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Long-Term Recovery of South Indian Creek Following Interstate Construction

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ABSTRACT

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The expansion of Interstate 26 from Erwin, TN to the North Carolina border was a project that potentially adversely impacted South Indian Creek because of the steep landscapes and potential for erosion. Several studies have shown the short-term, negative effects of road construction on the water quality of nearby water bodies. Non-point source pollution is the major source of water pollution in the United States. The primary objective of this research is to evaluate the long-term effects of the construction of Interstate 26 on South Indian Creek to see if there has been any ecological recovery. The Environmental Health Sciences Laboratory of East Tennessee State University was contracted by the Tennessee Department of Transportation to collect data from before construction (1991-1992), during construction (1993-1994), and postconstruction (1995-1996). Comparison of microbial enzyme activities and other parameters to present-day (2012-2013) water quality conditions indicate that South Indian Creek has not fully recovered from the effects of the construction of the interstate.

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CHAPTER 1

INTRODUCTION

Construction of Interstate 26 between Johnson City, Tennessee and Asheville, North Carolina was an extreme modification of the mountainous terrain of Appalachia. It was "one of the largest and most environmentally sensitive projects ever undertaken by the Tennessee Department of Transportation" (Fish and Wildlife Associates, n.d., p.1). The Appalachian Development Highway System first proposed construction of the interstate in the 1960s to help foster economic development and public health access to the rural communities. This study evaluates water quality in five sites on South Indian Creek expected to have the heaviest impact 18 years after completion of road construction. This section of the construction area includes mostly headwater streams from Flag Pond, TN to the North Carolina state line part of the expansion of I-181 that began in 1990 and was completed in 1996. South Indian Creek is a third order headwater stream in the upper reaches of the Nolichucky watershed. It begins in Flag Pond, TN at approximately 2,400 feet in elevation with the convergence of Upper Higgins Creek and Sam's Creek. South Indian Creek runs parallel to the old highway 81 and highway 19 until it empties into the Nolichucky River just south of Erwin, TN at approximately 1,750 feet (Fish and Wildlife Associates, n.d., p.9). Headwater streams typically have little buffering capacity, so any disturbance heavily influences downstream waters.

Major Challenges

Because of the steep terrain and erosion potential, the refuge for black bears and trout, and the presence of pyretic rock, the construction was undoubtedly a major challenge. Other concerns included loss and fragmentation of wildlife habitat, noise, air pollution, and aesthetics. The interstate was constructed through areas of ridges and valleys, ranging from 1,700 to 3,800

feet in elevation. Approximately 1,146 acres of land were cleared for the new road that crosses five major streams draining into South Indian Creek and its tributaries. Two stream sections were relocated and 22 bridges were installed in the 15.3-mile section from Erwin, TN to the North Carolina State Line.

Designated Uses

The Tennessee Department of Environment and Conservation (TDEC) Division of Water Pollution Control is required to classify surface waters by their designated usage. South Indian Creek and its tributaries (including Sam's Creek, Higgins Creek, and Rocky Fork) are designated for use by fish and aquatic life, recreation, livestock watering and wildlife, irrigation, and naturally reproducing trout stream. Each designated usage has associated water quality criteria that must be met in order to remain unimpaired. Some are specific; fish and aquatic life streams must have a dissolved oxygen (DO) content less than 5.0 mg/L, the pH must be between 6.0 and 9.0, and the water temperature should not exceed 3 degrees relative to upstream control. Some criteria are more ambiguous, i.e. "the quality of downstream waters should not be detrimentally affected" (TDEC, 2008).

Construction Concerns

Erosion was considered a major environmental concern and management was of utmost priority. Erosion mitigation included sediment ponds and traps, slope drains, silt fences, stone check dams, temporary berms, rock drainage ditches, geotextile fabric, sodding/mulching, and brush barriers and ended up costing over $6.5 million by the end of construction. Large amounts of cut and fill were relocated and 90,000 cubic yards of pyretic rock were encapsulated (Fish and Wildlife Associates, n.d.). According to the Fish and Wildlife Associates, who were contracted by the Tennessee Department of Transportation (TDOT) to evaluate erosion related concerns

associated with Interstate 26 construction, “The Design Office had no experience in designing erosion control for such steep, mountainous areas of the magnitude encountered on this four lane interstate type highway. The degree of erosion and the velocity of the runoff were severely underestimated” (Fish and Wildlife Associates, n.d., p.48). Erosion and turbidity were of continuous concern because of the lack of proper stabilization mechanisms combined with the steep terrain and large-scale vegetation clearance. Because most residences and commercial operations were located in the floodplain of South Indian Creek, changes in the frequency and magnitude of flooding events due to road construction were of concern.

Acid Leaching

Construction cut through pyretic rock, which when exposed, interacts with bacteria, oxygen, and water to form iron hydroxide and sulfuric acid. The following equations illustrate the reactions responsible for acid leaching from pyrite (Stunn & Morgan, 1996).

$$FeS_2(s) + 7/2O_2 + H_2O = Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad \text{(Eq. 1)}$$

$$Fe^{2+} + ¼\ O_2 + H^+ = Fe^{3+} + ½\ H_2O \quad \text{(Eq. 2)}$$

$$Fe^{3+} + 3H_2O = Fe(OH)_3(s) + H^+ \quad \text{(Eq. 3)}$$

$$FeS_2(s) + 14Fe^{3+} + 8H_2O = 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad \text{(Eq. 4)}$$

Acid drainage will lower the downstream pH and can reduce survival of aquatic organisms. Precautions to lower acid rock drainage included locating the pyrite before excavation and analyzing the rock for potential acid, potential alkalinity, percentage pyretic sulfur, net acid/base potential, and paste pH. If net acid/base values were between -5.0 and 0.0 , the excavated rock was treated with agricultural lime (approximately 200,000 cubic yards total) and at values -5.0 or lower they were encapsulated (90,000 cubic yards total) (Fish and Wildlife Associates, n.d.). Encapsulation included a clay liner method and the newly developed geomembrane method, which seemed to work well and became the preferred method.

Water Quality Monitoring Program

The Tennessee Department of Transportation (TDOT) contracted East Tennessee State University's Environmental Health Sciences Laboratory (EHSL) to evaluate these sites prior to, during, and immediately following the construction of Interstate 26 because of the obvious sedimentation/turbidity problems, as well as the potential of acid rock drainage or ARD (TDOT). Scheuerman, Farris, Cherry, and Curie (1995) developed the water quality monitoring program that included measurement of 26 physical, chemical, and biological variables at 60 sites (Appendix N). The EHSL began the monitoring program on the lower projects (sites from Erwin to Ernestville, TN) in March 1990 and on the upper projects (sites from Ernestville, TN to the North Carolina border) in April/May of 1991 for preconstruction data. Results from the EHSL monitoring program indicated that the most impacted streams were first-order, headwater streams that have little buffering capacity, slopes were steep, and flow was too low to flush out the accumulated sediments. The larger streams had a better recovery rate because of their ability to flush out the sediments. In the final report of a 6-year water quality monitoring study by Scheuerman et al. (1997), certain sites had not ecologically recovered due to suspended solids and toxicants from the interstate construction activities. The upper sites continued to show erosion impacts through 1996, such as high solids, alkalinity, and conductivity. The low water conditions in 1995 were partially responsible for the slow recovery (Scheuerman et al., 1997).

Previous Microbial Enzyme Studies

Microorganisms use enzymes to break down organic matter into useable forms for metabolism and growth. Enzymes produced in microorganisms and are used for internal processes are referred to as endoenzymes. Exoenzymes are produced by the organism but are used for external processes. Microorganisms that produce exoenzymes have an advantage in

competition for resources in aquatic ecosystems (Chrost, 1990). In a typical aquatic environment, substrate concentration is usually low and variable, and/or the substrate may be tied up with another compound or insoluble in water. This may prevent exoenzyme and substrate coupling. Also, the exoenzyme may be lost from the original cell, denatured, or exposed to inhibitors in the water. If the exoenzyme is able to pass these environmental variables, optimum conditions for catalysis must also be available, such as optimum pH, temperature, and ionic strength.

Scientists continue to search for rapid and simple methods to understand microbial enzyme activities and their responses to environmental conditions. Methods should be sensitive enough that microbial growth is unnecessary and the measurement easy and rapid (Godsey, Matteo, Shen, Tolman, & Gohike, 1981). Microbial enzyme activity profiling is one opportunity. In this study the microbial enzyme activities are measured in order to identify activities and responses of the microorganisms to external processes. The use of bacterial counts and microbial enzyme activities as an indicator of environmental conditions is reviewed later in the Literature Review.

In 1996 Gu statistically analyzed data collected between 1991and 1995 by ETSU from upper South Indian Creek. Parameters included MEAs and Acridine Orange Direct Counts (AODCs), or microscopic bacteria counts (Gu, 1996). Gu attempted to evaluate MEAs as an indicator of both stream health and the river continuum concept on headwater streams. Gu found that MEAs were adversely affected by road construction because MEA activity was lowest during 1993, the main construction period. This decline in activity was not displayed at the control in the Doe River, 15-20 miles from construction. Gu also found that enzyme activity is a more sensitive parameter and more indicative of environmental conditions than AODCs, which

remained relatively constant and independent of MEAs. MEAs differentiate based on stream order with enzyme activity higher and more variable in the upper segment of South Indian Creek headwaters, which are primarily stream orders 1 through 3. The lower, more stable activity occurs in the lower segment of the stream, which are primarily orders of 4 through 6 (Gu, 1996)

Ecological Stoichiometric Theory

Microorganisms are essential in ecosystems because of their ability to cycle nutrients and energy required for life on Earth. Microorganisms attempt to maximize their use of nutrients and use catabolic and anabolic processes to break down organic substrates to gain energy and re-form cellular components for growth. Microorganisms differ by use of carbon sources; for instance, autotrophs use inorganic carbon in the form of carbon dioxide, while heterotrophs use organic carbon sources. Among heterotrophs, differences lie in the major source of carbon and how they metabolize it. Chemoorganoheterotrophs use carbon in the form of carbohydrates, fats, and proteins from plant and animal sources. Photoorganoheterotrophs can use sunlight coupled with the oxidation of organic substances to form their carbon source. Chemolithoautotrophs use carbon dioxide as their carbon source. Microorganisms can also differ by energy source, as chemotrophs use chemical energy and phototrophs use light energy. Differences also occur based on sources of electron donors, as lithotrophs use inorganic electron donors and organotrophs use organic electron donors.

A microbial cell is typically made of 50% carbon, 5%-15% nitrogen, and 0.5%-1.5% phosphorus and sulfur. Therefore, a typical restrictive ratio is about 100:10:1:1 for Carbon:Nitrogen:Phosphorus:Sulfur. Nutrients are limiting to growth, and without the proper ratio, microorganisms are unable to further metabolize and reproduce. The ecological stoichiometric theory suggests when the ratio changes (for instance, when storm-water runoff

delivers a large input of nutrients and organic matter), metabolism and activity by microorganisms will shift accordingly. Other nutritional limitations include growth factors, as some microorganisms cannot produce them, and may include certain amino acids, purines, pyrimidines, and vitamins.

Several environmental factors are involved in microbial metabolism including microbial populations, nutrients, oxygen, pH, temperature, and water and soil composition (Margesin, Zimmerbauer, & Schinner, 2000). Microorganisms typically have a unique, species-specific optimal environment for survival and growth based on both abiotic and biotic factors. Abiotic factors include temperature, salinity, water concentration, pressure, pH, and oxygen. At the optimal growth temperature, there is a maximum growth rate for the species. Psychrophiles are microorganisms that grow at temperatures close to freezing, thermophiles grow at an optimum between 50-70ºC, mesophiles grow at a temperate range (20-49 ºC), stereothermophiles are those that only grow at temperatures in their optimum range, and eurothermal species can exist in a wide range of temperatures and therefore are typically more environmentally successful.

Microorganisms can be aerobic where they require oxygen in the environment to survive, anaerobic where they require an oxygen deficient environment, or facultatively anaerobic where they can use anaerobic metabolism when oxygen is limited.

Environmental salt concentrations of 1.8% to 80% are necessary for halophilic microorganisms where below this concentration they would lyse, whereas nonhalophiles survive in environments of less than 2.5% salt concentrations. Specific water saturation percentages are also required for certain microorganisms to survive.

Dynamic Environments

Population dynamics change due to birth rate, death rate, emigration, immigration,

competition, pollution, and limited nutrients. Because organisms require a specific niche, they have a specific purpose that affects the entire ecosystem. Organisms depend on each other for the cycling of energy and nutrients. The interaction among the community and its environment determines the composition. Dynamic environments such as those with high physical variability generally reflect a more diverse community because of the wide variety of niches available (Lee et al., 2004; Vannote, Minshall, Cummins, Sedell, & Cushing, 1980). Interaction within a community greatly influences the composition. The interaction may be negligible, adverse, or beneficial. Competition can greatly change population dynamics and can occur between different species or within the same species. The theory of competitive exclusion states that for two species to coexist, they need to use different resources. Only one species can fill a specific niche in an ecosystem, the presence of each niche is determined by available resources, how the species metabolizes the resources, and the specific habitat. Community structure may stabilize due to debris dams and nutrient cycling or, conversely, destabilize during floods, temperature fluctuations, and epidemics.

The River Continuum Concept

An understanding of the River Continuum Concept is important for the evaluation of lotic systems because each variable in a flowing body of water affects the whole system. The River Continuum Concept describes the dynamic equilibrium between physical, chemical, and biological variables in a river ecosystem. These factors tend to fluctuate to maximize energy use efficiently and at a uniform rate (Vannote et al., 1980). The habitat, the abiotic components, and the biotic community characterize an ecosystem. The river balances physical parameters such as temperature, flow, width, depth, and sedimentation with chemical parameters such as inorganic matter, organic matter, and biological parameters such as the microorganisms,

macroinvertebrates, and other aquatic organisms. Energy is transferred and nutrients are cycled throughout the food web. The first trophic level includes primary producers, which contains the largest amount of biomass and includes microorganisms and photosynthetic organisms, which can produce organic compounds from inorganic substances and light. These organic compounds are then used by successive trophic levels for nutrients in order to survive. Energy and nutrients that are not used upstream will leak downstream for use by other organisms (Vannote et al., 1980). As surface water constantly travels downstream, it interacts with the bank and is heavily influenced by surrounding land use. The River Continuum Concept characterizes three segments of a typical river depending on size, because as the size of the river changes, so do the associated characteristics. Comparison of expected patterns versus observed patterns in an ecosystem can be useful as an indication of human influence. An overview of the characteristics described by the River Continuum Concept is provided in Table 1.

