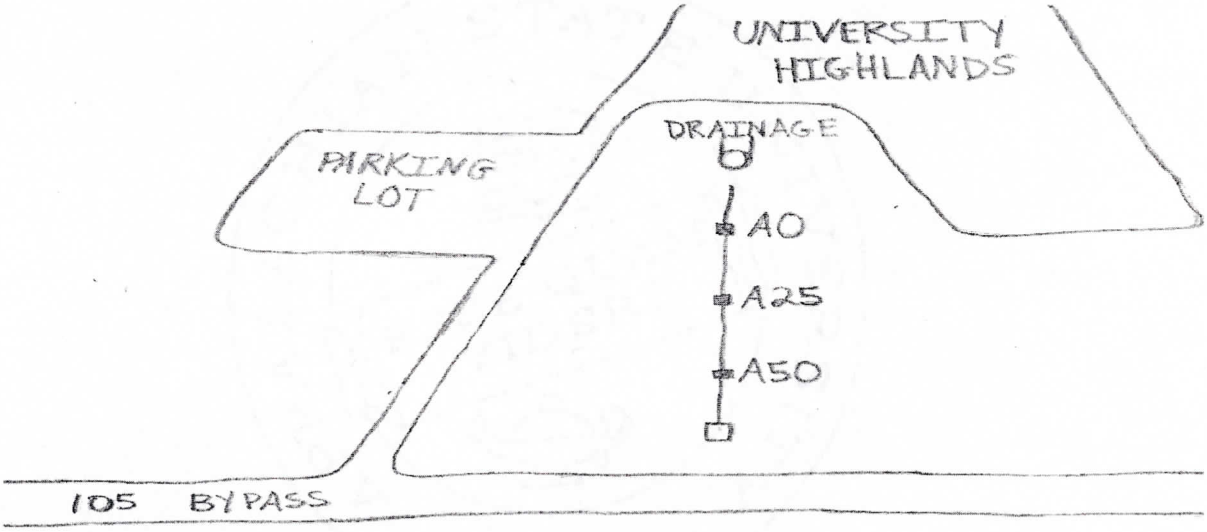

Figure 5. Map of University Highlands Site (A). Sampling locations and drainages are indicated. The sampling locations are labeled A0 (first visible portion of seep emanating from ground), A25 (approximately 25 meters downstream of A0), and A50 (approximately 50 meters downstream of A0).

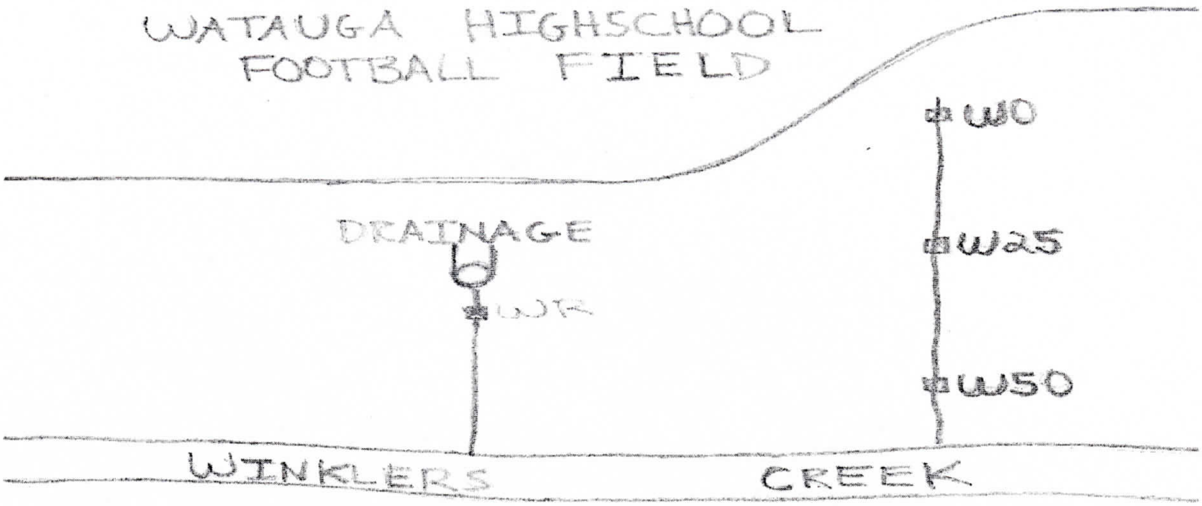

Figure 6. Map of Watauga High School Site (W). Sampling locations and drainages are indicated. The sampling locations are labeled W0 (first visible portion of seep emanating from ground), W25 (approximately 25 meters downstream of W0), and W50 (approximately 50 meters downstream of W0).

Water and Sediment Chemistry

Temperature, pH, and conductivity of each stream were measured using an Oakton pH/CON meter (Vernon Hills, Il). Dissolved oxygen (DO) was measured using a LaMotte #54183 water chemistry kit (Chestertown, MD). Water samples were collected during winter 2006 at all sampling locations by submerging the mouth of 750 ml flasks in the midpoint of the stream channel until the flasks were full. The concentration of Fe, Mn, and Zn in these samples was quantified by Water Quality Services Laboratory in Banner Elk, NC.

Sediment samples were collected during winter 2006 from each sampling location by tapping a 2" PVC core sampler 10cm into the sediment with a rubber mallet. Two sediment samples were collected from each sampling location, dried with a NAPCO 310 drying oven on maximum temperature for two days, and sent to Clemson Agronomy Laboratory at Clemson University for Fe, Mn, and Zn analysis.

Biofilm Sampling

Biofilm was sampled in winter 2006 by selecting two rocks from the stream bottom at each sampling location. A square centimeter quadrat was placed on each piece of substrata at four locations. Biofilm was scraped at each location using a spatula sterilized in ethanol. Samples were pooled in sterilized 1.5 ml microcentrifuge tubes and taken back to the lab for DNA extraction.

Genetic Analysis

DNA extraction was accomplished using UltraClean™ Soil DNA Kits (Mobio Carlsbad, CA) following manufacturers intructions. All biofilm samples used for DNA extraction weighed between 0.5 and 1 gram. Extracted DNA was used in building 16S ribosomal RNA gene (rDNA) libraries as described by Amann et al (1995) where one µl of extracted DNA was used as template for polymerase chain reaction (PCR). Primers P0Mod-1 and PC-5-1 were designed for amplification of 16S rDNA as described in Wilson et al (1990). A modification in each primer consisted of addition of the underlined portions to the 3' end as shown here: P0 Mod-1 5' AGAGTTTGATCMTGGCTCAG 3'; PC-5-1 5' TACCTTGTTACGACTTCACC 3'. PCR reactions using P0 Mod-1 and PC-5 primers amplified all 16S bacterial DNA. Contamination was a problem in initial reactions because bacteria in the air, on counter surfaces, and in pipetting equipment were being amplified in PCR experiments. In order to circumvent PCR contamination filtered pipette tips and autoclaved millipore water were used in PCR reaction set up. Also, all reactions were set up in an ultraviolet UVP PCR workstation that was turned on 30 minutes prior to setting up PCR reactions.

PCR amplification of the 1500 base pair16S rDNA was accomplished using an Eppendorf Gradient Mastercycler (Hamburg, Germany) and Bullseye Taq DNA Polymerase (Bullseye St. Louis, MO) under the following cycling conditions: an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 59°C for 30 seconds, and an extension at 70°C for 2 minutes. A final extension step at 65°C for 10 minutes was also incorporated at the end of each run. PCR products were separated by gel electrophoresis using a 1% agarose gel stained with ethidium bromide and visualized under UV light. A 1kb molecular weight ladder (PROMEGA Madison, WI) was used as a size marker to determine the length of the amplified products. The 1500 bp amplified products corresponding to the 16S rDNA were excised from the gel using a sterile razor blade. DNA was extracted from the gel was using QIAEX II Gel Extraction Kit (QIAGEN Valencia, CA).

Cloning

DNA retrieved from the gel was cloned into pGEM®-T vector following the manufacturer's instructions (PROMEGA Bridgeport, NJ) and electroporated into *Escherichia coli* DH5-alpha cells. The transformants were plated on Lauria-Bertani (LB) - agar plates containing 150 µg/ml ampicillin and 40 µl of 40 mg/ml X-Gal and allowed to incubate overnight at 37ºC. Ampicillin resistance and blue/white colony screening were used as selectable markers to identify transformants containing the pGEM-T vector and cloned 16S rDNA insert. White colonies from the plates were transferred to LB-Agar numbered master plate also containing 150 µg/ml ampicillin and 40 µl of 40 mg/ml X-Gal and incubated overnight at 37°C for a second round of colony screening. The next day, white colonies were chosen from the master plate and used as DNA template in a colony PCR reaction using vector specific primers (M13 forwards, M13 reverse) to check for the presence of an insert. DNA template was obtained from the master plate by touching the white colonies on the plate with a toothpick and dipping the toothpick in 100 µl of distilled water in a microfuge tube. The tube was then vortexed briefly, heated at 100° C for 10 minutes in a heat block, and centrifuged at 5000 rpm for one minute to removed unlysed cells and cell debris. 20 µl of the centrifuged sample was used in each 50 µl PCR reaction. PCR products were visualized using gel electrophoresis to determine if the colony contained the 1500 base pair 16S rDNA segment of interest.

Plasmids that contained a 1500 base pair segment were isolated from 20 colonies within each sampling location (SR, S0, S25, S50, I0, I19, & I38) using standard protocols (Engebrecht *et al.* 1997). Briefly, colonies were picked with a sterile toothpick; the tooth pick was placed into a test tube containing 2 ml of LB broth inoculated with 150 µg/ ml amipicillin and incubated on a shaker at 37° C for 19 hours. The next day cells were harvested by centrifugation at 14,000 RPM for 1 minute. The supernatant was discarded being careful not to disturb the pellet, and 100 µl of ice cold GTE (5 ml of 1 M Glucose; 2.5 ml of 1 M Tris (pH 8); 2 ml 0.5 M EDTA) was added to each tube. The pellet was resuspended by pipetting up and down several times until cell clumps were no longer visible. After a 5 minute incubation at room temperature, 200 µl of SDS/NaOH solution (20% SDS; 0.5 ml of

4M NaOH) was added to each tube, and the contents were mixed by inverting the tubes several times. The tubes were placed on ice for five minutes before 200 µl of ice cold 3M KOAc was added to each tube. Tubes were then returned to ice for 5 minutes and centrifuged at 14,000 RPM for five minutes. The supernatant was removed from each tube and placed in clean microfuge tubes. To each tube of supernatant, 400 µl of isopropanol was added and the contents were mixed vigorously by rapidly inverting tubes. This step was done quickly to avoid precipitating proteins along with DNA. Tubes were incubated at room temperature for two minutes, and centrifuged to pellet nucleic acids. The supernatant was poured from tubes and the tubes were tapped gently on paper towels to thoroughly drain them. Ethanol (200 µl of 100%) was added to each tube and flicked several times to wash pellets and the tubes were then centrifuged for 3 minutes. Supernatant was poured from tubes taking care not to disturb pellet and the tubes were tapped gently on paper towels to thoroughly drain them. Remaining ethanol was removed by placing tubes in a speed vacuum until no ethanol odor was detected. Depending on the size of the pellet, 30-50 µl of TE (Tris/EDTA) was added to each tube, and pellets were resuspended by smashing them with the pipette tip and pipetting up and down vigorously.