Table 1*: Overview of River Continuum Concept*

River Segment	Headwater	Midreaches	Mouth
Primary production/respiration ratio	<1	>1	<1
Organic Particulate Matter	Coarse	Fine	Fine
Impact of Riparian Vegetation	Significant	Less Significant	Insignificant
Macroinvertebrate Community	Shredders and Collectors	Collectors and Grazers	Collectors

Microbial Enzyme Activities

Metabolism by microorganisms varies widely. Microorganisms use enzymes to break down organic matter and polymers into inorganic forms so that the smaller molecules are available for use by bacteria. Productivity measured by organic carbon sequestration is governed

by the availability of nutrients (Hill et al., 2006). Microbial activity is affected by the amount of organic material in the system and the residence time of the water in the river or stream basin. Nitrogen limitation may be more pronounced in freshwater systems where anoxic sediment environments limit the electron acceptor to the availability of nitrates or because eutrophication has made phosphorus more available (Hill et al., 2006). Low biofilm respiration and enzyme activities may reflect low nutrient concentrations and dense canopy closure (low sunlight and/or lower water temperature).

Other factors that affect the response of microorganisms include salts and electronegative ions, which can change the conductivity of the environment. Toxins can also affect microbial metabolism, and some microorganisms are better than others at detoxification or deactivation of deleterious compounds.

Evaluation of Environmental Conditions

Conventional indicators of anthropogenic alteration of lotic systems include bacterial count and total biomass. These methods provide a way to estimate growth and the structure of microbial communities but provide no information on the function of microbial activity. Microbial growth may not be a good indication of anthropogenic alterations to the water body because microbial composition is extremely diverse (Frossard, Gerull, Mutz, & Gessner, 2011). When overarching ecosystem processes are measured, such as respiration or nitrogen mineralization, they typically are too coarse a measure to reflect a specific and significant response and may instead reflect simply an average response to environmental conditions. (Frossard et al., 2011).

A new development for indication of pollution includes measurement of microbial enzyme activities (MEAs). Because microbial enzymes regulate energy, carbon, and nutrient

dynamics, they are sensitive to anthropogenic alterations (Hill et al., 2006). Microorganisms respond quickly and sensitively to shifts in environmental condition. Measuring microbial metabolism may indicate growth, activity, and response to pollution. Microbes significantly alter both dissolved and particulate organic matter and are major competitors for reduced carbon in aquatic environments (Chrost, 1990). Because microorganisms are small organisms, tend to proliferate quickly, and have high metabolic activity per unit biomass, they have great potential for reduction of large carbon molecules (Chrost, 1990). Microbes must use enzymes to depolymerize molecules that may be too large to be readily used. Even though they operate at a molecular level, microbial enzymes contribute a large role to the function of aquatic ecosystems.

Measurement of MEAs may be a less expensive and quicker method to evaluate environmental conditions. In addition to being contained in microorganisms living in the ecosystem, these enzymes may be dissolved in water or attached to particles in the water or the sediment. Enzyme activities commonly used in environmental studies include: Dehydrogenase, Acid phosphatase, Alkaline phosphatase, Glucosidase, and Galactosidase. The various levels of microbial enzyme activity can be affected by several factors such as the sample type, pH, temperature, oxygen, buffer composition, substrate concentration, and incubation time. Increased runoff from roads causes an increase of nutrients in surface waters, called "nutrient loading" or "shock loading", and thereby changes the enzyme activity.

Dehydrogenase

Oxygen consumption, or respiration, may be measured using the relative levels of dehydrogenase enzymes. Dehydrogenase catalyzes oxidation-reduction reactions and the rate of this reaction, Dehydrogenase Activity, (DHA) is a measure of the total respiration as a rate of oxygen uptake and carbon dioxide production. DHA is generally present in aquatic systems

because of the aerobic environment. An increase in DHA indicates sufficient oxygen and carbon to support aerobic metabolism and active electron transport. Dehydrogenase activity is affected by substrate concentration, incubation time, extraction procedure, buffer composition, pH, temperature, oxygen conditions (aerobic versus microaerobic), sediment storage time, sediment type, and sediment volume (Burton & Lanza, 1985). An example of dehydrogenase activity is the conversion of lactate to pyruvate by lactate dehydrogenase (Figure 1). Lactate loses two electrons, or is oxidized, to become pyruvate.

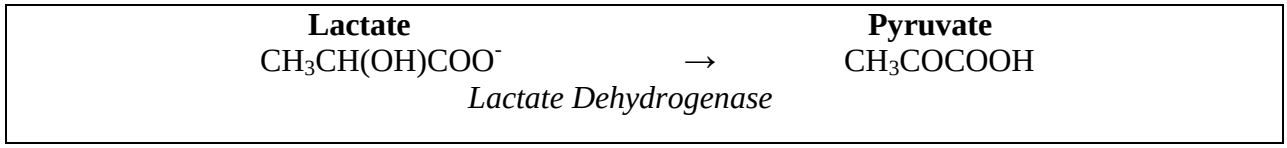

Figure 1: Example Reaction with Dehydrogenase

Phosphatase

Phosphatases hydrolyze organic phosphate into orthophosphate or alcohol. Alkaline phosphatase (AP) is repressed by inorganic phosphate and has maximum activity at a high pH (Chrost, 1990). Alkaline phosphatase activity is an indicator of phosphorus deficiency. Acid phosphatase activity, which increases at low inorganic phosphorus concentration, is repressed by inorganic phosphate, and has maximum activity at a low pH. The major difference between acid and alkaline enzyme activity is that acid phosphatase can hydrolyze *O*-substituted monoesters and alkaline phosphatase can hydrolyze *S*-substituted monoesters.

Because phosphorus is the rarest nutrient in the system, low phosphatase activity usually indicates anthropogenic eutrophication. An increase in phosphatase activity indicates phosphorus as the limiting nutrient. Studies have shown a dose-response relationship between phosphatase activity and toxicant exposure, with activity inhibition with rising toxicant concentration (Burton & Lanza, 1985). Figure 2 is an example reaction with alkaline phosphatase.

Orthophosphoric monoester	**+**	**Water**		**Alcohol**	**+**	**Phosphoric Acid**
$R\text{-}O\text{-}OP(OH)_3$	+	H_2O	→	R-OH		H_3PO_4
		Alkaline Phosphatase				

Figure 2: Example Reaction with Alkaline Phosphatase

Glucosidase

Heterotrophic bacteria are prominent in lotic system metabolism evidenced by their involvement in nutrient cycling, organic matter transformation and mineralization, energy flux, and influence on toxic compounds in the ecosystem. Both Glucosidase (GLU) and Galactosidase (GAL) are glycosidases involved in glycerine-glucose metabolism. Glucosidase is a broad-range substrate enzyme with specificity over aryland alkyl- ß-glucosides produced mainly by heterotrophic bacteria. Glucosidase activity increases with a growth in bacterial abundance, organic carbon loading, heterotrophic uptake of glucose, and bacterial production. ß-glucosidase catalyzes the hydrolysis of ß-linked disaccharides of glucose. It is also involved with phosphorylation leading to glucose 1-phosphate and transglycosylation leading to cellotriose. There is typically a temporal and spatial pattern associated with glucosidase in aquatic systems. An increase in glucosidase activity corresponds to an increase in heterotrophic uptake of glucose and bacterial production. Figure 3 is an example reaction with glucosidase.

Glucoside	**+**	**Water**		**Glucose**	**+**	**Alcohol**
$C_7H_{14}O_6$	+	H_2O	→	$C_6H_{12}O_6$	+	R-OH
			Glucosidase			

Figure 3: Example Reaction with Glucosidase

Galactosidase

Galactosidase (GAL) is a type of glycosidase involved in the glycerine-glucose metabolism, which cleaves lactose into galactose, glucose, and galactoside. An increase in galactosidase activity occurs with sudden inputs of cellulose, such as from autumn leaves. A

decrease in enzyme activity can occur when vegetation is removed, which causes a loss of habitat and substrate for microorganisms, an increase in water temperature, and a decrease in dissolved oxygen. Elevated microbial enzyme activity in the absence of fecal contamination is indicative of a large volume of plant matter entering the stream. Figure 4 is an example reaction with galactosidase.

Galactoside	+	**Water**		**Galactose**	+	**Alcohol**
$C_7H_{14}O_6$	+	H_2O	$\xrightarrow[Galactosidase]{}$	$C_6H_{12}O_6$	+	R-OH

Figure 4: Example Reaction with Galactosidase

Long-Term Studies on Road Construction

There are few long-term studies on the effects of road construction on nearby surface water. However, several studies have shown that an increase in impervious surfaces reflects an increase in erosion potential and a decrease in indices of biotic integrity (Angermeier, Wheeler, & Rosenberger, 2004; Arnold & Gibbons, 1996; Wang, Lyons, & Kanehl, 2001). Many studies have defined three temporal stages in order to assess the impacts of road construction on nearby river systems (Angermeier et al., 2004; Wheeler et al., 2005). The first stage, "Road or Highway Construction" includes the short-term and acute effects on the local stream due to construction (Angermeier et al., 2004; Wheeler et al., 2005). The most serious of these effects is aggradation, or increased fine sediment pollution, which alters the habitat structure, the macroinvertebrate community, and interferes with an organism's ability to breathe, feed, and reproduce (Angermeier et al., 2004; Wheeler et al., 2005). Streambed habitat is reduced and silt-tolerant species replace the intolerant, more sensitive species, altering the community composition.

Continuous impacts of road presence include pollutants from traffic runoff and channel alterations (Angermeier et al., 2004; Wheeler et al., 2005). Heavy metals from runoff include lead oxide and zinc from tire wear, as well as iron, cadmium, nickel, copper, and chromium

(Angermeier et al., 2004; Forman & Alexander, 1998; Tsihrintzis & Hamid, 1997). Pollutants tend to accumulate in sediments and biota and the concentrations increase with increasing traffic volume and intensity. Other factors include the number of antecedent dry days, rainfall patterns and volume, land use, geographic/geologic features, maintenance practices, and drainage (Tsihrintzis & Hamid, 1997). Other chemical pollutants from roads include deicing salt, which greatly increases conductivity and alters chemical interactions necessary to facilitate a healthy surface water body. Petroleum products are often found in leaking oil and gasoline from vehicles and runoff into surface waters with precipitation. Hazardous waste spills have extremely detrimental effects and occur most often on bridges crossing streams because they are prone to ice over in cold seasons (Forman & Alexander, 1998).

Hydraulic alteration and channel incision caused by road construction replaces coarse substrate with finer sediment, reduces riparian cover, and changes the natural depth, velocity, and pool-riffle sequences. These effects negatively impact the aquatic communities that rely on specific substrate, habitat, and other environmental factors such as temperature to survive. Reduction in riparian cover causes an increase in water temperature, disrupts the sediment bed, and reduces food sources, which allows for more intolerant biota (Paul & Meyer, 2001). Leaching, especially in mountainous regions, can cause acid drainage.

The third phase, "Landscape Urbanization", includes the extensive and chronic impacts of urbanization. Highways are known as "magnets for decentralized growth" and are a direct cause for urban sprawl. Urbanization is the leading cause of water-body impairment (Angermeier et al., 2004). Even small amounts of urban cover can impact biota compared with the large amounts of agricultural area. There is evidence of a threshold value for impervious surface cover that if exceeded causes detrimental effects on aquatic biota (Wang, Lyons, &

Kanehl, 2001). A 10%-20% increase of impervious surface cover causes runoff to double, a 35%-50% increase causes runoff to triple, and a 75%-100% increase causes runoff to quintuple (Arnold & Gibbons, 1996). Runoff causes sedimentation, as well as pollutant and nutrient loading. A small amount of impervious cover in a watershed of 5%-15%, results in tolerant macroinvertebrates replacing more sensitive species (Angermeier, Wheeler, & Rosenberger, 2004). At 10%-12% imperviousness, the integrity of a given fish species community is consistently poor (Wang et al., 2001).

Headwater streams are greatly influenced by riparian vegetation and the streamside terrestrial setting. When alterations occur, such as an increase in impervious surfaces, large quantities of organic matter and other toxicants can inhibit microbial enzyme activity. Most exoenzyme activity is repressed when dissolved organic matter is readily usable. However, when the concentration of low-molecular-weight molecules fall below a critical level, exoenzymes are expressed. Microorganisms use this as a strategy to save energy and prevent production of enzymes that are not useful when their growth is not limited. Microbes have specific environmental conditions for optimum growth and enzyme activity, so changes in these conditions, such as pH, temperature, oxygen level, and salinity reflect changes in enzyme activity. Naturally alkaline waters and acidic waters generally have the highest exoenzyme activity at the pH range of 7.5- 8.5 and 4.0-5.5, respectively (Chrost, 1990). Both high and low salinity concentrations inhibit substrate decomposition. Toxicants tend to inhibit enzyme activity, although some research shows that this inhibition may decrease with time by desensitization (Burton & Lanza, 1985).

Out of the 60 sites evaluated by ETSU on SIC, those predicted to have the most impact by the road construction are included in Table 2. These are areas near bridges, culverts, and

other anthropogenic alterations associated with the Interstate 26 construction. Figure 5 is a map of the sites with the approximate locations.