All plasmid isolates had to be purified before being sent for sequencing. A QIAquick PCR purification kit was used in accordance with manufacturer's instructions to purify plasmid (QIAGEN Valencia, CA).

Sequencing

Twenty plasmids from sampling locations SR, S25, S50, I19, & I38 were sent to Cornell University DNA Sequencing Facility (Ithaca, NY). Sequences obtained were analyzed using a sequence match program: NCBI Ribosomal Database Project (RDBP, www.rdp.cme.msu.edu) Sequences were uploaded into the sequence match program to search for the nearest bacterial neighbor 16S rDNA sequences. RDBP results consisted of taxonomic hierarchies of potential matches extending to bacterial strain with corresponding sequence name and similarity scores. Analysis of this data was accomplished by creating spreadsheets and histograms representing taxonomic hierarchies using the sequences with the highest similarity scores.

Macroinvertebrate sampling

Benthic macroinvertebrate samples were taken during the winter, spring, and summer 2006 from each sampling location at the S and I sites. Square meter sampling quadrats were kick sampled for two minutes using a square meter kick net located downstream. Benthic macroinvertebrates were picked from the net, preserved in 95% ethanol, and taken to the lab for identification. All leaf pack debris was also preserved in ethanol and examined later for macroinvertebrates. Specimens were identified to family level using a Cambridge Instruments (StereoZoom® 4) dissecting microscope.

Results

Stream Morphology

The stream channel morphology for all sites is provided in Table 2. These dimensions represent an average width and depth of all sampling locations in each site. Channel width is much smaller (87 cm) at the downstream Sorrento (S) locations than at the Sorrento Reference (SR; 210 cm) or Ingles (I ; 211 cm) locations. Depth of 17 cm at the S locations is higher than the other sites.

Table 2. Average stream dimensions for all sites for 2006.

Site	Width (cm)	Depth (cm)
SR (Sorrento Reference)	210	11
S	87	17
I	211	11
WR (Watauga Reference)	86	6
W	81	6
A	65	4

Assessment of Water Chemistry

Table 3 presents the water quality values for the SR, S, and I sites. No significant differences in dissolved oxygen or temperature were found when comparing SR with S and I locations. SR pH was significantly higher than S sampling locations (p=0.036; df=8), and significantly lower (p=0.059; df=8) than the I sampling locations. Conductivity differences were highly significant (p=0.0001; df=8) between SR and both the S and I sites. However, the I sampling locations (207.34 mS/cm) had much higher mean conductivity than either SR (34.33 mS/cm) or S (49.63 mS/cm).

Table 3. Comparison of water chemistry parameter means (± standard error) for 2006.

Site	Dissolved Oxygen (ppm)	Temperature (°C)	pH	Conductivity (mS/cm)
SR	7.93 (0.640)	10.87 (2.236)	6.65 (0.217)	34.33 (1.124)
S	7.41 (0.888)	13.91 (3.576)	6.25* (0.115)	49.63* (0.958)
I	7.33 (0.425)	12.91 (2.123)	6.92 (0.054)	207.34* (9.704)

Table 4 lists the metal concentrations in stream water, and indicates several important differences between reference sites, SR, the Watauga Reference (WR), and other sampling locations. S0 has the highest concentrations of Fe and Mn (5.780 mg/L and 0.350 mg/L, respectively) of the S and I sampling locations. Fe concentrations at the I19 and I38 locations were 3.98 mg/L and 2.96 mg/L respectively, compared to SR (0.323 mg/L). It is notable that Mn concentrations are higher than SR (0.015 mg/L) in all other S and I sampling locations. The S25 location has the highest Zn level (0.340 mg/L) of all sampling locations. All Watuaga High School (W) sampling locations exhibit an average twenty fold increase in Fe and Mn compared to WR. The University Highlands (A) sampling locations were higher in Fe (sixteen fold increase) and Mn (eight fold increase) compared to WR.

Table 4. Summary of the concentrations of Fe, Mn, and Zn in stream water from February 2006.

Sample ID	Fe (mg/L)	Mn (mg/L)	Zn (mg/L)
SR	0.323	0.015	0.010
S0	5.780	0.350	0.025
S25	0.360	0.085	0.340
S50	0.630	0.060	0.060
I0	0.650	0.226	0.010
I19	3.980	0.216	0.010
I38	2.960	0.233	0.030
WR	0.800	0.251	0.140
W0	22.720	5.110	0.010
W25	26.200	5.530	0.010
W50	10.600	4.720	0.010
A0	32.700	1.880	0.010
A25	13.700	2.310	0.010
A50	5.100	1.930	0.010

Table 5 shows the total average Fe, Mn, and Zn in the SR, S, and I sites along with the North Carolina Department of Water Quality standard limits of Fe, Mn, and Zn. The total concentration of Fe, Mn, and Zn in the SR location was below the standard limits for these metals. However, the concentration of Fe was more than double the standard limit in the S (2.26 mg/L) and I (2.53 mg/L) sites. Also notable was the average concentration of Zn in the S site (0.14 mg/L), almost three fold that of the standard limit for Zn.

Table 5. Water quality standard limits of Fe, Mn, and Zn in freshwater resources compared to means at the SR, S, and I sites for 2006.

Metal	NC DWQ (mg/L)	Concentration In Water (mg/L)		
		SR	S	I
Fe	1.00 [a]	0.02	2.26	2.53
Mn	0.20 [b]	0.02	0.17	0.03
Zn	0.05 [a]	0.01	0.14	0.02

[a] Standard for freshwater supporting aquatic life
[b] Standard for use in water supply for consumption of either fish or water. (This is the only NCDWQ requirement for Mn).

Table 6 lists the stream sediment concentrations of Fe, Mn, and Zn in all sampling locations. All S and I sampling locations had at least a two fold increase in Fe, Mn, and Zn compared to SR. Most notable is an Fe concentration of 539.0 mg/L at S0. The highest Zn levels of the stream sediments, ranging from 20.8 – 27.6 mg/L, are found at I sampling locations. The W and A sampling locations all have higher Fe concentrations than WR. Mn levels fluctuate considerably in the W and A sites compared to WR. Most notable at W and A sites is an extremely high Mn level at W50 (1623 mg/L). Zn levels were much higher at WR (144 mg/L) than any other location.

Table 6. Concentration of Fe, Mn, and Zn in stream sediments from February 2006.

Sample ID	Fe (mg/L)	Mn (mg/L)	Zn (mg/L)
SR	52.0	83.0	3.6
S0	539.0	382.0	7.9
S25	247.0	186.0	5.6
S50	363.0	338.0	9.0
I0	188.0	156.0	21.5
I19	239.0	191.0	20.8
I38	226.0	200.0	27.6
WR	132.0	374.0	144.0
W0	176.0	191.0	12.0
W25	681.0	748.0	15.0
W50	298.0	1623.0	67.0
A0	342.0	169.0	6.9
A25	295.0	345.0	9.0
A50	522.0	626.0	11.3

Assessment of Biofilm Bacterial Communities

DNA was successfully isolated from biofilm samples taken from the SR, S0, S25, S50, I0, I19, and I38 sampling locations. Listed in Table 10 is the concentration and purity of the DNA extracted from biofilm samples from each location. In order to isolate and amplify the 16S rRNA gene from the extracted DNA, PCR reactions using primers P0-Mod-1 and PC-5 were set up to target this region of the bacterial genome. PCR experiments using DNA template taken from SR, S25, S50, I0, and I19 sampling locations produced DNA fragments the approximate length of the 16S rRNA gene (1,500 base pairs) based on electrophoretic mobility of the PCR product run on agarose gels (Figures 14 and 15). Repeated PCR reactions with DNA from the S0 and I38 sampling locations did not produce DNA fragments of 1,500 base pairs, indicating that bacterial 16S rDNA was not successfully amplified. DNA from these locations was not used in cloning experiments. PCR products of approximately 1,500 base pairs were excised from the agarose gels (Figures 14 and 15) to obtain only the amplified 16S rDNA to use in cloning experiments. The concentration and purity of DNA extracted from the excised portions of the gel are listed in Table 10.

DNA extract taken from electrophoresis gels was cloned into pGEM®-T vector and electroporated into *Escherichia coli* DH5-alpha cells to order to create five clone libraries representing DNA extracted from biofilm samples in locations S0, S25, S50, I0, and I19. Plasmid DNA was isolated from twenty clones from each clone library and screened using PCR to check for the presence of the cloned 16S rRNA gene. Figures 16 and 17 show gel electrophoresis images of PCR amplified plasmid DNA (using M13 primers to amplify the inserted 16S rDNA segment) from locations SR, S25, S50, I0, and I19. The plasmids containing a cloned DNA fragment of approximately 1,500 base pairs was sent to Cornell University's DNA Sequencing Facility for sequencing. Sequence information obtained from Cornell is listed in Appendix A.

Table 7. Volume and purity of DNA extract from biofilm samples (collected February 2006) and agarose gels.

Site	Concentration of DNA extracted from biofilm (ng/µl)	260/280
SR	3.2	2.24
S0	20.8	1.66
S25	8.6	1.74
S50	13.3	2.48
I0	13.6	1.81
I19	21.3	1.58
I38	20.2	1.90
Site	**Concentration of DNA extracted from gel (ng/µl)**	**260/280**
SR	6.70	1.48
S25	5.90	1.22
S50	4.10	0.90
I0	63.4	1.47
I19	9.70	1.76

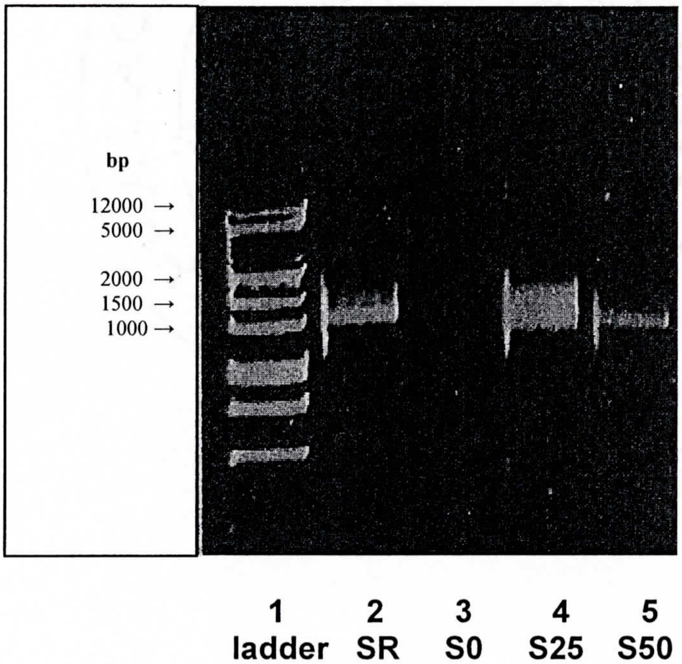

Figure 7. Electrophoresis gel showing the 16S rRNA gene amplified from biofilm DNA taken from the Sorrento site. The bands in lanes 2, 4, and 5 correspond to a DNA fragment of approximately 1,500-1,600 base pairs.

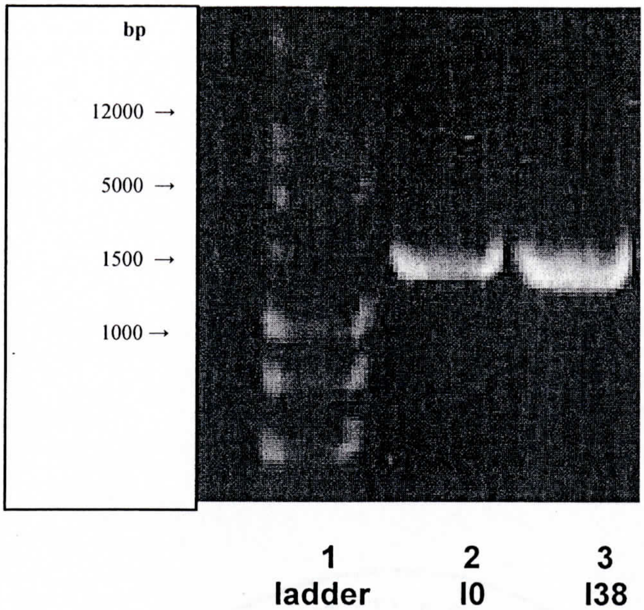

Figure 8. Electrophoresis gel showing the 16S rRNA gene amplified from biofilm DNA taken from the Ingles site. The bands in Lanes 1, 2, 4, and 5 correspond to a DNA fragment of approximately 1,500 base pairs.

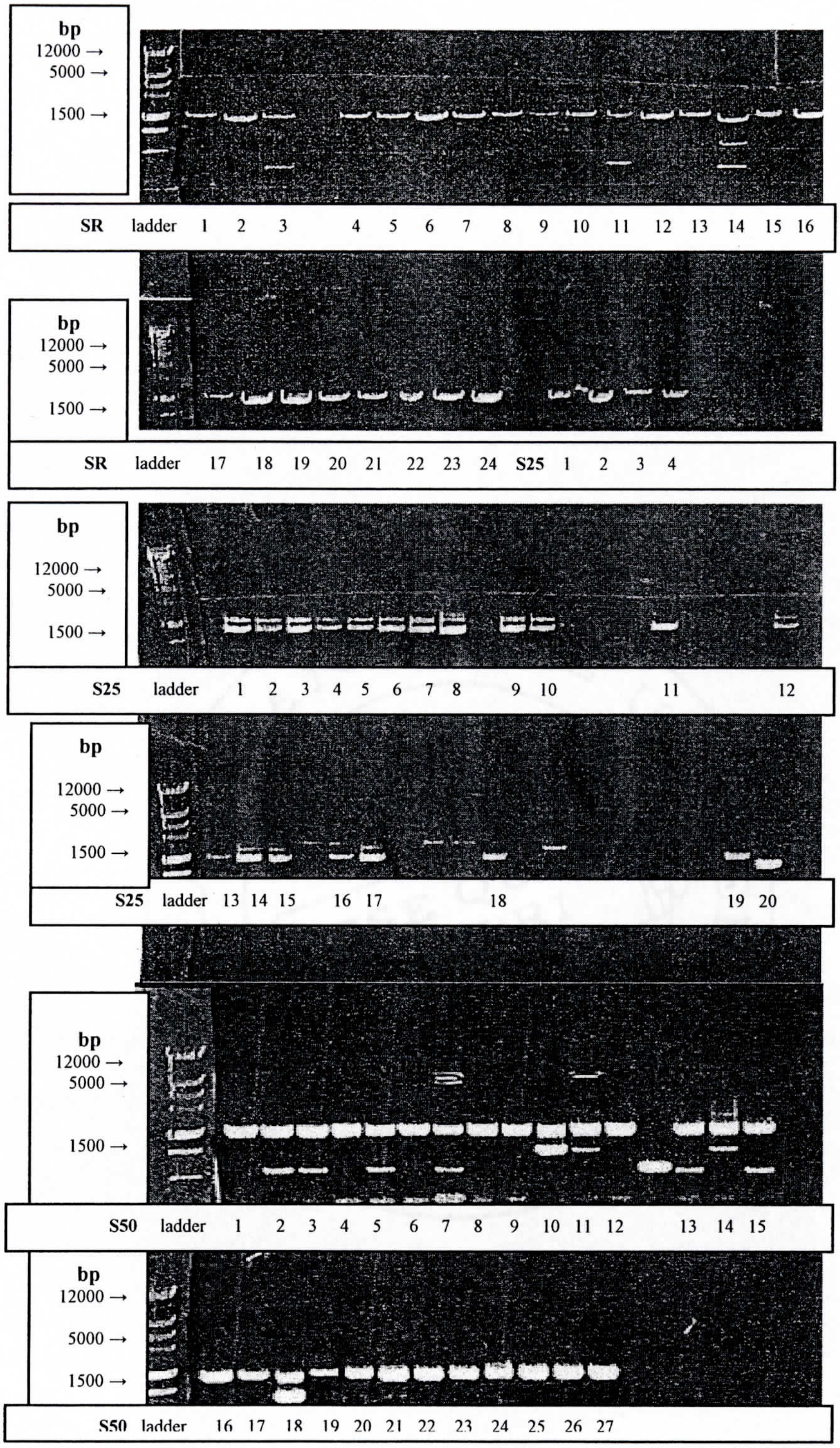

Figure 9. Electrophoresis gels showing the products of a PCR to amplify plasmid DNA from clones containing the 16S rRNA gene. The bands in numbered lanes represent clone libraries from the Sorrento site.

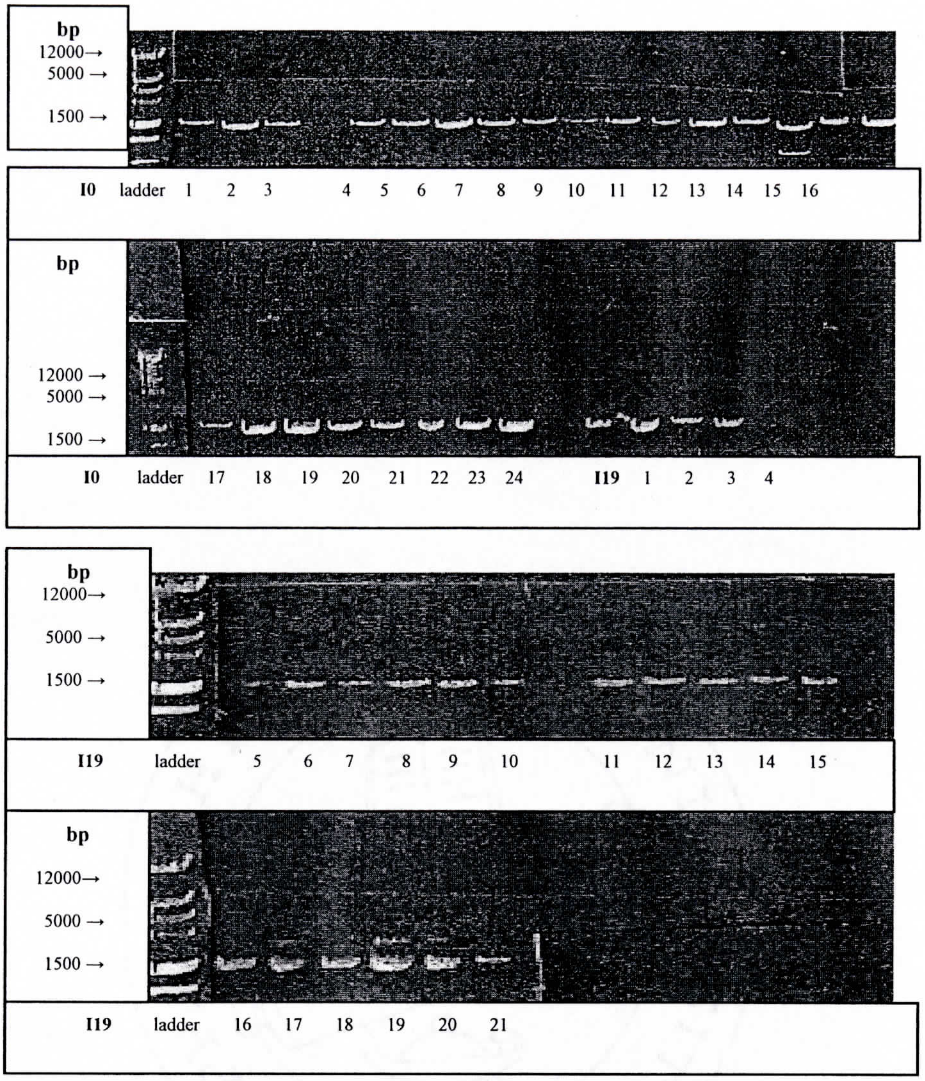

Figure 10. Electrophoresis gels showing the products of a PCR to amplify plasmid DNA from clones containing the 16S rRNA gene. The bands in numbered lanes represent clone libraries from the Ingles site.