Table 2*: South Indian Creek Sites*

Station	Stream Order	Depth	Width	Description	Location
2	2nd	2.7	1.0	Rocky/muddy	In Sam's Creek downstream of retaining walls
6	2nd	4.7	2.0	Rocky/silty	Stream along Carver Road downstream of construction
16	2nd	19.7	6.1	Rocky	Upper Higgins Creek downstream of box culvert
22	2nd	8.0	2.0	Rocky/silty	Clear Branch between farm and community center, across from Clear Branch Baptist Church
26	3rd	39.3	6.7	Rocky	Rocky Fork, station above bridge on Rocky Fork Road (control site)

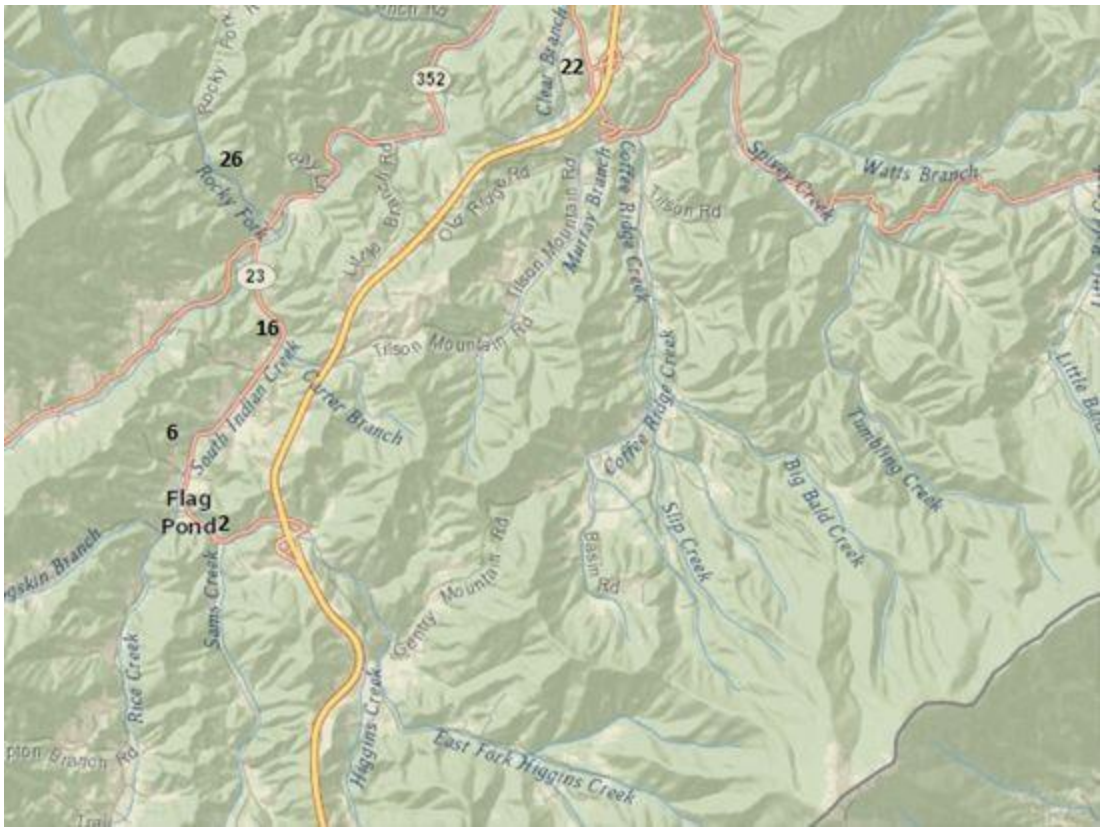

Figure 5: Map of South Indian Creek Area

(Esri ArcGIS)

CHAPTER 2

MATERIALS AND METHODS

Habitat Assessment using USEPA Rapid Bioassessment Protocols

An initial habitat assessment was performed in February 2012 to qualitatively evaluate the condition of the sites. This was compared to the 1998 habitat assessment performed by Scheuerman et al. A final Habitat Assessment was performed in May 2013 to reflect present-day conditions of the sites and was a useful comparison to the past. A habitat assessment was performed on the 10 sites to measure the following parameters: 1. bottom substrate/available cover, 2. embeddedness, 3.velocity/depth, 4. channel alteration, 5.bottom scouring and deposition, 6. pool/riffle-run/bend ration, 7. bank stability, 8. bank vegetative stability, and 9. streamside cover. These are the same habitat assessment parameters measured in the 1998 survey of postconstruction recovery performed by Scheuerman et al. Each station was ranked using US EPA Rapid Bioassessment Protocols (US EPA, 1989). The comparison of the habitat assessments provides a glimpse of the stage of long-term recovery.

Monthly Water Quality Data Collection

All procedures have followed closely to the Environmental Health Science Laboratory (EHSL) Standard Operating Procedures (SOPs).

Field Parameters

These parameters were measured with every sampling event for any confounding effects of ambient environmental conditions on measured parameters. Monthly sampling trips began in May 2012 and ended in June 2013. Water quality parameters measured in the field at each sampling site include dissolved oxygen, conductivity, pH, temperature (air and water), water stage (depth and width), and flow. Field sampling procedures followed closely to the

EHSL SOP # 35 (see Appendix A). Dissolved oxygen and air temperature were measured using the Thermo Orion YSI Model 115 dissolved oxygen meter. Conductivity was measured using the YSI Model 115 Conductivity meter. Water temperature and pH were detected using the Fisher Scientific Accumet Portable AP61 pH meter. Width was measured using a fiberglass measuring tape. Flow was measured using the Global Water Model FP101 flow meter. These parameters are the same water quality parameters performed by Scheuerman et al., and can therefore be used in comparison to the previous data.

Laboratory Water Quality Parameters

Water samples were collected in acid washed two-liter high density polyethylene bottles (Fisher Scientific, Atlanta, GA). Additional water samples were taken in sterile plastic sample Whirl-Paks for both standard plate counts and acridine orange direct counts. Samples were collected midstream along a standard transect at each site. Samples were kept on ice until they arrived in the laboratory, where they were transferred to a refrigerator at 4ºC. Sediment samples were collected by scraping the upper sediment surface with a spoon and placed into presterilized, high-density plastic bags. All samples were processed within the appropriate time period as described by USEPA methods.

Biological Water Quality

Biological water quality measures included acridine orange direct counts for water and sediment, standard plate counts for water, and microbial enzyme activities for sediment (Dehydrogenase, Glucosidase, Galactosidase, Acid Phosphatase, and Alkaline Phosphatase). AODCs and standard plate counts were used to enumerate microorganisms and estimate the relationship with microbial enzyme activities. They were also used in comparison to past data to evaluate long-term recovery. Biological parameters were assessed for both water as well as

sediment and include the following: Acridine Orange Direct Counts, EHSL SOP # 15 (see Appendix E), standard plate counts, EHSL SOP # 72 (see Appendix D), and microbial enzyme activities: Dehydrogenase Activity, EHSL SOP # 14 (see Appendix G), Glucosidase Activity, EHSL SOP # 34 (see Appendix H), Galactosidase Activity, EHSL SOP # 36 (see Appendix I), and Alkaline phosphatase, EHSL SOP # 16 (see Appendix F).

Acridine Orange Direct Counts (AODCs) were used to enumerate bacterial cells in water and sediment. The Acridine Orange (AO) stain interacts with nucleic acids, forming bonds with both DNA and RNA. Once the two are electrostatically bound, they fluoresce at different wavelengths and can be counted by viewing with ultraviolet light. Sediment samples were placed into Phosphate Buffered Saline and Tween 80 (polyoxyethylene sorbitan monoleate), vortexed, and left overnight for sediment to settle. Water samples and sediment suspension were added to a small amount of deionized water and Acridine Orange stain and vacuum filtered through 0.2 micron polycarbonate filter paper. This was then mounted on a microscope slide. Triplicate counts of fluorescent microorganisms were obtained.

Heterotrophic plate counts were used to estimate bacteria on R2A agar using the pour plate method. A small water sample (between 0.1 and 0.5 mL) is aseptically pipetted onto the bottom of a sterile Petri dish before the heat-tempered agar is added and mixed. After the agar has solidified, the plates are inverted and incubated at room temperature for 48 hours. Colonies were then counted using the Quebec colony counter.

Microbial enzyme activities were conducted by placing 1 gram of sediment into small test tubes containing the buffer used for the respective enzyme. For each site, there were three triplicate samples and one blank. A negative control for each enzyme activity batch was also used to control for substrate interferences. Buffers include Phosphate buffer with pH of 9.0 for

both Galactosidase and Glucosidase, and TRIS buffer 8.6 for Alkaline Phosphatase. The substrate with added indicator for the specific enzyme was then added to the respective tubes and incubated for 18 hours. TRIS Buffer with phosphatase substrate (pH 7.6) was added to both the Alkaline Phosphatase sample tubes and the negative control (not the blanks). Phosphate Buffer (pH 7.6) with 4-nitrophenyl-ß-D-glucopyranoside (PNPGlu) was added to the Glucosidase sample tubes and the negative control. Glucosidase hydrolyzes PNPGlu and releases the yellow compound, *p*-nitrophenol. Phosphate Buffer (pH 7.6) with p-nitrophenyl-ß-D-galactopyranoside (PNPGal) was added to the Galactosidase sample tubes and the negative control. When galactosidase hydrolyzes PNPGal, it releases *p*-nitrophenol. Using the spectrophotometer the following day, the relative amounts of activities were measured by reading the absorbance at 418 nm. If a sample absorbance was measured as 0.6 or above, samples were diluted with deionized water.

For dehydrogenase activity, the buffer solution is a 0.1 M phosphate buffer with a pH of 7.6. One gram sediment samples were placed into each tube and sample tubes were given the additional substrate, INT solution (2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phynyl 2H-tetrazolium chloride). Tubes are vortexed and incubated for 45 minutes in the dark. A 1-mL aliquot of each tube is then vacuum-filtered through a sterile 0.45 micron membrane filter, dried, and then dissolved in DMSO. These tubes are then incubated overnight and read at 460 nm using the spectrophotometer the following day.

Chemical Water Quality

Total Carbon was measured using the High Temperature Total Organic Carbon / Total Bound Nitrogen (TOC/TN_b) Analyzer, LiquiTOC (Elementar Analysensysteme GmbH), SOP # 90 (see Appendix C). Water samples are injected into a high temperature furnace with a

platinum or cobalt catalyst. An acid is added to the sample to convert the inorganic carbon to carbon dioxide. The remaining inorganic carbon-free sample is oxidized and the generated carbon dioxide is a measure of Total Organic Carbon. Carbon is the major nutrient source for microorganisms, and measurement of total carbon is to determine potential relationships with microbial enzyme activities, Glucosidase, Galactosidase, and Dehydrogenase.

Total Phosphorus was measured using the ICS-1000 Ion Chromatograph (Dionex), SOP #92, (see Appendix B). Because phosphorus is often a limiting nutrient for microorganisms in freshwater systems, this potentially forms a relationship to microbial enzyme activities, Alkaline and Acid phosphatase. Samples are passed through the ion chromatograph matrix with an eluent in order to stabilize the ions and mobilize them at different speeds based on their relative physiochemical characteristics.

Experimental Variables

Time

The temporal factor under investigation is the primary experimental variable. Long-term recovery is determined using comparison of data from approximately 20 years ago.

Seasonal variation and Climate

Changes in local climate patterns such as droughts and flooding most likely yield influence on the recovery and/or rate of recovery.

Erosion

Due to the steep terrain, the primary concern of construction activity is the alteration of the stream due to erosion. These changes were physical, chemical, and biological.

Population Changes

There was an increase in the Unicoi County population from 17,666 in April 2000 to 18,313 in 2010. The April 2012 population decreased to 18,235. The population percent change was -0.4% (U.S. Census, 2012).

Transportation

There was a decrease in transportation on Highway 352, the old highway adjacent to I-26 and a corresponding decline in the slower movement of traffic on the old highway. There was also an increase in transportation on I-26 and a corresponding increase in the faster movement of traffic on the interstate.

Confounding Variables

Factors that may have caused complexity in the data analyses include the following:

Determination of adequate control site

It is difficult to find an adequate control site that is completely free from any effects from the interstate construction but is similar enough to compare to the other sites.

Anthropogenic alterations

Anthropogenic alterations include agriculture, construction of homes, bridges, and the associated runoff from all the roads.

Dynamic Environment

Because environmental conditions are interactive and constantly shifting, it is difficult to assess the effect of a single environmental parameter in situ (Singleton, Attwell, Jangi, & Colwell, 1982).

Quality Assurance and Quality Control

Field Procedures

A field log book was kept with the following information provided for each sample event: date and time of sampling, sample location, sample type, parameters analyzed, preservation of sample, field conditions, and signature of samplers. At each site dissolved oxygen, conductivity, air temperature, water temperature, pH, depth, flow, and width of stream were measured. All meters were calibrated prior to the sample event according to SOP #35 (see Appendix A). For each sampling event triplicate samples were taken for each parameter measured. For each event a field blank and a trip blank were taken to ensure that no contamination occurred.

Laboratory Procedures

Analyses were conducted using written procedures described in the EHSL Standard Operating Procedures for each parameter (see Appendix A-I). Bench sheets including all analyses and QA/QC data were filled out and taken to the QA/QC officer, Brian Evanshen. Samples were rejected if they were improperly stored, improperly preserved, held in a leaking container, transport/storage times were too long, invalid sampling technique, did not meet acceptable precision, did not meet acceptable accuracy, or if the Chain of Command was not continuous.

Analytical QA/QC Checks

For every analytical batch or 10 samples, whichever is smaller, a standard and reagent blank were analyzed. On 10% of the samples, a spike was run to determine recovery. On 10% of the samples, a duplicate set of analyses were run. The instrument detection limit and the method detection limit were detected to determine the minimum concentration to provide

reproducible results. This was determined by creating a standard curve for each of the different enzyme activities. If measurements were below the minimum detection limit or below the standard curve, the lowest detection limit was used in its place.

Data Analyses

Data were first qualitatively evaluated using graphical methods to determine any observable trends or differences. Time series plots illustrate the four major time periods: before-construction (1991-1992), during- construction (1993-1994), immediately postconstruction (1995-1996), and present-day (2012-2013). Environmental conditions were evaluated at the same sites (2, 6, 16, 22, and 26) and compared pair-wise across years to determine if recovery has occurred in South Indian Creek following Interstate 26 construction. Tests for normality were performed using IBM SPSS Statistical Software (Norusis, 2003). The data for the microbial enzyme activities, acridine orange direct counts, and standard plate counts were not normally distributed, so geomeans were used instead of averages for data analyses. The data were transformed using natural log to create a normal plot of each MEA (Appendix K). Multivariate Analysis of Variance determined there was a statistically significant difference in all MEAs based on time period and year. All parameters were subject to the time-series plot and paired *t*-tests across the four time periods using Minitab. Alpha levels were set to 0.05.