The majority of clones from libraries created using biofilm bacterial DNA (collected February 2006) in locations SR, S25, S50, I0, and I19 belong to the Phylum Proteobacteria (Figure 11). Of the 20 clones sequenced from each location, the number of Proteobacteria ranged from 12 - 16. Cyanobacteria were the second most frequently occurring phyla ranging from 4 – 8 clones in each location. Figure 12 shows that in all locations Alphaproteobacteria, Betaproteobacteria, and

Cyanobacteria were the most dominant bacterial classes found. Gammaproteobacteria was a dominant taxon at location S50 where four clones were identified as belonging to this class. The SR clone library was the only library with no Gammaproteobacteria. The Orders Sphingomonadales, Burkholderiales, and Cyanobacteria are present in clone libraries of all sampling locations (Figure 13). Bacteria belonging to the Order Burkholderiales were a dominant taxon at I0 (7 clones) and I19 (13 clones).

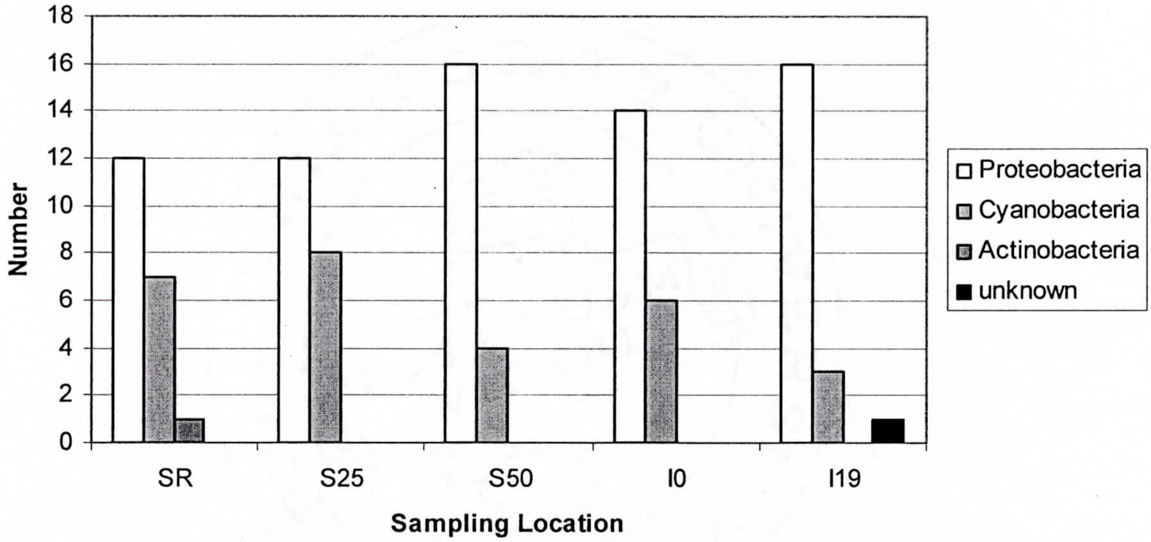

Figure 11. Comparison of bacterial phyla identified from clone libraries.

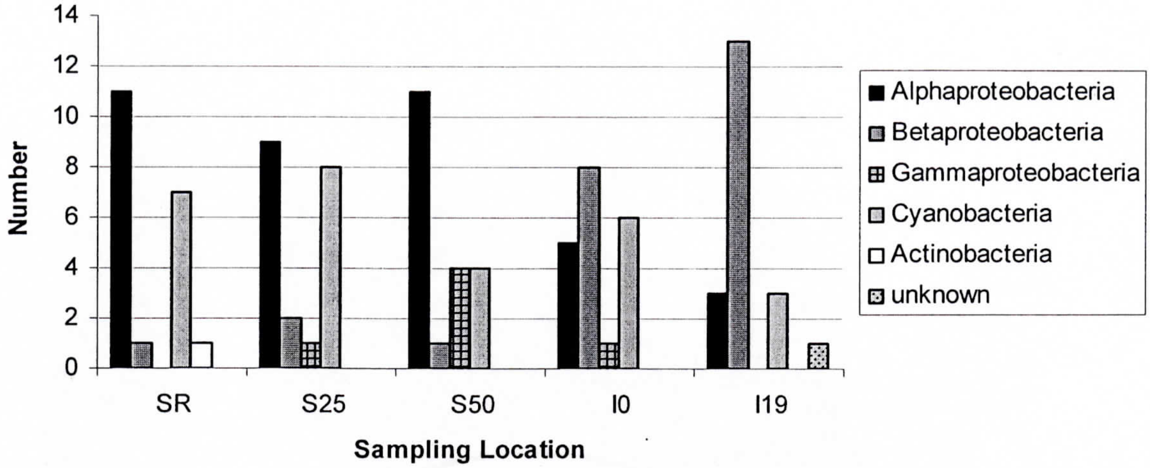

Figure 12. Comparison of bacterial classes identified from clone libraries.

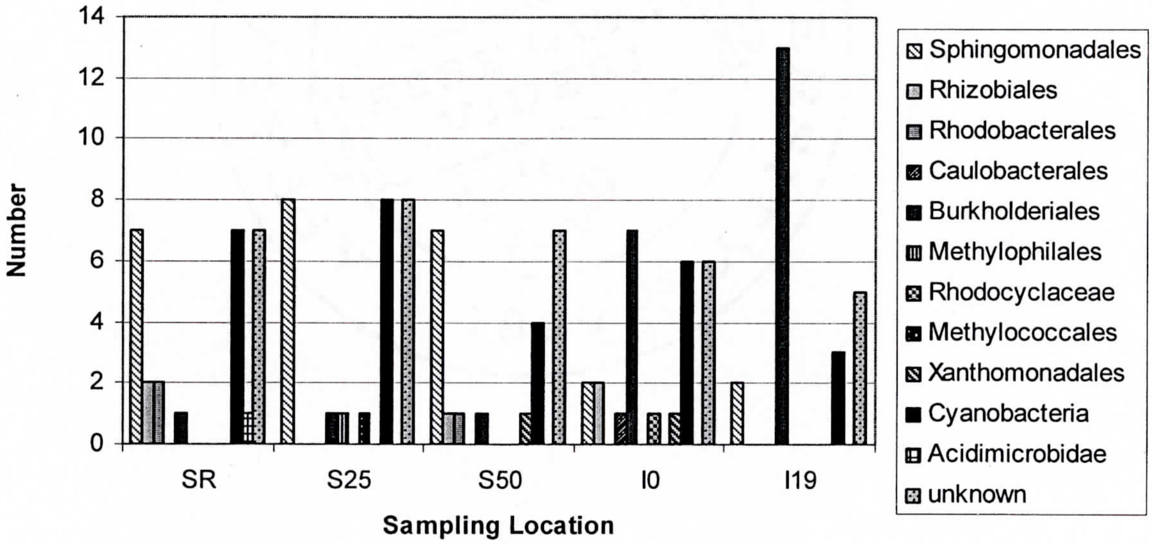

Figure 13. Comparison of bacterial orders identified from clone libraries.

Assessment of Macroinvertebrate Communities

Figure 14 presents the average number of benthic macroinvertebrates collected from each site. In the 2006 samples SR had the highest mean sample abundance (193), while S and I sites averaged 117 and 57, respectively. SR abundance is only significantly higher than I abundance (p=0.007; df=2).

A significantly greater diversity of macroinvertebrate families exists in the SR sampling location compared to any S or I locations (Figure 15). SR had an average of 19 families collected in 2006 while samples from the S and I locations ranged from 6-11 families in each collection. Macroinvertebrate community composition of each collection (winter, spring, and summer) from 2006 is shown in Tables 8, 9, and 10.

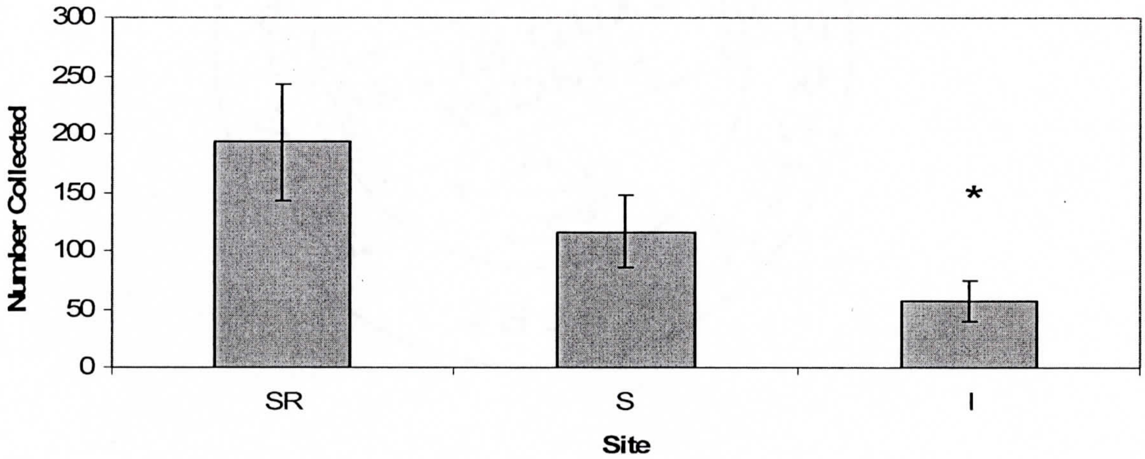

Figure 14. Comparison of mean abundance (± standard error) of benthic macroinvertebrates collected in 2006.

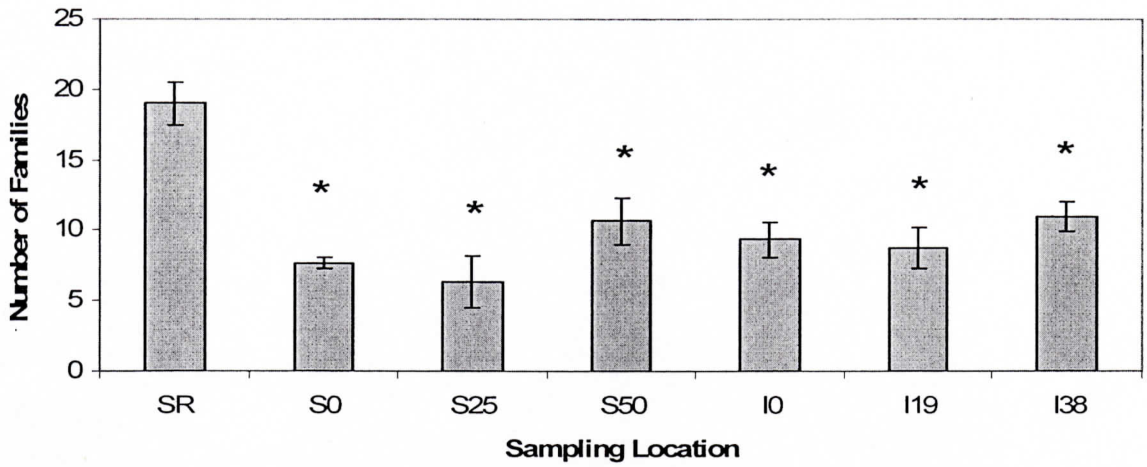

Figure 15. Comparison of macroinvertebrate mean taxa richness (± standard error) in all sampling locations collected in 2006.

Table 8. Number of each macroinvertebrate family collected at each sampling location in winter 2006.

		Sampling Locations						
Order	Family	SR	S0	S25	S50	I0	I19	I38
Ephemeroptera	Heptageniidae	44	0	0	0	1	0	0
Ephemeroptera	Leptophlebiidae	29	15	3	3	0	0	0
Ephemeroptera	Ephemerellidae	14	6	1	1	9	2	10
Ephemeroptera	Ameletidae	2	0	0	0	0	1	0
Ephemeroptera	Beatidae	0	0	0	6	8	3	20
Plecoptera	Peltoperlidae	4	0	0	0	0	0	0
Plecoptera	Perlodidae	7	0	0	1	2	4	1
Plecoptera	Nemouridae	2	0	0	1	0	0	0
Plecoptera	Leuctridae	7	0	4	3	0	0	0
Plecoptera	Taeniopterygiidae	0	0	0	0	1	0	0
Tricoptera	Hydropsychidae	48	13	26	6	3	1	8
Tricoptera	Limnephilidae	3	0	0	0	0	0	0
Tricoptera	Rhyacophilidae	3	0	7	1	0	0	0
Tricoptera	Philpotamidae	8	0	2	1	0	0	0
Diptera	Simulidae	6	6	98	56	0	0	1
Diptera	Chironomidae	67	68	67	102	8	6	4
Diptera	Empididae	0	0	0	2	2	0	0
Diptera	Tipulidae	8	0	2	2	1	0	9
Diptera	Ceratopogonidae	0	2	9	4	0	0	0
Megaloptera	Corydalidae	0	3	0	0	0	0	0
Odonata	Gomphidae	1	0	0	0	0	0	1
Coleoptera	Elmidae	0	0	0	0	1	0	0
	TOTAL	**255**	**113**	**219**	**189**	**36**	**17**	**54**

Table 9. Number of each macroinvertebrate family collected at each sampling location in spring 2006.

		Sampling Locations						
Order	Family	SR	S0	S25	S50	I0	I19	I38
Ephemeroptera	Heptageniidae	8	0	0	1	0	0	1
Ephemeroptera	Leptophlebiidae	28	11	7	1	0	0	0
Ephemeroptera	Ephemerellidae	13	0	0	0	0	0	5
Ephemeroptera	Beatidae	5	1	2	1	21	2	39
Plecoptera	Capniidae	9	0	0	0	1	1	1
Plecoptera	Peltoperlidae	1	0	0	0	0	0	1
Plecoptera	Perlodidae	11	0	0	0	0	0	0
Plecoptera	Nemouridae	8	0	0	0	0	0	1
Plecoptera	Leuctridae	12	0	0	0	0	0	0
Plecoptera	Perlidae	2	1	0	0	0	0	0
Plecoptera	Taeniopterygiidae	1	0	0	0	0	0	0
Plecoptera	Chloroperlidae	3	1	0	1	0	0	0
Tricoptera	Hydropsychidae	86	0	0	0	8	3	8
Tricoptera	Philpotamidae	9	0	0	0	0	1	0
Tricoptera	Limnephilidae	5	0	0	0	0	0	0
Tricoptera	Phryganeidae	1	0	0	0	0	0	0
Tricoptera	Rhyacophilidae	4	1	0	0	0	0	0
Diptera	Simulidae	0	0	0	53	0	0	0
Diptera	Chironomidae	4	17	51	38	43	31	86
Diptera	Tipulidae	2	0	0	0	2	2	1
Diptera	Ceratopogonidae	0	4	1	2	1	1	0
Odonata	Empididae	5	0	0	2	0	1	3
Coleoptera	Elmidae	11	2	0	1	0	0	0
	Pleuroceridae	0	0	0	0	4	2	3
	TOTAL	**229**	**38**	**61**	**100**	**80**	**44**	**150**

Table 10. Number of each macroinvertebrate family collected at each sampling location in summer 2006.

		Sampling Location						
Order	Family	SR	S0	S25	S50	I0	I19	I38
Ephemeroptera	Heptageniidae	10	0	1	0	0	0	1
Ephemeroptera	Leptophlebiidae	12	1	0	2	0	0	1
Ephemeroptera	Ephemerellidae	0	0	0	0	0	1	0
Ephemeroptera	Beatidae	1	4	0	2	5	2	6
Plecoptera	Capniidae	7	0	0	1	3	1	0
Plecoptera	Perlodidae	5	0	0	0	2	2	13
Plecoptera	Nemouridae	1	0	0	0	0	0	0
Plecoptera	Leuctridae	6	2	0	6	1	1	3
Plecoptera	Chloroperlidae	2	0	0	0	0	0	0
Tricoptera	Hydropsychidae	27	9	9	12	1	1	13
Tricoptera	Philpotamidae	5	0	0	0	3	2	3
Tricoptera	Limnephilidae	3	0	0	0	0	0	0
Tricoptera	Rhyacophilidae	2	0	0	0	0	0	0
Diptera	Simulidae	0	3	18	7	1	0	0
Diptera	Chironomidae	5	57	117	57	10	9	13
Diptera	Tipulidae	1	0	0	0	1	5	3
Diptera	Ceratopogonidae	1	0	0	0	0	0	1
Odonata	Empididae	1	1	9	7	0	2	8
Coleoptera	Elmidae	5	1	0	0	0	0	0
	Pleuroceridae	0	0	0	0	4	2	6
	TOTAL	**95**	**78**	**154**	**97**	**32**	**28**	**72**

Figure 16 shows that the average % EPT taxa richness at the SR sampling locations was 81.3% compared to a range from 13-53% at other locations. SR was significantly higher than S0 (p=0.04; df=2), S25 (p=0.001), and S50 (p=0.002). Shannon index and evenness scores are shown in Figure 17. Shannon index scores ranging from 0.95 - 1.82 were found at the S and I sampling locations compared to an average score of 2.31 at location SR. Significant differences from SR were found at locations S0 (p=0.04; df=2), S25 (p=0.001; df=2), and S50 (p=0.002; df=2). Evenness scores varied less from a score of 0.45 at SR compared to a range from 0.19 – 0.50 at S and I locations. SR evenness scores were significantly higher than those at S25 (p=0.01; df=2) and S50 (p=0.017; df=2).

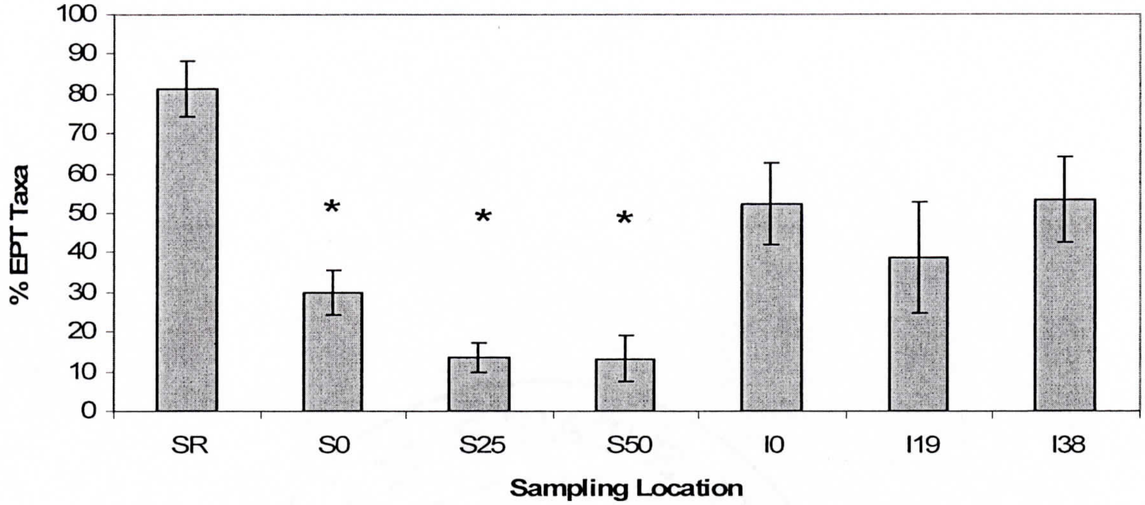

Figure 16. Comparison of mean %EPT taxa (± standard error) of macroinvertebrates at all sampling locations in 2006 (n=3).

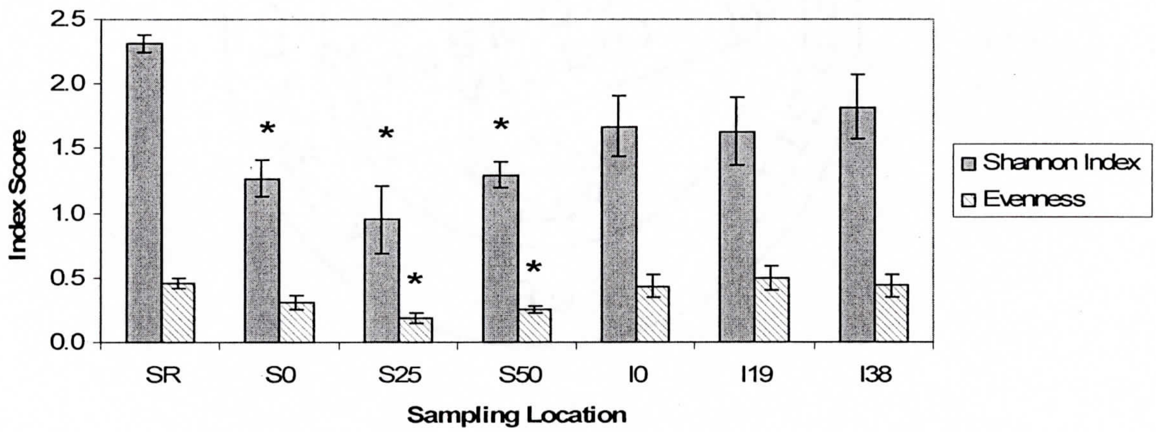

Figure 17. Comparison of mean Shannon Index and Evenness scores (± standard error) of macroinvertebrates at all sampling locations in 2006 (n=3).