Parameters that were measured but not included in data analyses because there was not enough data points include Total Organic Carbon, Total Phosphorus, and Acid Phosphatase Activity.

CHAPTER 3

RESULTS

Tables 3 and 4 are the results from the 1998, 2012, and 2013 habitat assessments.

Table 3*: A Comparison of the 1998 Habitat Assessment to the 2012 Habitat Assessment using USEPA Rapid Biological Protocol Classifications are E (>90% as excellent), S (75-89% supporting), P (60-74% as partially supporting), and N (<59% as nonsupporting)*

Site	1998 Score	Class	2012 Score	Class	Comments
2	107	S	101	S	Lack of adequate riparian vegetation and bank stability on right bank. Most upstream site, downstream from culvert. High erosion potential.
6	94	P	90	P	Channelization appears extensive, high erosion potential, lack of adequate vegetation and bank stability.
16	119	E	105	S	Bank is moderately unstable, lack of riparian vegetation especially on right bank. Interstate ~ ½ mile up the road, by gate and farms.
22	76	N	89	P	Lack of riparian vegetation on left bank, Located by community and small neighborhood, gardens, septic tanks. Pollution, such as oil plumes, was visible.
26	130	E	129	E	Reference site. Appears stable, with adequate habitat for mixed colonization

Table 4*: June 2013 Habitat Assessment using USEPA Rapid Biological Protocol*

Site	2013 Score	Class	1998 Score	Class	2012 Score	Class	Comments
2	133	E	107	S	101	S	Channelization may be extensive, lack of adequate vegetative protection
6	55	N	94	P	90	P	Epifaunal substrate / available cover marginal to poor, lack of adequate riparian vegetation
16	112	S	119	E	105	S	Bank is moderately unstable, lack of riparian vegetation and vegetative protection
22	61	N	76	N	89	P	Lack of riparian vegetation, available cover marginal, bank moderately unstable.
26	142	E	130	E	129	E	Reference site. Appears stable, with adequate habitat for mixed colonization

Between Time-Period Differences

Time series plots illustrate the four major time periods: before-construction (1991-1992), during- construction (1993-1994), immediately postconstruction (1995-1996), and present-day (2012-2013). Environmental conditions were evaluated at the same sites (2, 6, 16, 22, and 26) and compared pair-wise across years to determine if recovery has occurred in South Indian Creek following Interstate 26 construction. The null hypothesis, H_0, is that South Indian Creek sites have recovered to before-construction conditions; i.e. before-construction and present-day environmental conditions are not significantly different. The alternative hypothesis, H_A, is that there is a significant difference between before-construction and present-day conditions, and the South Indian Creek sites have not recovered.

To check if outlier tests would be mandatory, any possible outlier was identified using box plots. Outlier tests were performed and those data were either removed or recognized and respected, according to the magnitude of the difference and the circumstance. Quantitative analyses began with Multivariate Analysis of Variance (MANOVA) using SPSS. Multivariate tests between years supplied the Wilk's Lambda significance value of 0.000, implying there is a statistically significant difference in MEA concentration based on year. Univariate tests found a statistically significant difference in *all* MEA concentrations based on year (Wilk's Lambda: 0.000). Tukey's post-hoc test identified specific between-year significant differences. These between-year differences were then analyzed using student's paired *t*-tests. Multivariate Tests between sites provided a Wilk's Lambda significance value of 0.782, which indicates that there is not a significant difference in MEA concentration based on site.

Microbial Enzyme Activities

Galactosidase

Figures 6 and 7 illustrate time series plot of galactosidase across time periods and years.

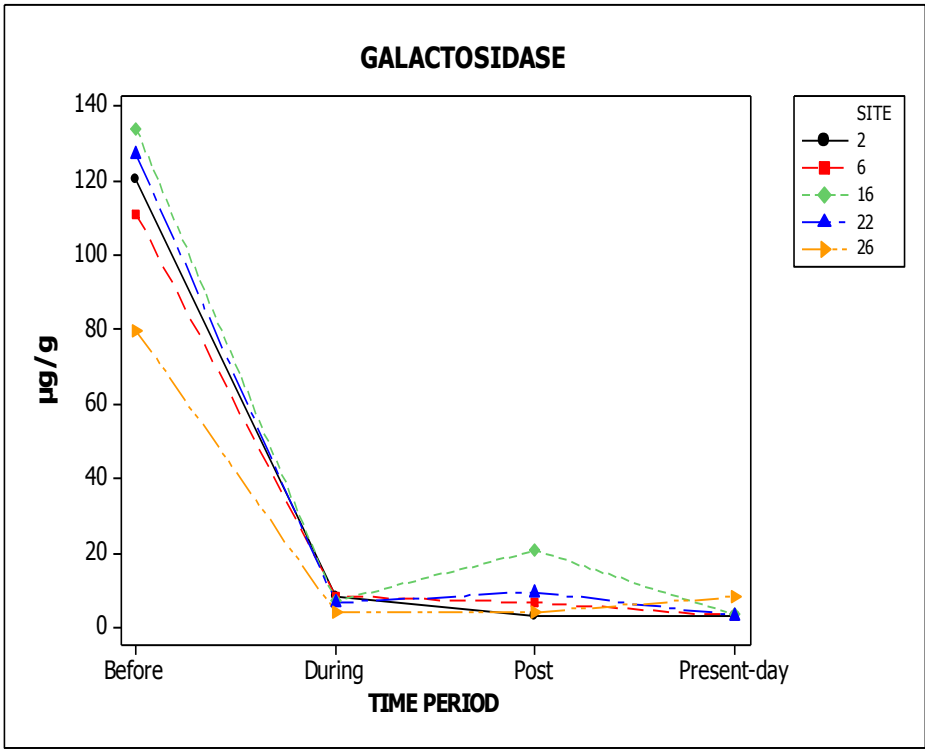

Figure 6: *Time Series Plot of GAL across Time Periods*

All parameters were subject to the time-series plot and paired *t*-tests across the four time periods using Minitab. Alpha levels were set to 0.05. Significant differences in Galactosidase activity were found between before-construction and during construction (p=0.000), before-construction and postconstruction (p=0.000), and before-construction and present-day (p=0.000). No significant difference was found between during-construction and postconstruction (0.559), during construction and present-day (0.221), or postconstruction and present-day (0.276). Before-construction data are significantly different from all following years, which indicates that

the Galactosidase levels have not recovered to prior-construction conditions. Galactosidase levels are approximately the same during construction, post-construction, and present-day.

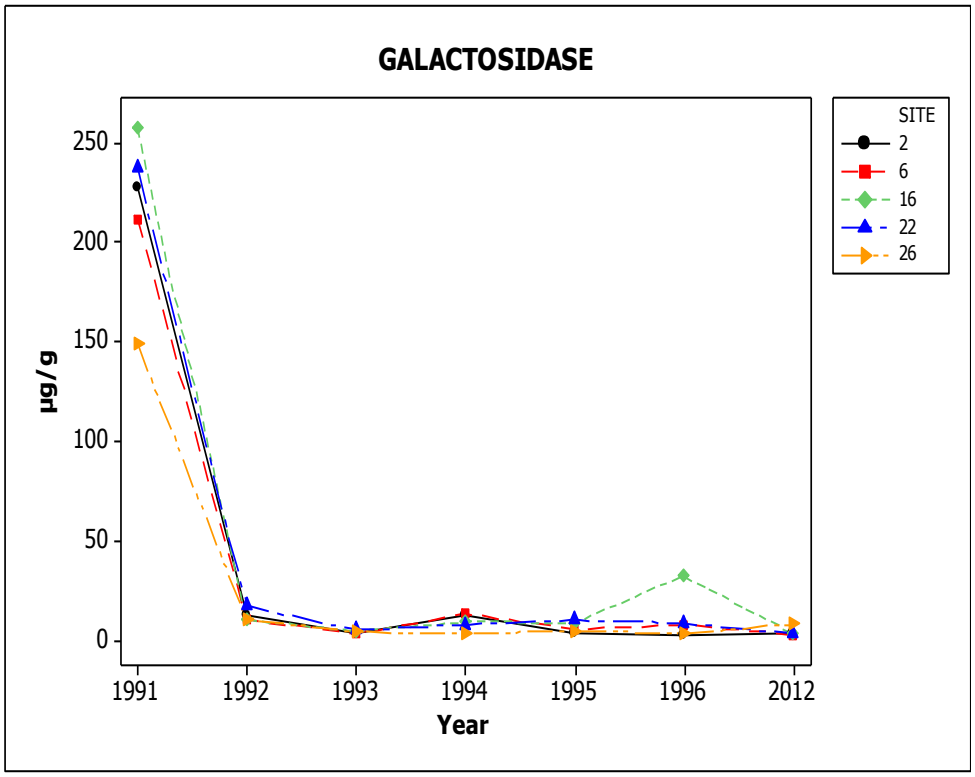

Figure 7: Time Series Plot of GAL across years

1991 Galactosidase is significantly different from all other years (p=0.000). Significant differences also occur between 1992 and 1993 (p=0.002), 1992 and 1995 (0.012), and 1992 and 2012 (0.014). Galactosidase levels have not recovered to before-construction conditions.

Glucosidase

Figures 8 and 9 illustrate time series plot of glucosidase across time periods and years.

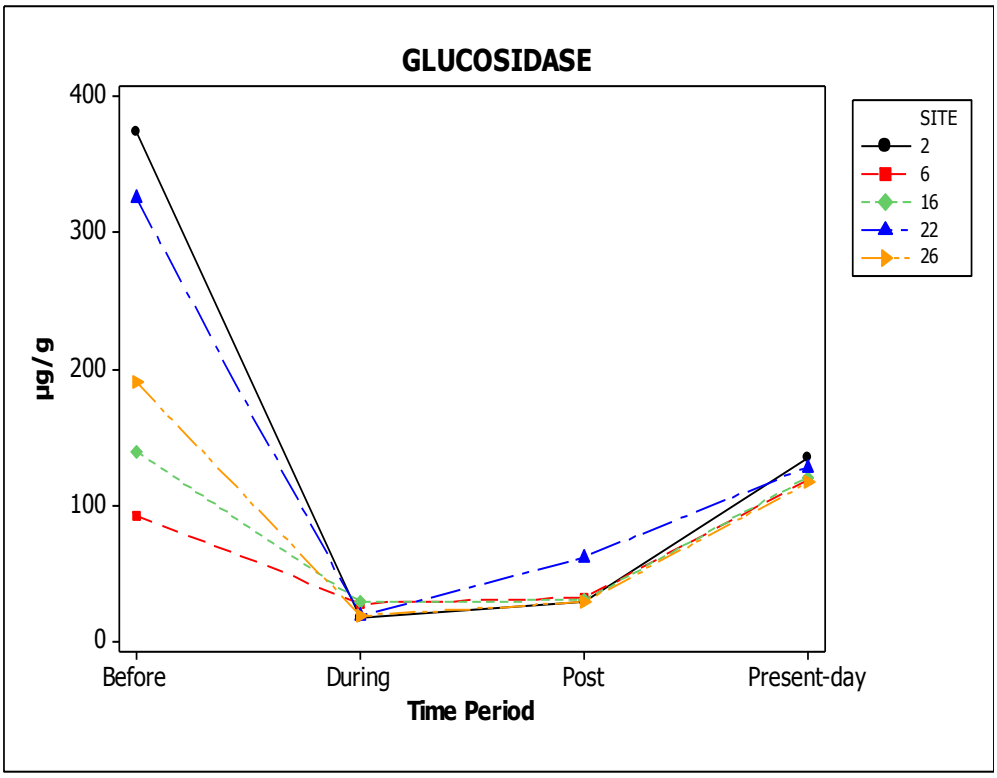

Figure 8: Time Series Plot of GLU across Time Periods

Significant differences in glucosidase were found between before-construction and during-construction (p=0.023), before-construction and postconstruction (p=0.022), during-construction and present-day (p=0.000), before construction and present-day (0.000), and postconstruction and present-day (p=0.000). No significant difference was found between during-construction and postconstruction (p=0.133). Glucosidase activities have increased slightly over the postconstruction and present-day time periods, but have not recovered to the same levels as before construction.

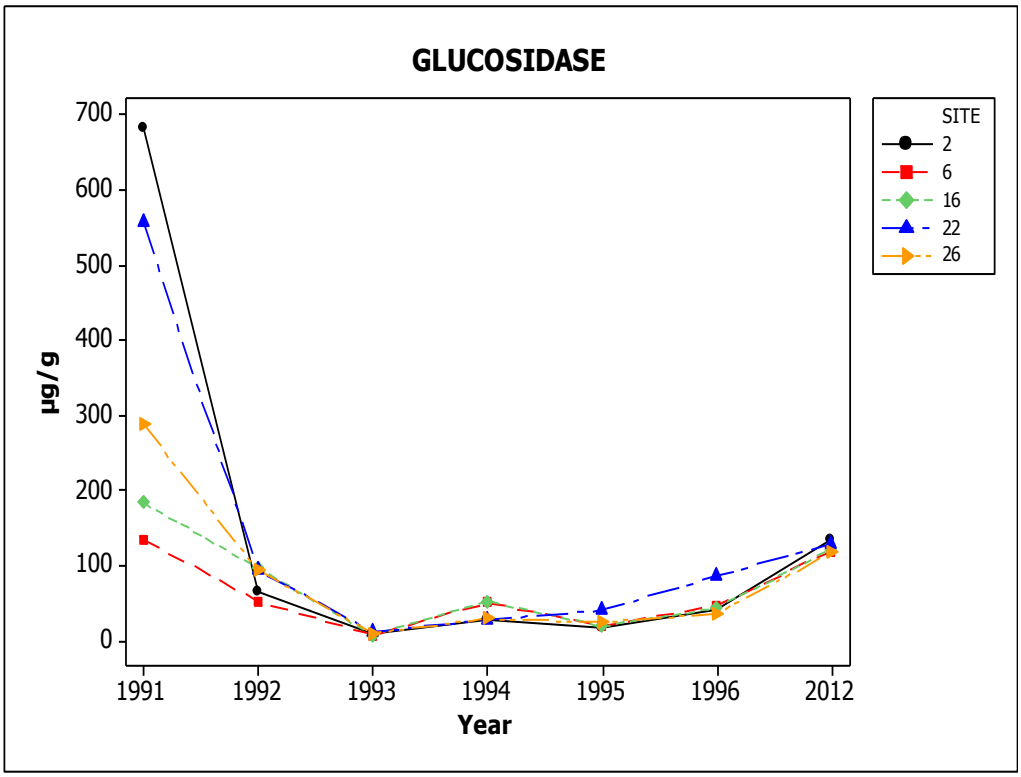

Figure 9: Time Series Plot of GLU across Years

Glucosidase levels were significantly different between all years except 1991 versus 1992, 1991 versus 2012, 1992 versus 1996, 1994 versus 1995, and 1994 versus 1996. There is not significant different between 1991 versus 2012, which may indicate recovery.

Dehydrogenase

Figures 10 and 11 illustrate time series plot of dehydrogenase across time periods and years.

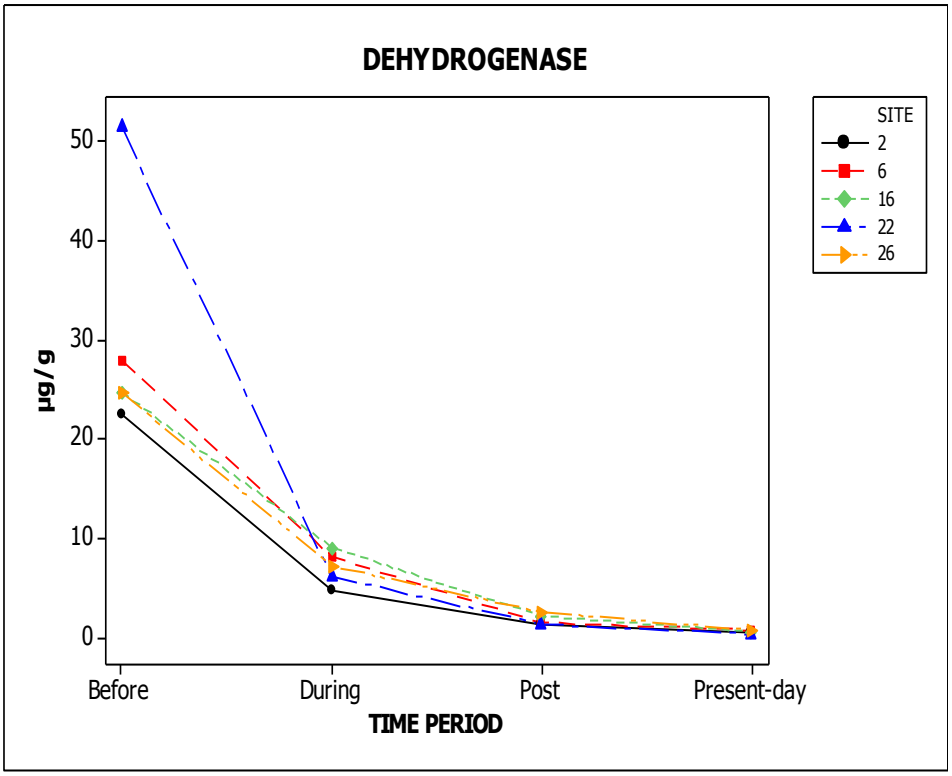

Figure 10: Time Series Plot of DHA across Time Periods

Significant differences in dehydrogenase activity were found between before-construction and during-construction (p=0.014), before-construction and postconstruction (p=0.006), before-construction and present-day (p=0.005), during-construction and postconstruction (p=0.001), during-construction and present-day (p=0.001), and postconstruction and present-day (p=0.004). Before-construction data are significantly different from all following years, which indicates that the dehydrogenase levels have not recovered to prior-construction conditions. Dehydrogenase levels are approximately the same during-construction, postconstruction, and present-day.

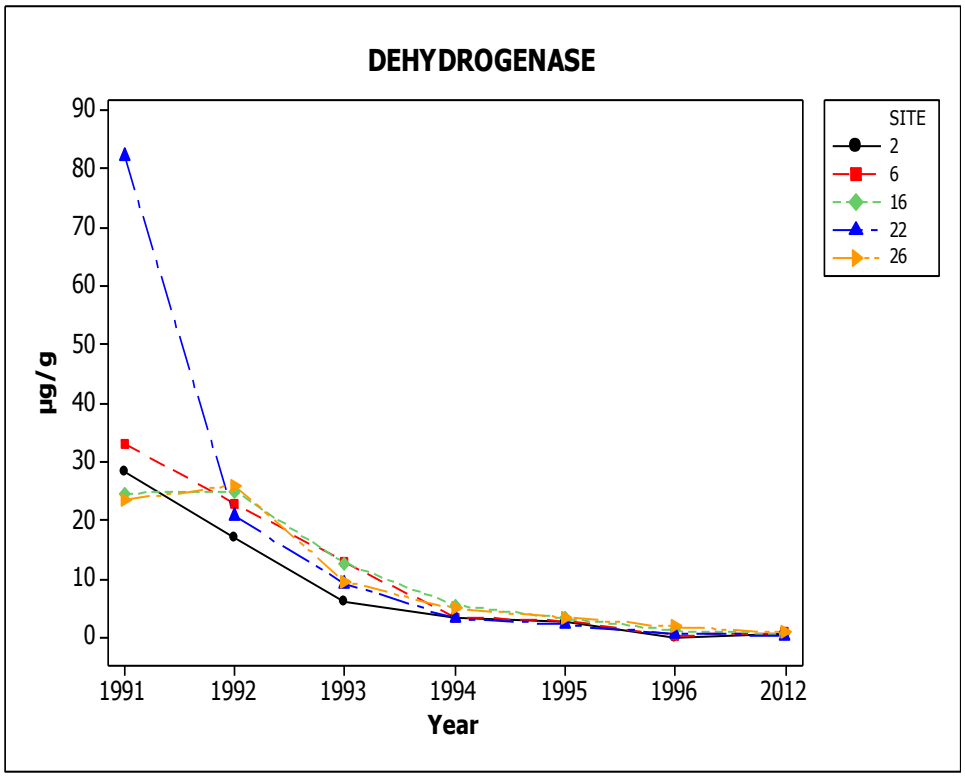

Figure 11: Time Series Plot of DHA across Years

Dehydrogenase levels were significantly different between all years except 1991 versus 1992, 1991 versus 1993, and 1996 versus 2012. Dehydrogenase levels have not recovered to before-construction levels.

Alkaline Phosphatase

Figures 12 and 13 illustrate time series plot of alkaline phosphatase across time periods and years.

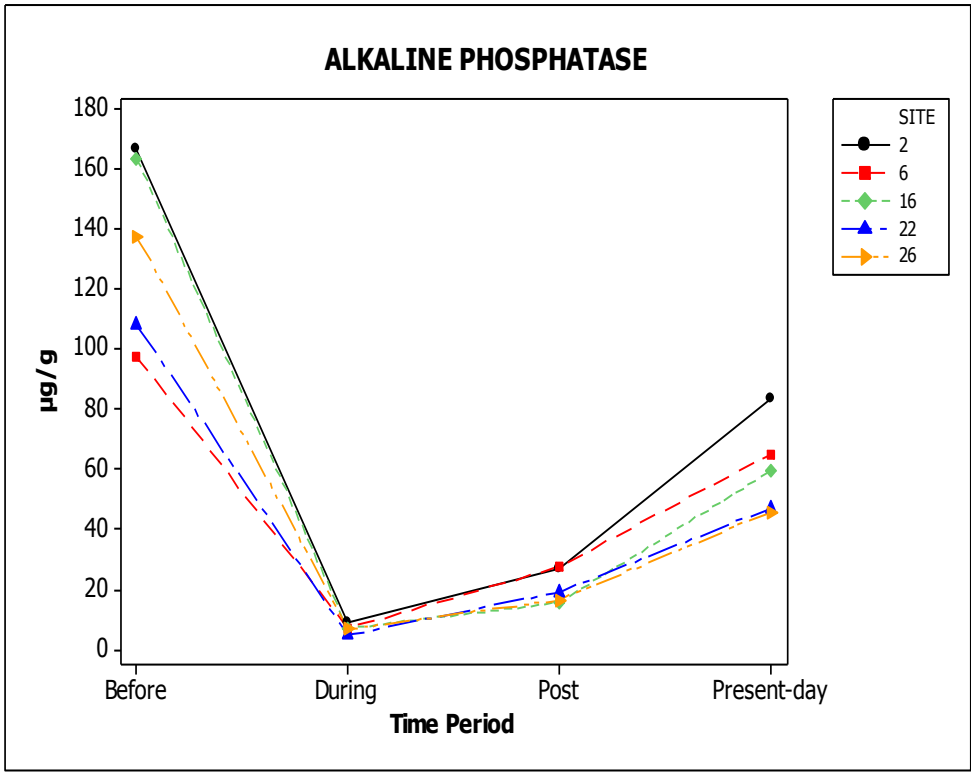

Figure 12: Time Series Plot of AP across Time Periods

Significant differences in Alkaline phosphatase activity were found between before-construction and during-construction (p=0.001), before-construction and postconstruction (p=0.002), before-construction and present-day (p=0.004), during-construction and postconstruction (p=0.003), during-construction and present-day (p=0.001), postconstruction and present-day (p=0.002). It appears that AP activities have increased slightly since before construction, but have not recovered to the levels that existed before construction.

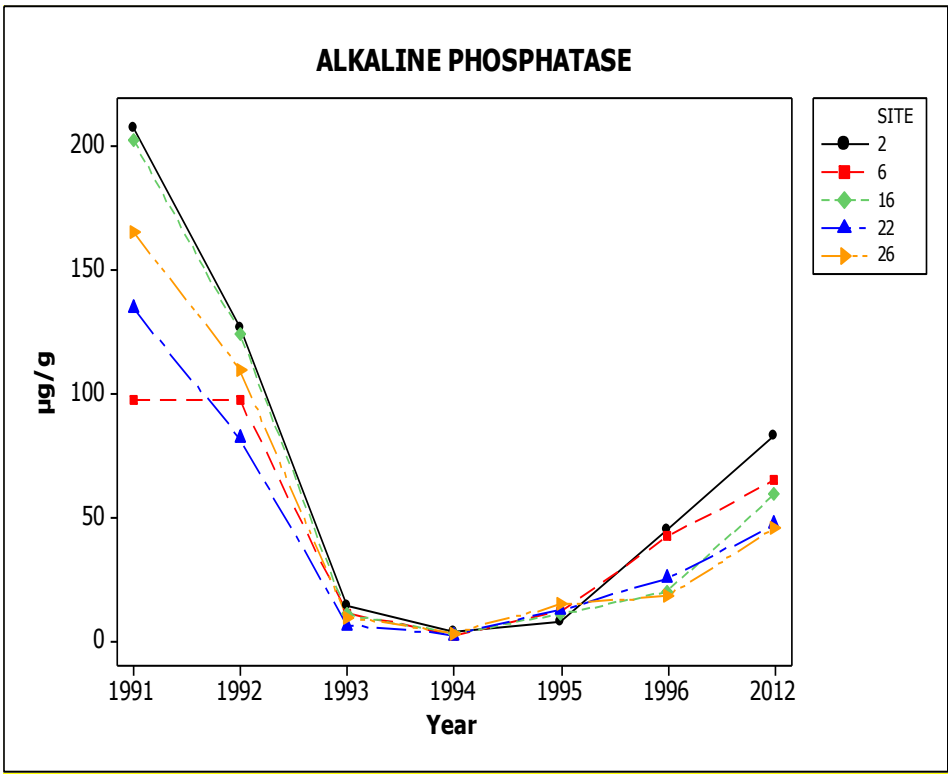

Figure 13: Time Series Plot of AP across Years

Alkaline phosphatase levels were significantly different between all years except 1993 versus 1995. It appears that present-day alkaline phosphatase levels have recovered slightly but have not reached before-construction levels.

Acridine Orange Direct Counts (AODCs)

Acridine Orange Direct Counts in Water

Figures 14 and 15 illustrate time series plot of acridine orange direct counts for water across time periods and years.

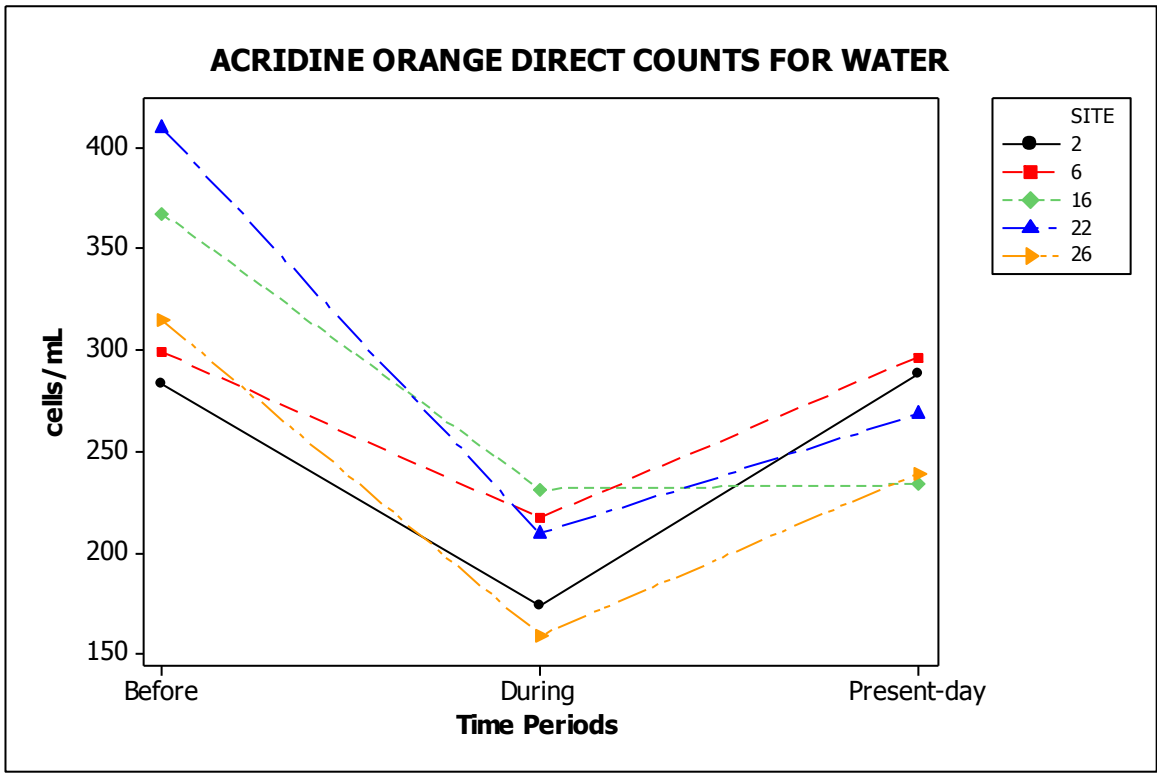

Figure 14: Time Series Plot of Acridine Orange Direct Counts for Water across Time Periods

There is a significant difference between before-construction and during-construction AODCW (p=0.002) and between during-construction and present-day AODCW (p=0.022). There is no significant difference between before-construction and present-day AODCW (p=0.088), which indicates that AODCW has recovered to before-construction levels.