Mean Family Biotic Index (FBI) scores for 2006 are shown for all sampling locations in Figure 18. S0 (p=0.008; df=2), S25 (p=0.004; df=2), and S50 (p=0.002; df=2) scores were significantly higher than the mean SR FBI score of 3.53. S and I sampling locations all had higher scores than SR ranging from 5.19 - 6.85.

The average percentage of three major functional feeding groups is presented in Figure19. SR had a lower percentage of collector-filterers (44.3%) than any sampling locations in the other S and I sites, and significantly lower than the S25 (p=0.0006; df=2) and S50 (p=0.0012; df=2) locations. A greater average percentage of the macroinvertebrate community at SR was composed of shredders (13.0%) than any other sampling locations, but statistically more than locations S0 (p=0.0087; df=2), S25 (p=0.0091; df=2), and S50 (p=0.0374; df=2). The percentage of scrapers also were significantly lower at S0 (p=0.04), S25 (p=0.006), and S50 (p=0.006; df=2) than at SR. Percent scrapers at I0 were significantly higher (p=0.0084; df=2) than SR.

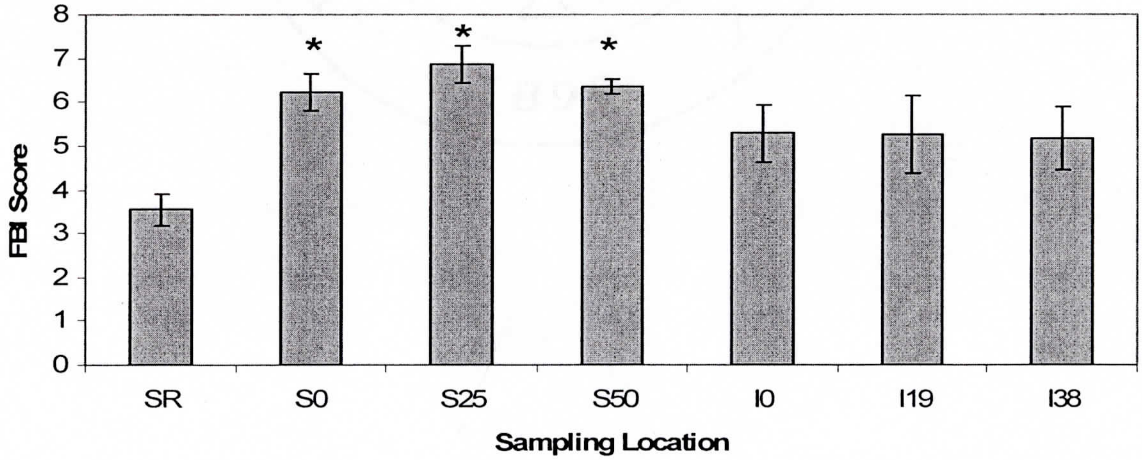

Figure 18. Comparison of mean Family Biotic Index (FBI) scores (± standard error) of macroinvertebrates at all sampling locations in 2006 (n=3).

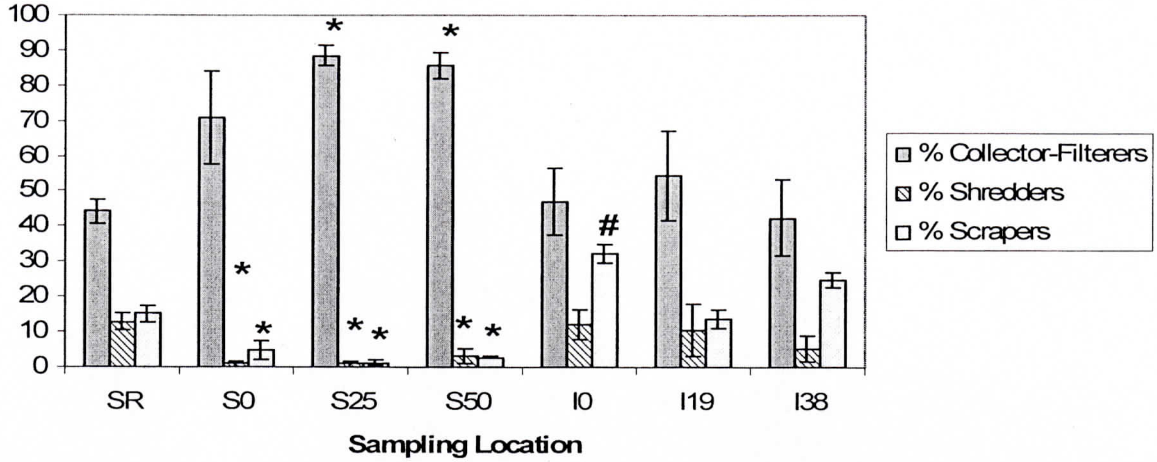

Figure 19. Comparison of mean % collector-filterers, % shredders, and % scrapers (± standard error) of macroinvertebrates at all sampling locations in 2006 (n=3).

Figure 20 shows the average levels of Fe, Mn, and Zn in water samples collected from the sampling locations at the SR, S, and I sites. Also shown is the average number of Heptageniids collected from each site. Notice that the logarithmic scale is used for the y-axis. This figure shows low concentrations of Fe (0.023 mg/L), Mn (0.015 mg/L), and Zn (0.01) in the SR location compared to much higher levels in the S (Fe-2.26 mg/L; Mn- 0.17 mg/L; Zn- 0.14) and I (Fe- 2.53; Mn- 0.23; Zn- 0.02) sites. The average number of Heptageniids at the SR location was much higher (20.7) than that at the S (0.2) and I (0.3) locations.

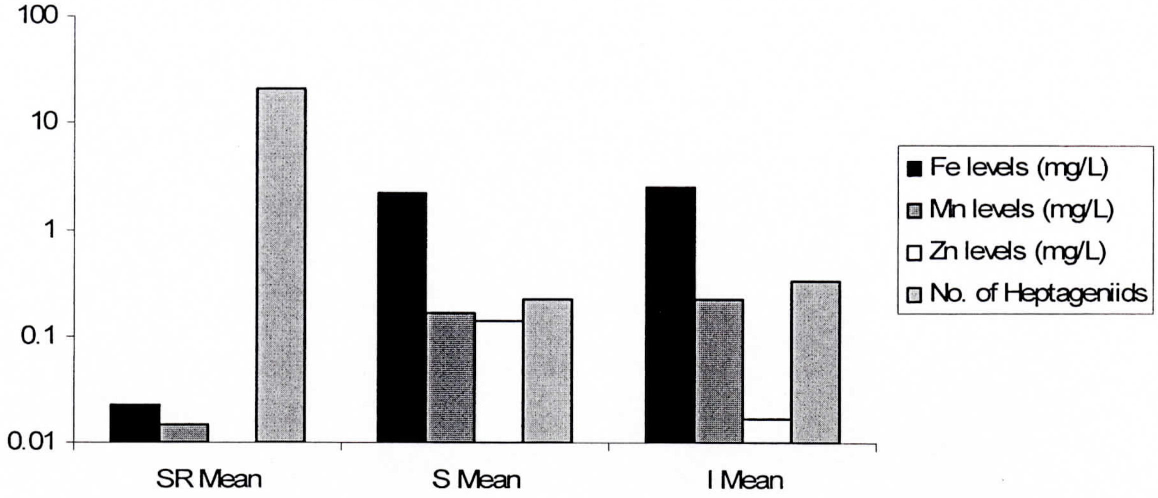

Figure 20. Average Fe, Mn, and Zn concentrations in water samples along with the average number of Heptageniid mayflies collected in 2006 at SR, S, and I sampling locations.

Discussion

Avoidance of anthropogenic impacts on small mountain streams requires a reduction in land use activity that impacts the physical habitat (Allan 2004). The results presented here indicate that human development has the ability to disrupt groundwater inputs into high elevation streams and wetlands. In the tributaries examined in this study, these disruptions have produced metal-rich seeps that are negatively influencing the diversity and distribution of biotic communities. Maintaining a large forested buffer zone between small mountain streams and wetlands and anthropogenic activities may be the best way to maintain a physical habitat and corresponding water quality capable of supporting ecologically healthy biotic communities (Hauer and Lamberti 1996).

The purpose of this thesis research was to addresses physical disturbance as it relates to Fe, Mn, and Zn pollution, concurrent biofilm production, and alterations in macroinvertebrate community composition.

Are the concentrations of Fe, Mn, and Zn in the water and the sediment of two disturbed mountain streams different than those of an undisturbed reference stream?

Initial water chemistry and macroinvertebrate surveys at the Sorrento site (S) in 2005 indicate that Fe and Zn levels may be influencing poor diversity of macroinvertebrate communities at this location (Greco 2005). For comparison a macroinvertebrate sample was collected in a pristine portion of the stream in a sampling location surrounded by a large riparian buffer. Not surprisingly, the sample had a much richer taxonomic diversity (Greco 2005). The initial macroinvertebrate surveys, the overall appearance of the stream channel (i.e. lack of rust colored biofilm), the lack of any orange seeps, and the presence of a large forested buffer indicate that the SR sampling location serves as a suitable reference site for comparing biological diversity with the other sampling locations in this study. Table 5 shows water quality standard limits of Fe, Mn, and Zn for freshwater resources. Also listed are the average levels in water samples collected at the SR, S, and I sampling locations. Average levels of Fe, Mn, and Zn at the SR location are well under the standard limits of the NCWQ confirming that SR is a suitable reference site for the purposes of this study.

The portions of the streams at Sorrento (S) and Ingles (I) with elevated levels of Fe, Mn, and Zn occur most likely because land use history at these sites was such that the physical habitat was impacted by anthropogenic activities. At the S site a portion of a wetland was filled with soil and retention ponds were constructed upstream of the S0, S25, and S50 sampling locations. The upstream corridor of the I site has been developed into a shopping center and adjoining parking lot. Therefore, both sites have been impacted by anthropogenic activity that may have altered groundwater flow and produced metal rich seeps. In both the S and I sites, several small groundwater seeps heavily coated with an orange biofilm (indicative of Fe/Mn polluted groundwater entering aerobic environments (Emerson & Revsbech 1994)) were releasing water into the stream channel. All water and sediment samples from sampling locations downstream of seeps have higher Fe and Mn concentrations than those at SR, and the majority of locations have higher Zn levels

(Tables 4 and 6). The lack of seeps in the SR location indicates that they could be the source of Fe, Mn, and Zn in the other sampling locations.