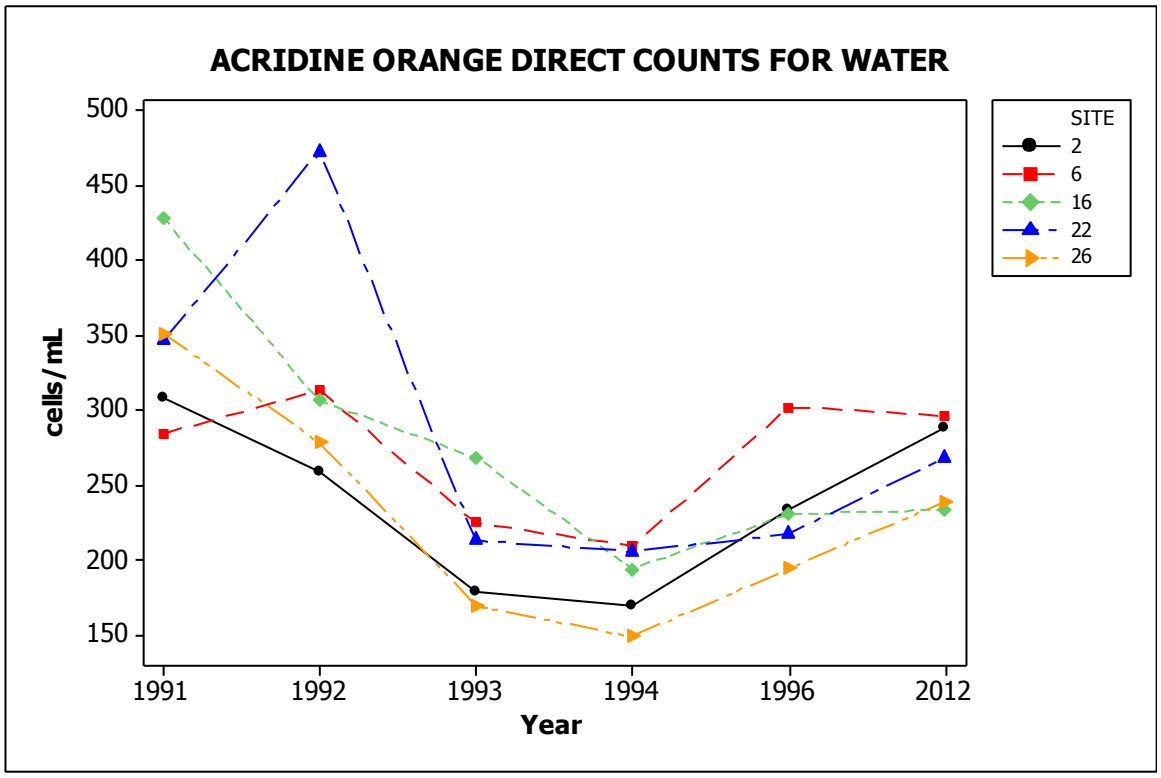

Figure 15: Time Series Plot of Acridine Orange Direct Counts for Water across Years

There were significant differences in AODCW in all of the years except for 1991 versus 1992, 1991 versus 2012, 1992 versus 1996, 1992 versus 2012, and 1996 versus 2012. It appears that AODCW has recovered to levels in proximity with before-construction levels.

In order to evaluate for the effects of discharge (width x depth x flow), total mass (bacteria counts x discharge) was calculated using 1994 and 2012 data because there was not enough flow and/or AODCW data from the other years. There was no significant difference of total mass using AODCW between 1994 and 2012 ($p=0.174$). This indicates that the effects of discharge and flow are null; however, there is a not a sufficient amount of comparable data to confirm this conclusion.

Acridine Orange Direct Counts in Sediment

Figures 16 and 17 illustrate time series plot of acridine orange direct counts for sediment across time periods and years.

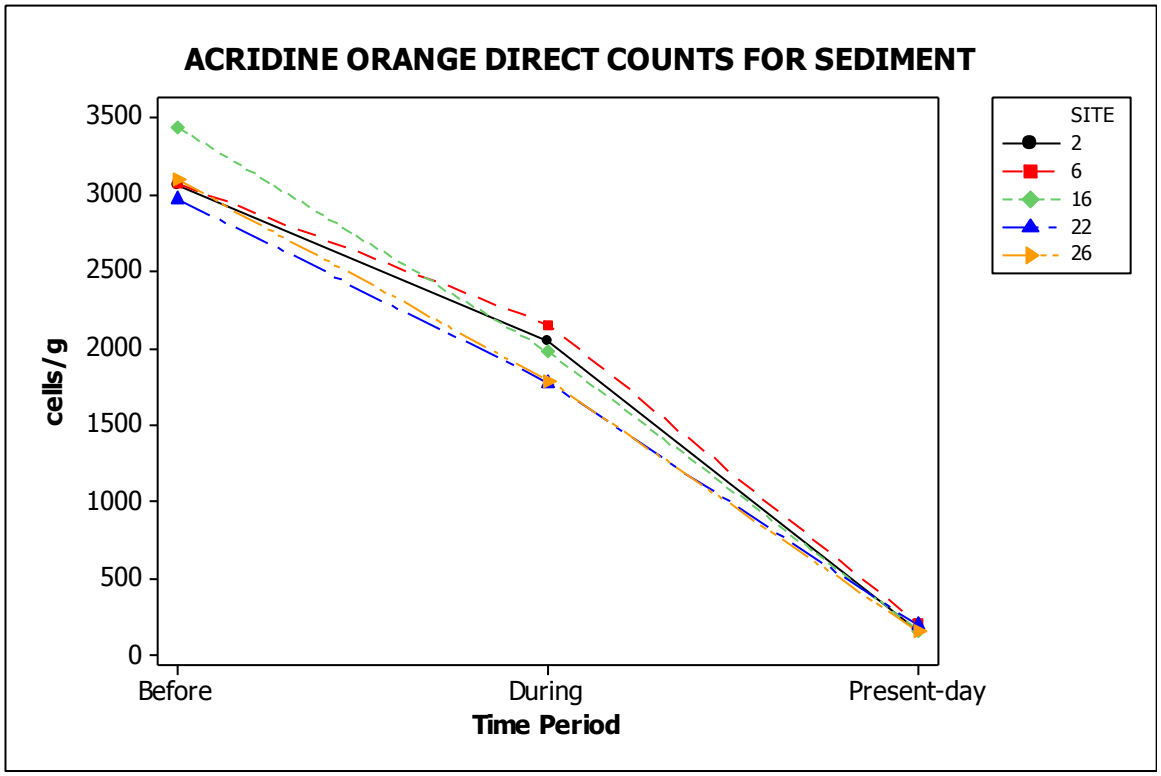

Figure 16: Time Series Plot of Acridine Orange Direct Counts for Sediment across Time Periods

Acridine Orange Direct Counts in Sediment- There is a significant difference between before-construction and during-construction AODCS (p=0.000), between before-construction and present-day AODCS (p=0.000), and between during-construction and present-day AODCS (p=0.000). This indicates that AODCS have not recovered to before-construction levels.

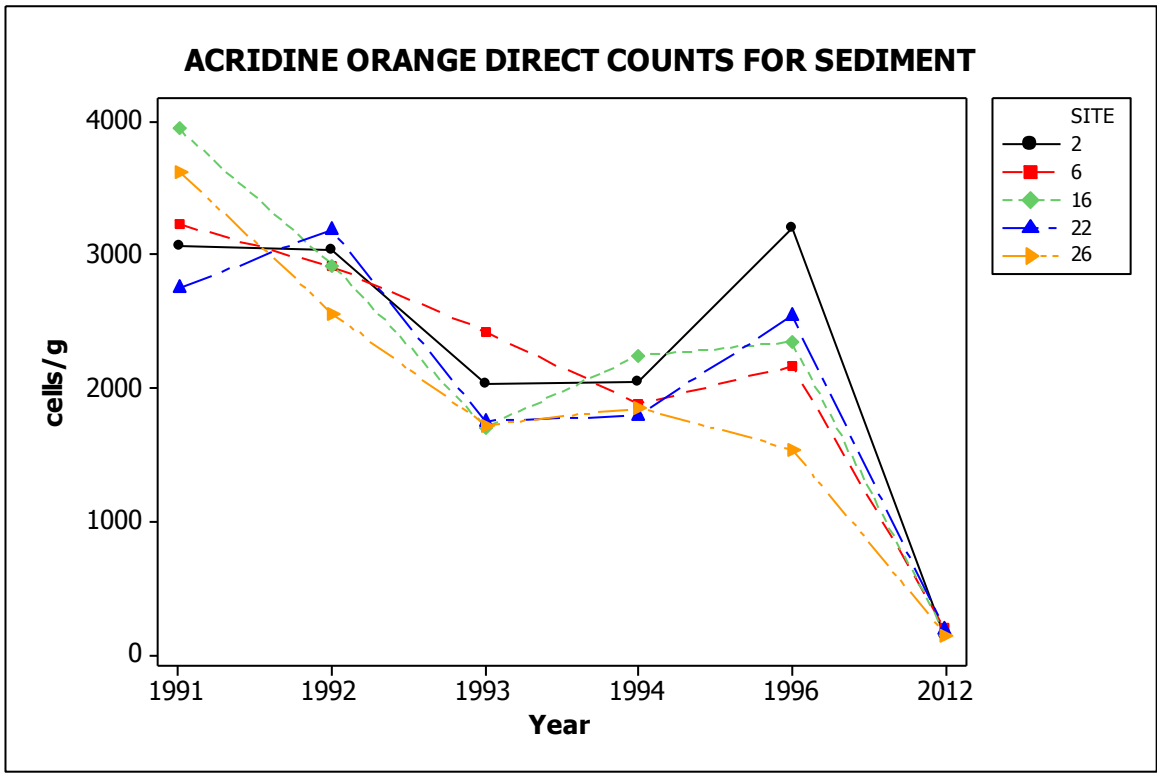

Figure 17: Time Series Plot of Acridine Orange Direct Counts for Sediment across Years

Significant differences were between all years except for 1991 and 1992, 1993 and 1994, 1993 and 1996, and 1994 and 1996. Present-day levels of AODCS are significantly different from all of the years and are of lower levels than all previous years tested.

Tables 5 and 6 include the significance values between time periods and years.

Table 5: *Significance Values between Time Periods*

Significance values at alpha=0.05	**GAL**	**GLU**	**DHA**	**AP**	**AODCW**	**AODCS**
Before vs. During	0.000	0.023	0.014	0.001	0.002	0.000
Before vs. Post	0.000	0.022	0.006	0.002	---	---
Before vs. Present-day	0.000	0.000	0.005	0.004	0.088	0.000
During vs. Post	0.559	0.133	0.001	0.003	---	---
During vs. Present-day	0.221	0.000	0.001	0.001	0.022	0.000
Post vs. Present-day	0.276	0.000	0.004	0.002	---	---

Table 6: *Significance Values between Years*

Significance values at alpha=0.05	**GAL**	**GLU**	**DHA**	**AP**	**AODCW**	**AODCS**
1991 vs. 1992	0.000	0.054	0.243	0.021	0.703	0.236
1991 vs. 1993	0.000	0.028	0.068	0.002	0.003	0.008
1991 vs. 1994	0.000	0.041	0.040	0.002	0.005	0.001
1991 vs. 1995	0.000	0.031	0.035	0.002	---	---
1991 vs. 1996	0.000	0.037	0.029	0.004	0.044	0.080
1991 vs. 2012	0.000	0.078	0.028	0.006	0.096	0.000
1992 vs. 1993	0.002	0.001	0.000	0.000	0.039	0.003
1992 vs. 1994	0.256	0.021	0.000	0.000	0.012	0.002
1992 vs. 1995	0.012	0.002	0.000	0.000	----	---
1992 vs. 1996	0.844	0.054	0.000	0.001	0.106	0.045
1992 vs. 2012	0.014	0.012	0.000	0.002	0.198	0.000
1993. vs. 1994	0.066	0.011	0.007	0.002	0.108	0.840
1993 vs. 1995	0.086	0.011	0.004	0.624	---	---
1993 vs. 1996	0.274	0.008	0.001	0.017	0.287	0.198
1993 vs. 2012	0.978	0.000	0.001	0.001	0.087	0.000
1994 vs. 1995	0.312	0.187	0.015	0.002	---	---
1994 vs. 1996	0.777	0.336	0.000	0.009	0.021	0.197
1994 vs. 2012	0.131	0.000	0.001	0.001	0.004	0.000
1995 vs. 1996	0.406	0.008	0.001	0.045	---	---
1995 vs. 2012	0.280	0.000	0.000	0.004	---	---
1996 vs. 2012	0.310	0.001	0.555	0.001	0.082	0.001

Between Time-Period Differences in Other Environmental Parameters

Figures 18 and 19 illustrate the National Oceanic and Atmospheric Administration Annual Means of precipitation and air temperature for Erwin, TN.