For a more complete understanding of the effects of water chemistry on biotic communities at the SR, S, and I sites, water and sediment sampling and analysis should be conducted throughout the year. Water samples should be analyzed for Fe, Mn, and Zn in fall, winter, spring, and summer to determine seasonal effects on metal concentrations. The lack of seasonal Fe, Mn, and Zn is a severe limitation of this research since previous research has shown that metal concentrations may be highest in summer months (Sheldon & Wellnitz 1998). However, the cost of metal analysis was very high, and one sample from each sampling location was all that the budget would allow for this study.

In addition to metal analysis, in the future all water samples should be analyzed for total phosphorus, nitrates, and total suspended solids (TSS). Phosphorus, nitrates, and TSS commonly increase in streams and wetlands impacted by anthropogenic activity, and are also likely contributing to the degradation of biotic communities at the S and I sites (Hauer & Lamberti 1996).

Are the bacterial biofilm communities of two disturbed mountain streams different than those of an undisturbed reference stream?

In an attempt to understand if bacteria play a role in Fe and Mn oxidation, and thereby contribute to the degradation of macroinvertebrate communities, the bacterial communities of the SR, S25, S50, I0, and I19 sampling locations were described using 16S rRNA clone library analysis. It was difficult to see any trend, or causal relationship between Fe, Mn, and Zn levels and bacterial community structure when comparing sequence information from the SR clone library with the other sampling locations (Figures 11, 12, and 13). One of the major reasons for this could be that only twenty clones from each sampling location clone library were sequenced, thereby identifying only twenty different bacteria using the RDBP sequence blast program. Initially, sequence information from twenty clones seemed like a reasonable number considering that it is well established that in pH neutral environments, many types of bacteria are capable of Fe and Mn oxidation including *Leptothrix, Siderocapsa, Sphaerotilus*, *Cladothrix* and *Gallionella* (Wetzel 2001); and previous aquatic research indicates that in systems with large concentrations of Fe and Mn deposits, metal oxidizing species from the genera *Leptothrix* and *Gallionella* are often dominate members of the bacterial community. However, research in these systems has typically characterized the microbial community using microscopic examination of microscope slides placed in stream or microbial mat cores (Sheldon & Skelly 1990; Emerson & Revsbech 1994; Emerson & Weiss 2004). Although studies using16S rRNA analysis have found *Leptothrix* and *Gallionella* in Fe and Mn polluted systems (Stein *et al.* 2001; Bruneel *et al.* 2006), the analysis of sequences from the 16S rRNA clone libraries in this study revealed that no commonly recognized metal oxidizing bacteria were dominant in any metal polluted sampling location in this study.

One of the few recognizable trends in bacterial communities in sampling locations with elevated Fe, Mn, and Zn was the presence of Gammaproteobacteria and bacteria from the Family Comamonadaceae. 16S rRNA phylogenetic analyses of the major species of Comamonadaceae have shown that the closest relatives of the family do in fact include *Leptothrix* (Wen *et al.* 1999). Twenty-two of the eighty sequences obtained from the metal impacted sampling locations were identified as members of the Family Comamonadaceae, and only one of those sequences was identified to a higher taxonomic level (*Polaromonas*) by the sequence analysis program (RDBP). The probability that Comamonadaceae bacteria occur in the Fe, Mn, and Zn polluted locations because of their ability to oxidize Fe or Mn is most likely very low. However, it is plausible that Comamonadaceae bacteria identified in this study could share similarities in their genome with *Leptothrix* other than in the 16S region (such as the *mof*A gene that codes for physiological machinery necessary to oxidize Fe or Mn (Siering & Ghiorse 1997)), or Comamondaceae sequences may represent *Leptothrix* bacteria with more variable 16S genotypes than those in the RDBP database.

Ironically, one sequence from the SR clone library, identified as *Hyphomicrobium,* has been previously associated with Fe and Mn rich sediments in Wisconsin and is thought to facilitate Mn oxidation (Stein *et al.* 2001). However, the concentration of Mn in the SR location was well below the standard limits (Table 1) indicating that *Hyphomicrobium* can be found in aquatic environments with low concentrations of Mn.

The I0 clone library had two sequences that shared similarities to a ferromanganous micronodule bacterium described in metal rich sediments in Green Bay, WI (Stein *et al.* 2001). RDBP placed these two sequences in the order Rhizobiales. The only other sequence with similarities to ferromanganous micronodule bacterium was again in the SR clone library, but this sequence was seated in the Order Burkholderiales. Considering that one *Hyphomicrobium* sequence, and three sequences with similarities to ferromanganous micronodule bacteria are the only sequences with any similarity to any metal oxidizing taxa, and that two of the four sequences occurred in the SR clone library, Fe, Mn, and Zn levels may not be significantly influencing bacterial community diversity and distribution.

Clone libraries from all sampling locations contained sequences identified as Cyanobacteria. Of the one-hundred sequences obtained in all sampling locations, twenty-eight were identified as belonging to the class Cyanobacteria. This was the second most frequently occurring class of bacteria in all sampling locations. Only Alphaproteobacteria in the S locations and Betaproteobacteria in the I locations were more common (Figure 12). The universal presence of Cyanobacteria in all sampling locations regardless of the concentration of Fe, Mn, and Zn indicates that Cyanobacterial growth can persist in aquatic environments with elevated levels of Fe, Mn, and Zn. Previous research also shows that Cyanobacteria can persist in high Fe environments or even become dominate members of the bacterial community in aquatic habitats with elevated Fe concentrations (Emerson & Weiss 2004).

The most notable limitation of the bacterial community work in this study was the lack of the S0 biofilm community. This location had the highest levels of Fe and Mn in water samples, and the bacterial community may have therefore been influenced most at this location. Another limitation was sequence information for only 20 clones from each sampling location. This is due to the expense of sequencing a larger number of clones.

To more completely understand the functional role of bacterial communities at the S and I sites, sequence information from at least 100 members of the 16S rRNA clone libraries would be a good starting point. Also, in addition to biofilm samples from each sampling location, samples should also be taken from the groundwater seeps that are thought to be the source. Ideal habitat for species of Fe and Mn oxidizing bacterial species in neutral pH aquatic systems is typically in areas where the redox gradient is very steep. In these locations metal oxidizing species have an opportunity to gain energy from metal oxidation before it reacts spontaneously with oxygen (Wetzel 2001). Also, a study that would aid in determining if a difference exists between multiple samples taken from the same general location (riffle) would be useful in determining the amount of variation that exists in the biofilm community in a small area. Finally, a temporal study that examined seasonal effects on bacterial communities would be helpful in determining the relationship between seasonal metal concentrations and microbial community structure.

Are the macroinvetebrate communities of two disturbed mountain tributaries different than those of an undisturbed reference tributary?

Macroinvertebrate collections at SR are vastly different from those at all other locations as evident from multiple indices commonly used in analysis of the macroinvertebrate communities (Figures 7,8,9,10,11,12, and 13). Although not all index scores are significantly different, SR communities are much more abundant and diverse overall. Water and sediment chemistry assessment shows that Fe, Mn, and Zn in water and/or sediment samples taken from the S0, S25, S50, I0, I19, and I38 sampling locations is elevated compared to SR (Tables 4, 5, and 6). Fe and Mn can have deleterious effects on benthic macroinvertebrates when oxidized forms adhere to the body surfaces of some species. A few individual Heptageniid mayflies (Order Ephemeroptera) captured from the S0 location in 2005 were heavily coated with oxidized metal, a phenomena seen in previous research (Lemly & King 2000). Heptageniids were rarely seen in any of the locations with elevated metal concentrations because they have delicate gill filaments and caudal cerci that get coated with Fe and Mn oxides that interfere with oxygen transfer resultantly increasing mortality and macroinvertebrate drift (Lemly & King 2000).

Zn levels have also influenced the presence of Ephemeropteran taxa by inducing macroinvertebrate drift (Clements 2004; Courtney & Clements 2002; Clements *et al.* 2000), particularly in smaller streams (Kiffney & Clements 1993). Elevated Zn levels also have the largest impact on the presence of Heptageniid mayflies (Clements *et al.* 2000; Lemly & King 2000). This phenomenon is seen in the S and I metal polluted locations as well (Figure 20).

Benthic macroinvertebrate index scores including %EPT, Shannon Index, Eveness, and Family Biotic Index (FBI) from the S0, S25, and S50 locations are almost all significantly different from SR scores. However, the I locations appear similar to SR based on index scores (Figures 16, 17, and 18). Figure 14 shows that the overall abundance of macroinvertebrates collected at the I locations (57) was significantly lower than the SR location (193). With such low abundance, the I site index scores are more easily skewed by a single tolerant EPT taxa. For example, Beatid mayflies (Order Ephemeroptera) were collected at all I locations in all three collections (winter, spring, and summer), and make up an average of 21% of total macroinvertebrates and 46% of EPT taxa collected from the I site in 2006. The Family Biotic Index score for this family is 5, the highest score for Ephemeropteran taxa collected in this study. The presence of large numbers of Beatids in the I location indicates that they may show tolerance to elevated concentrations of Fe, Mn, and Zn.

Hydropsychid caddisflies (Order Tricoptera) were also a frequently occurring EPT taxa in the I and S sampling locations. They were present in all locations during winter and summer 2006, and made up an average of 21% of collections in the metal polluted S locations and 27% in the I locations in 2006. An average of 42% of EPT taxa collected at the S site and 20% of EPT taxa collected at the I site were Hydropsychids. Hydropsychids have an FBI score of 4 and are the second most tolerant Tricopteran taxa collected in this study. This indicates that Hydropsychids may show tolerance to elevated Fe, Mn, and Zn levels as well.

Continued seasonal macroinvertebrate sampling at these sites along with concurrent metal and microbial sampling is imperative in order to understand the relationships that exist between the physical and chemical environment and the biological community of these small mountain tributaries. Macroinvertebrate sampling in a large number of metal polluted aquatic habitats like the S and I sites could also help to create a Family Biotic Index for metal pollution. This type of index could be very helpful in determining the ecological health of a given habitat as it relates to metal concentrations without expensive metal analysis.

Concluding Interpretations of Data

Sheldon & Skelly (1990) and Sheldon & Wellnitz (1998) indicate that ferromanganous depositing bacteria are the source of Fe and Mn oxide deposits in small mountain tributaries; however, oxidation and deposition of Fe and Mn in this study seems to be a biologically independent process. The absence of any dominant groups of Fe or Mn oxidizing bacteria in any of the metal polluted sampling locations where oxidized forms of Fe and Mn abound indicates that reduced forms of Fe and Mn are primarily oxidized spontaneously (see equations 6 and 7: Wetzel 2001; Madigan & Martinko 2006) when reaching oxic zones of the stream.

Equation 6: $Fe^{2+} + \frac{1}{4} O_2 + 2\frac{1}{2} H_2O \rightarrow Fe(OH)_3 + 2H^+$

Equation 7: $Mn^{2+} + \frac{1}{2} O_2 + H_2O \rightarrow MnO_2 + 2H^+$

$\Delta G^{0'} = -68$ kJ

The accumulation of Fe and Mn oxides normally affect primary production through "bottom up control", where abiotic factors determine the distribution and diversity of biotic communities beginning with the lowest trophic level and moving up through the trophic cascade eventually impacting macroinvertebrate communities (Dodds 2002). Comparing bacterial community structure at the SR location with metal polluted locations indicates that bacterial community structure is largely unaffected by Fe and Mn levels, and therefore is having little influence on the structure of the macroinvertebrate community. However, Fe and Mn deposits may be having impacts on macrophyte community diversity. Although macrophytes were not assessed in this study, previous research has shown that oxidized forms of Fe and Mn adsorb phosphorus and can deprive macrophyte communities of this essential nutrient, resultantly decreasing the diversity of macrophyte communites (Sheldon & Wellnitz 1998; Wetzel 2001). Limitation of macrophyte diversity has direct influence on macroinvertebrate communities (Hauer & Lamberti 1996).

High concentrations of Zn in sediments (Figure 3) may be most influential in degradation of macrophyte communities in metal polluted sampling locations. Because Zn is a heavy metal and has low toxicity levels relative to Fe and Mn (Table 1), slightly elevated levels can have a large influence on the biotic community (Clements *et al.* 2000; Courtney & Clements 2002; Clements 2004; Morin *et al.* 2007). In oxygenated, neutral pH, aquatic environments the majority of Zn is adsorbed to particulate matter. However, free Zn^{2+} can form stable complexes with organic material allowing it to remain mobile in the water column. Zn solubility in groundwater and submerged soils also increases in environments where Mn and ferric Fe reduction results in production of organic complexing agents (Gambrell 1994; Wetzel 2001) indicating that soluble forms of Zn may be abundant in the Fe and Mn polluted S and I locations. Aquatic research has shown that as Zn levels increase the abundance and diversity of diatom communities decreases (Morin *et al.* 2007). Therefore, the degradation of macrophyte communities occurring in response to increased Zn levels results in a bottom up effect on macroinvertebrate communities (Clements *et al.* 2000; Courtney & Clements 2002; Clements 2004).

A clear indication that elevated Fe, Mn, and Zn levels influence the degradation of macrophyte communities is evident in the concurrent reduction of scraping and shredding functional feeding groups in the S0, S25, and S50 locations

(Figure 19). In collections from the S and I sites, one of the few taxa belonging to the scraping functional feeding group were members of the family Heptageniidae. This macroinvertebrate family was almost completely lost in metal polluted sampling locations (Figure 20), a phenomenon seen in other research as well (Clements *et al.* 2000; Courtney & Clements 2002; Clements 2004). Scrapers are feeding directly on macrophytes and shredders ingest macrophytes indirectly when feeding on organic leaf pack and detritus material (Thorp & Covich 2001), therefore both groups are affected when macrophyte communities are displaced by metal oxides. The I locations do not show a similar trend most likely because of the low abundance of macroinvertebrates coupled with the abundance of tolerant Beatid mayflies that feed by scraping (81% of scrapers collected at the I site in 2006 were Beatids), and the abundance of Dipterans (Family Tipulidae) that feed by shredding (62% of shredders collected at the I site in 2006 were Tipulids).

The results of this research indicate that Fe, Mn, and Zn concentrations increase in aquatic habitats that are not buffered from anthropogenic activity. While an increase in Fe, Mn, and Zn does not seem to determine the diversity and distribution of the bacterial biofilm community it severely limits macroinvertebrate community composition. The bottom up effect of Fe, Mn, and Zn on macroinvertebrates may occur when reduced forms of these metals reach oxic zones of the water column, are chemically oxidized forming immobile precipitates that adsorb phosphorus, thus depriving macrophyte communities of an essential nutrient, and in turn deprive scraping and shredding macroinvertebrates of suitable food sources. Therefore an end result of Fe, Mn, and Zn deposition is increased macroinvertebrate drift.

From the data collected from the SR, S, and I sampling locations it seems clear that the retention of a riparian buffer between anthropogenic activity and small order mountain streams and wetlands is necessary to retain physical and biological integrity. Data collected in this study indicates that if rural and urban developments continue to encroach upon these systems the physical habitat will be altered resulting in a bottom up effect on the biological community. Streams and wetlands and the biological communities therein are important functional and aesthetic resources, and the protection of these systems is imperative.

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APPENDIX A
16 rDNA Sequence Information

Each DNA sequence below represents partial 16S rRNA sequences from clone libraries created using biofilm DNA from sampling locations SR, S25, S50, I0, and I19. Each sequence is identified using the sampling location from which the sequence was found along with an individual identification number. For example, SR-1B was named clone 1B from the SR sampling location.

SR-1B
CTTTTAGGTGACCTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGT
CGACCTGCAGGCGGCCGCACTAGTGATTAGAGTTTGATCATGGCTCAGAACGAACGCTGGCGG
CAGGCTTAACACATGCAAGTCGAACGCCCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGCGT
GGGAATCTACCCAGAACTTCGGAACAACTGAGGGAAACTTCAGCTAATACCGGATACGCCCTAC
GGGGGAAAGATTTATCGGTTCTGGATGAGCCCGCGTTGGATAGCTAGTTGGTGGGGTAATGGC
CCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCAGTAGGGAAGATAATGA
CGGTACCTACAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGC
TAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGATCGTTAAGTCGGGGGTGAAA
TCCTGGAGCTCAACTCCAGAACTGCCTTCGATACTGGCGATCTTGAGTCCGGAAGAGGTGAGTG
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ACTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACTATGAGAGCTAGCCGTTGGAGGGTTTACCCTTCAGTGGCGCAGCTAA
CGCATTAAGCTCTCC

SR-23
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AGCGATTCCGCCTTCATGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGACTTTTGGAGA
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GCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGCTTATCACCGGCGGTTACC
TTAGAGTCCCCAACTAAATGATGGTAACTAAGGTCGAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTGTAGGTCCCCGAAGGGA
AGGAATCCATCTCTGGAAGCCGTCCTACCATGTCAAACGCTGGTAAGGTTCTGCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCAGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGC
GACCGTACTCCCCAGGCGGATAACTTAATGCGTTAGCTGCGTCACCGAAGCTCTAAGAGCCCCG
ACAACTAGTTATCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGC
TTTCGCACCTCAGCGTCAATACATGTCCAGTGAGCCGCCTTCGCCACTGGTGTTCTTTCCGAATA
TCTACGAATTTCACCTCTACACTCGGAAATTCCACTCACCTCTCCATGATTCTA

SR-32

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TGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGTCATGCTGTTACGCGA
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GGGATTAACCCATTGTCACCACCATTGTAGCACGTGTGTAGCCCAACCCGTAAGGGCCATGAGG
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CGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCGGT
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CTCGGAAATTCCGCTTACCTCTCTCGACCTCAANACCAGAAATTTTTGAA

SR-3B

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GAACGTACTCCCCAGGCGGGATACTTAACGCGTTAGCTACAGCACTGCACGGGTCGATACGCA
CAGCGCTTAGTATCCATCGTTTACGGCTAGGACTACTGGGGTATCTAATCCTGTTTGCTCCCCAC
GCTTTCGCACCTCAGCGTCAACAATCGTCCAGTGAGTCGCCTTCGCCACTGGTGTA

SR-5B

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SR-6B
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ACGAANTTCACCTCTATACTCGGAATTCN

SR-14
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SR-18
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SR-19
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GGGGCAAAGGGAACTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAAGAACATCGGTGGC
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SR-2
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SR-3
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SR-13
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SR-28
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CAGCTAGTTATCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT
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TACGAATTTCACCTCTACACTCGGAATTCCACTGAC

SR-33
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SR-8
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GTCTGCTTAACACATGCAAGTCGAACGGATGTAGCAATACATTAGTGGCGGACGGGTGAGTAAC
GCGTGAGAATCTGCCTTCAGGACGGAGACAACAGTTGGAAACGACTGCTAACCCCCGATGTACC
GCAAGGGAAAATATTTATAGCCTGAAAATGAGCTCGCGTCCGATTAGCTAGTTGGAGAGGTAAAA
GCTCACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGACGATCGGCCACACTGGGACTGAGAC
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SR-9
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SR-39
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SR-27
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SR-26
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SR-1
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S25-1
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TCTA

S25-10
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S25-10B
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S25-16
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S25-1B
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S25-2
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S25-2B
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S25-31
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S25-33
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S25-39
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S25-3B
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S25-41
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S25-42
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S25-4B
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S25-5B
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S25-6B
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S25-7B
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S25-8B
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S25-9B
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S50-22
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S50-6B
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S50-7B
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S50-8B
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S50-12
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S50-15
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S50-16
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S50-18
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S50-9
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S50-10
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S50-13
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S50-3

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S50-4

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S50-5

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S50-1
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S50-14
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S50-28
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S50-19
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S50-20
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S50-2
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I0-10
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I0-100
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I0-110
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I0-120
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I0-25
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I0-27
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I0-30
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I0-40
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I0-60
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I0-90
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I0-1
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I0-11
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I0-15
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I0-17
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I0-18
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I0-2
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I0-3
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I0-4
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I0-5
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I0-8
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I19-1
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I19-10
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I19-11
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I19-12
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I19-14
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I19-16
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I19-17
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I19-19
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I19-22B
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I19-23B
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I19-24B
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I19-25B
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I19-3
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I19-4
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I19-40
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I19-5
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I19-7
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I19-8
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I19-9
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I19-22
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AACCAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT
TCGTGCATGAAGCGTCGGTACAGGGTCCAGGGGAATTGCCTA

VITA

Jonathan Larry Pitchford was born in Dothan, AL 1979. He attended high school in Abbeville, AL where he graduated in 1997. He then attended Auburn University where he received a Bachelor of Science in Zoology in 2002. After working as a biology technician for in the Ouachita National Forest for Oklahoma State University and in Fort Benning, GA for Auburn University he accepted a graduate teaching assistantship at Appalachian State University where he would pursue a Master of Science in Biology.

Jonathan is currently working as an intern for the Canandaigua Lake Watershed Program in Canandaigua, NY. He lives in New York with his wife Genevieve Smith Pitchford. His address is 430 Wagner Street, Waterloo, NY 13165. His parents are Mr. and Mrs. Larry Pitchford of Abbeville, AL.