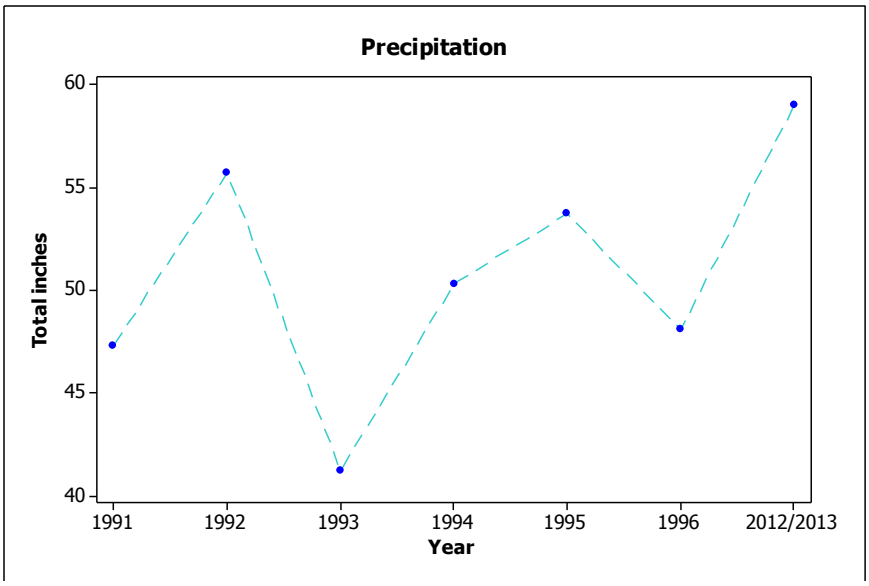

Figure 18: Time Series Plot of NOAA Total Precipitation across Years

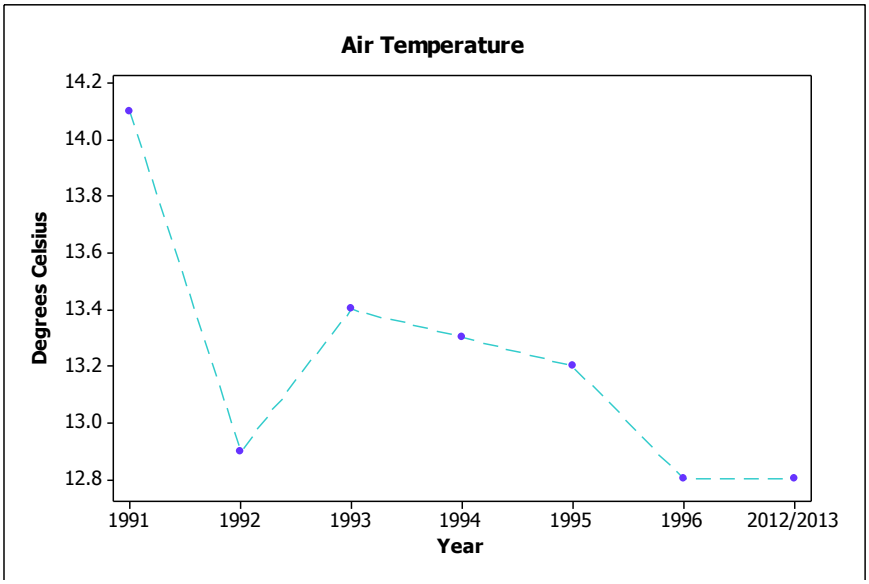

Figure 19: Time Series Plot of NOAA Air Temperature across Years

Data for the above time series plot of air temperature (in degrees Celsius) and total precipitation (in inches) is from National Oceanic and Atmospheric Administration's Climatological Summary of annual and monthly means taken from Erwin, TN, the closest monitoring station to the South Indian Creek Sites. Temperature appears to have decreased since 1991 with 1996 and 2012/2013 with the lowest averages. It appears that 2012/2013 had the highest amount of precipitation of all the time periods, closest to the 1992 average.

Air Temperature

Figures 20 and 21 illustrate the time series plot of air temperature across time periods and years.

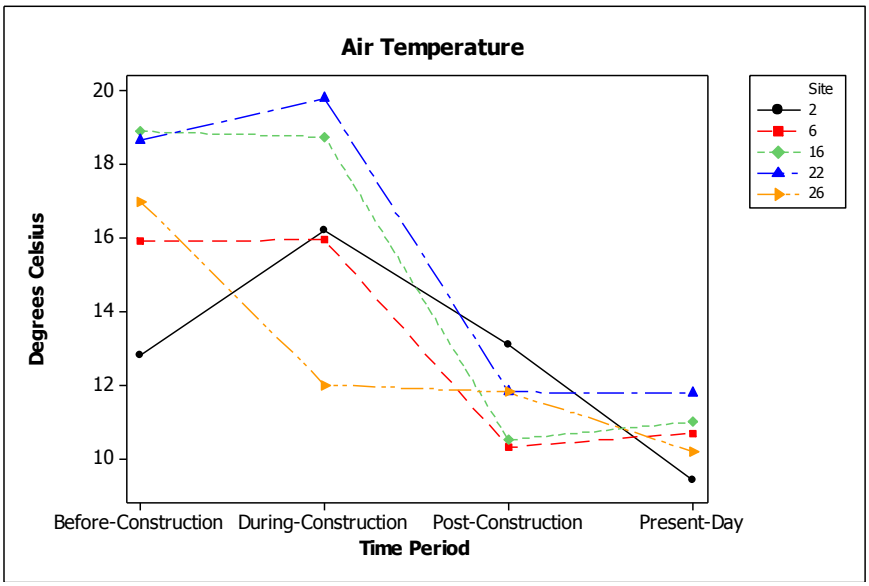

Figure 20: Time Series Plot of Air Temperature across Time Periods

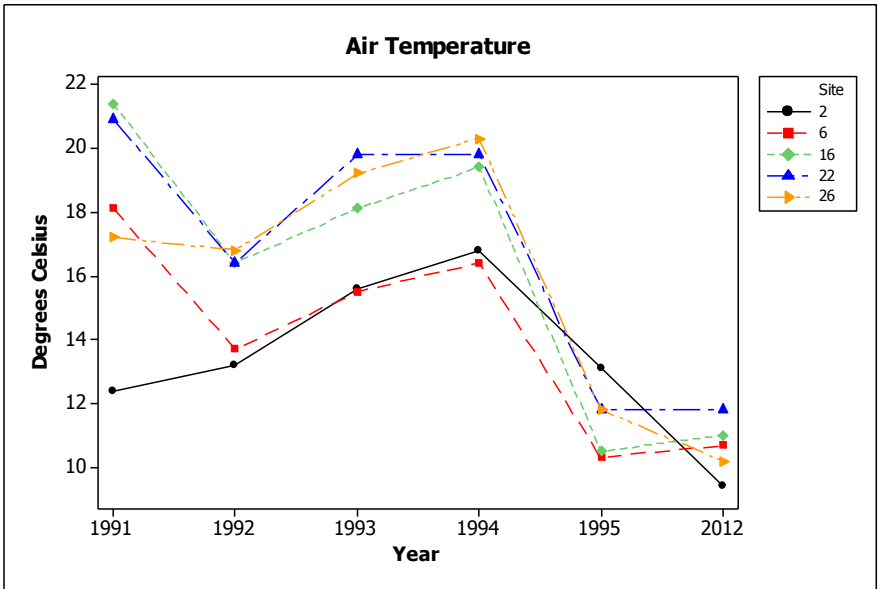

Figure 21: Time Series Plot of Air Temperature across Years

Air temperature means plotted by time period and site show an overall decrease in air temperature at all sites across time. This is similar to temperature averages taken from NOAA.

Water Temperature

Figures 22 and 23 illustrate the time series plot of water temperature across time periods and years.

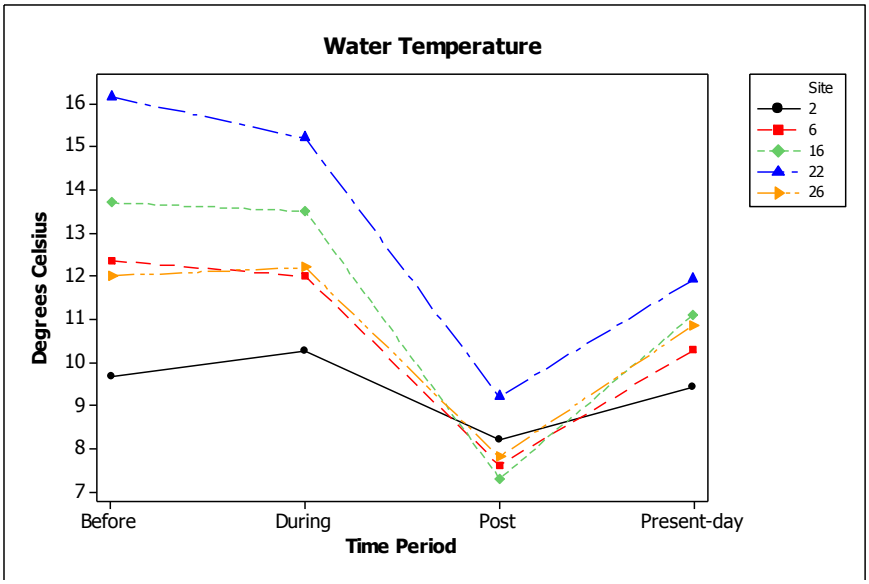

Figure 22: Time Series Plot of Water Temperature across Time Periods

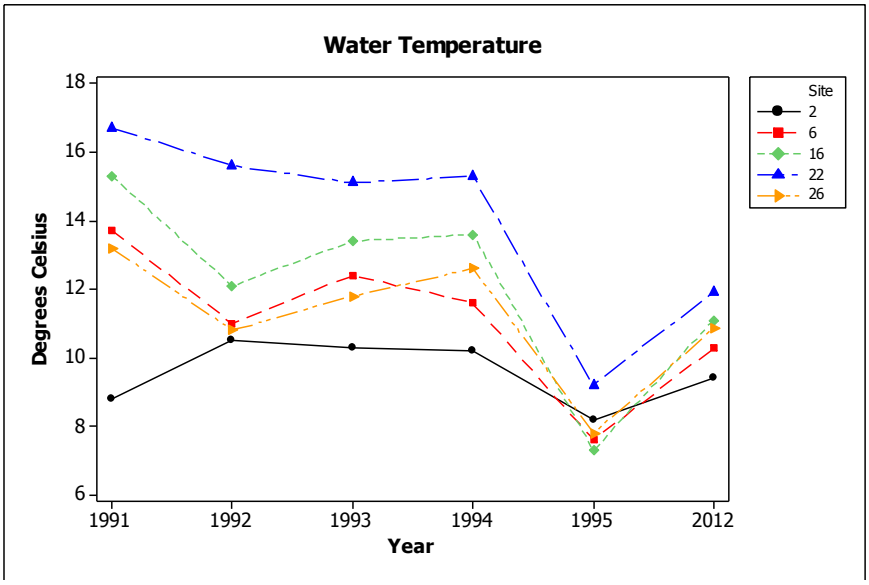

Figure 23: Time Series Plot of Water Temperature across Years

Water temperature means plotted by time show a sharp decline from during construction to postconstruction, followed by an increase in 2012-2013.

pH

Figures 24 and 25 illustrate the time series plot of pH across time periods and years.

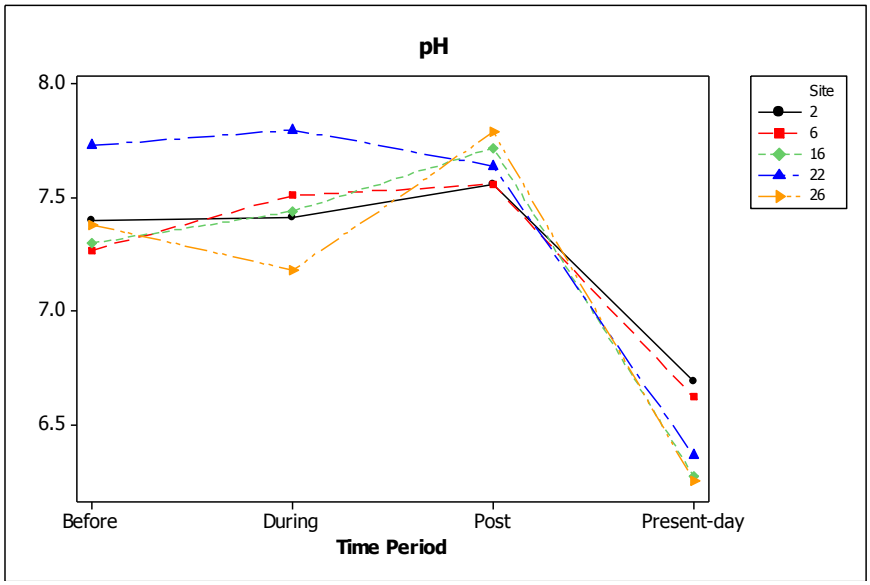

Figure 24: Time Series Plot of pH across Time Periods

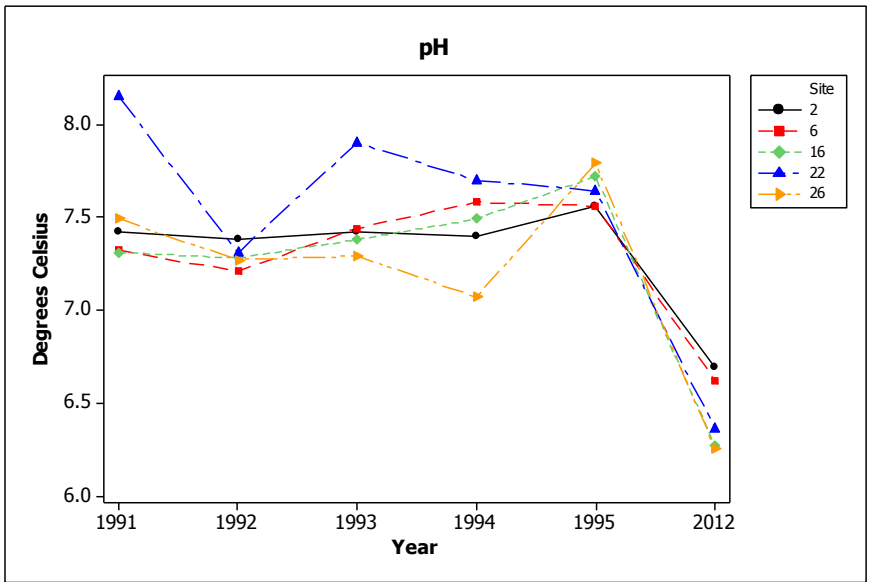

Figure 25: Time Series Plot of pH across Years

Present-day pH levels have significantly decreased from before-construction, during-construction, and after-construction pH levels.

Dissolved Oxygen

Figures 26 and 27 illustrate the time series plot of dissolved oxygen across time periods and years.

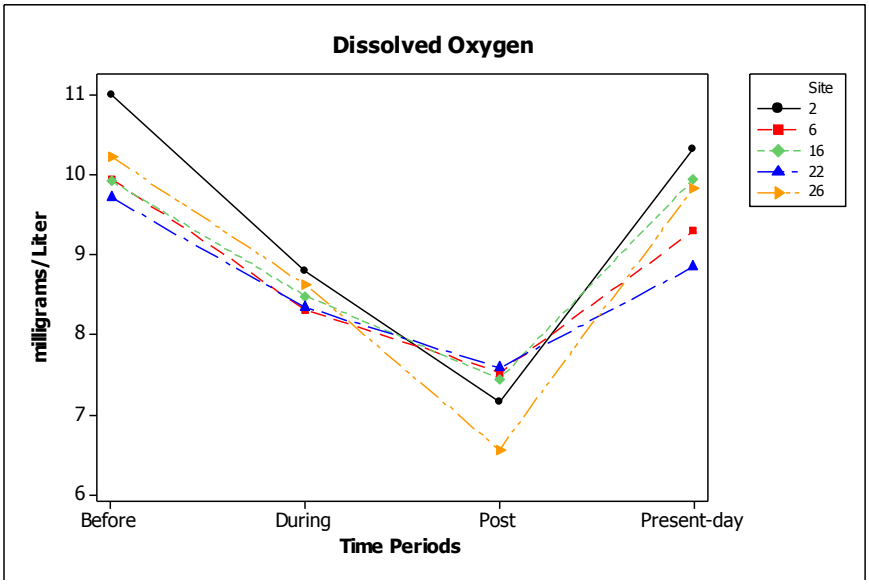

Figure 26: Time Series Plot of Dissolved Oxygen across Time Periods

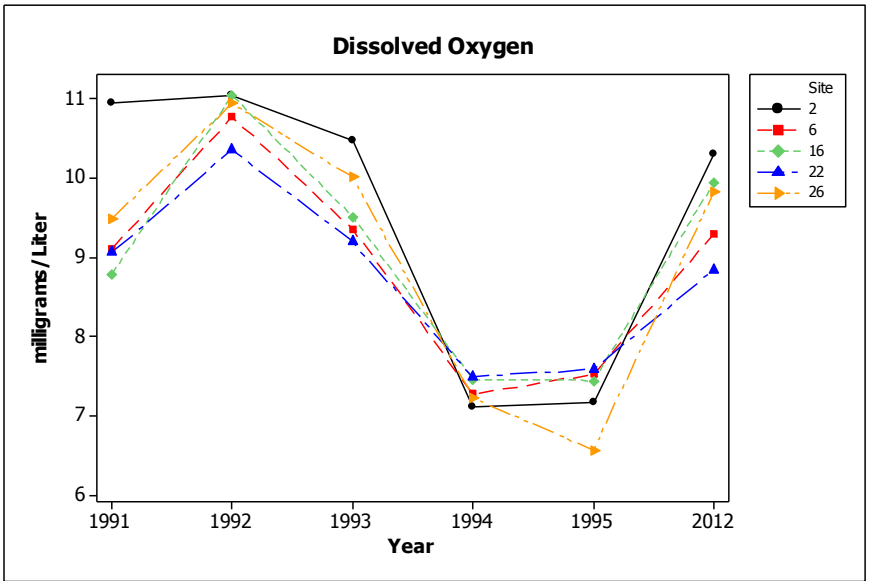

Figure 27: Time Series Plot of Dissolved Oxygen across Years

Dissolved Oxygen has increased to before-construction conditions.

Conductivity

Figures 28 and 29 illustrate the time series plot of conductivity across time periods and years.

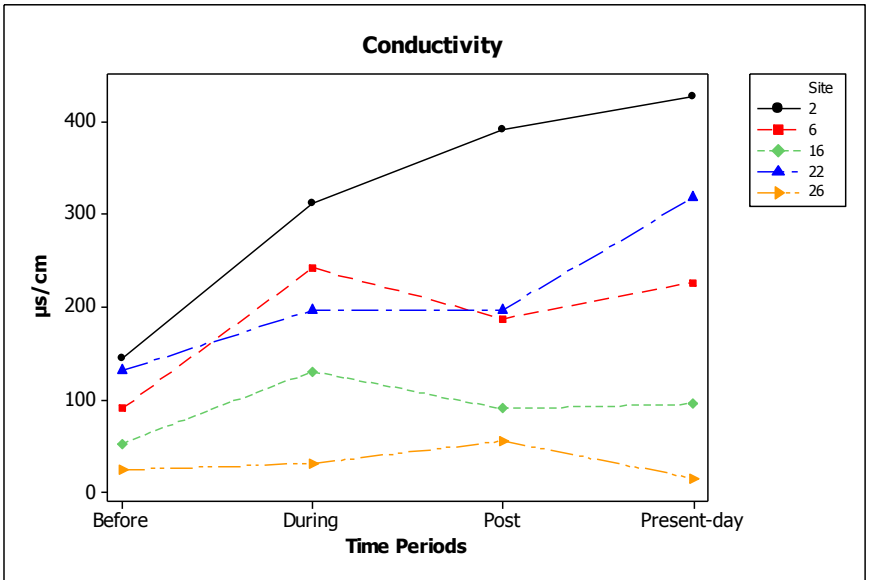

Figure 28: Time Series Plot of Conductivity across Time Periods

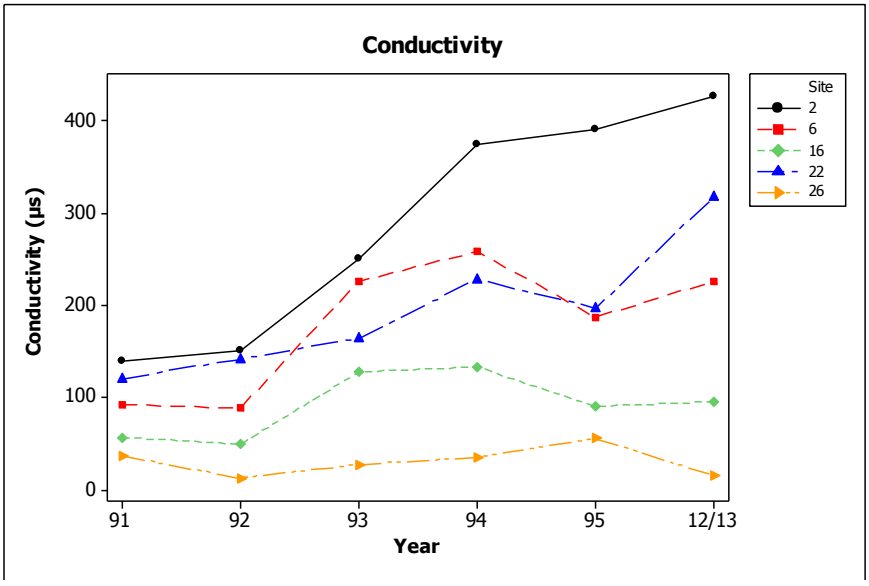

Figure 29: Time Series Plot of Conductivity across Years

Conductivity levels have increased across time. Site 2, not surprisingly, has the highest conductivity. Site 2 is the most upstream site sampled, and conductivity was greatest during the de-icing winter season. Salts and other ions have negatively affected the conditions of site 2.

CHAPTER 4
DISCUSSION

Since the construction of Interstate 26 between Tennessee and North Carolina, travel is more efficient than the small, winding highway that was previously the major link between the two states. South Indian Creek, which is the major drainage system for Unicoi County, Tennessee, meanders along the interstate. Because of the mountainous terrain and steep slopes in the area, runoff from the interstate continuously feeds into South Indian Creek. Because the interstate construction was an extensive alteration of the area, the landscape and its dynamic interaction with environmental factors will never be the same as before-construction.

The sites in this study are all headwater streams that are heavily influenced by riparian vegetation and have little buffering capacity. Habitat assessments have shown that many of the sites lack sufficient deep-rooted vegetation for support and prevention of erosion. The sites are at the foot of sloped hills and are in direct contact with runoff from roads/Interstate 26, farms, and residential neighborhoods. Excess nutrients, toxic agents, and sediments flow freely across impervious surfaces into the streams. These all affect the degree of recovery of the stream because aquatic microorganisms are sensitive to these changes and alter their enzyme activity in response.

South Indian Creek was negatively affected by the interstate construction activities (Scheuerman et al., 1999; Gu, S.,1996). More recent data collection and analyses uphold past studies and illustrate that all biological parameters dropped during the height of construction activity. Present-day conditions show that water quality conditions for most parameters have not recovered and that there are long-term impacts from the interstate construction. Galactosidase

activity, dehydrogenase activity, and acridine orange direct counts for sediment have not recovered. Glucosidase activity, acid phosphatase activity, and acridine orange direct counts for water, however, have recovered to levels in proximity with before-construction conditions.

The year 2012/2013 had the highest amount of precipitation of all the years, closest to the 1992 and 1995 averages. Precipitation is an important determination of how toxicants are removed from the system. Higher precipitation can help flush out the excess toxic materials. However, increased precipitation may also introduce large amounts of toxicants, organic matter, and nutrients into the system. As the ecological stoichiometric theory suggests, when the ratio of carbon: nitrogen: phosphorus: sulfur changes (typically around 100:10:1:1), microbial metabolism and activity alter in response.

Severe precipitation events can scour the sediments and dissemble habitats for microorganisms. This may cause a temporary decline in microbial population growth. Present-day pH levels have significantly decreased from before-construction, during-construction, and after-construction pH levels. This may be due to the higher amount of precipitation in 2012/2013 and may indicate larger concentrations of acid deposition. Acid leaching from pyrite may also explain the lower pH values and may indicate failing pyrite encapsulation and long-term mitigation strategies. The increase in conductivity levels in 2012/2013 supports the fact that pyrite might be leaching because of the increased ion levels in the water. Salt used on the roads and interstates during the winter might also explain the increase.

Regional environmental conditions show that average air temperatures have decreased with time. Air temperatures measured from past and present-day EHSL studies are in accord with the National Oceanic and Atmospheric Administration. The years 1996 and 2012/2013 had the lowest averages. The lower air temperature is typically associated with lower water

temperature; however, water temperature appears to have increased since 1995 while the difference between the air temperatures since 1995 is insignificant. Runoff from the interstate and other impervious surfaces may be responsible for the increase in water temperature, especially because precipitation was highest during 2012/2013. Loss of riparian vegetation and canopy cover may also explain the decline in water temperature. Warmer water temperature is associated with lower dissolved oxygen, but DO has increased since 1995, as well as water temperature. The solubility of oxygen, however, changes with atmospheric pressure and salinity as well as temperature.

The difference in response of the biological parameters may be attributed to various environmental conditions because microbial growth is dependent on other microbial populations, nutrients, oxygen, pH, temperature, and water and sediment composition. The increase in acridine orange direct counts in water implies an increase in bacteria in the water. Larger water column bacterial populations may be attributed to runoff from nearby agricultural land and/or failing septic tanks. The decrease in acridine orange direct counts in sediment suggests that perhaps the sediments have been scourged by heavy erosion and habitats are not available for the bacterial growth. The larger precipitation averages in 2012/2013 may be responsible, especially if these events were severe and washed away sediments.

The results indicate that Galactosidase activities have not recovered to before-construction levels. Before-construction GAL activities are significantly different from all following years and levels are approximately the same during-construction, postconstruction, and present-day. GAL is involved in metabolism of lactose, so the lower GAL activity may be indicative of the loss of riparian vegetation and canopy cover. The decrease in pH, the increase in

conductivity, the increase in precipitation (which may cause increased runoff from impervious surfaces), and the increase in water temperature may also explain the lack of recovery.

Present-day Glucosidase activities have increased to the proximity of before-construction activities. The increase in GLU activity typically indicates bacterial abundance, heterotrophic uptake of glucose, and bacterial production. However, the sediment bacterial counts dropped while GLU increased, so recovery may be attributed to organic carbon loading, which would provide more substrate per cell. Galactosidase declined because of loss of vegetation, while Glucosidase increased, suggesting that there are more degraded forms of carbon entering the stream, such as from soil.

Present-day Alkaline Phosphatase activities have increased to the proximity of before-construction levels. Between the years 1993-1996, alkaline phosphatase activities were extremely low, suggesting the activity was repressed by inorganic phosphate, such as from fertilizer use. Lower pH would also repress the activity of AP (Chrost), but data shows a lower pH in 2012, when alkaline phosphatase levels appear to recover. The increase in phosphatase activity indicates that inorganic phosphorus is the limiting nutrient. Alkaline phosphatase activity increases in order to scavenge more phosphate from organic phosphate molecules. This suggests a healthy stream, one that is not repressed by nutrient loading such as by fertilizers.

Before-construction Dehydrogenase activities are significantly higher than all following years, which indicates that the DHA has never recovered from the interstate construction. DHA levels are approximately the same during-construction, postconstruction, and present-day. The decrease in dehydrogenase activity indicates a decrease in aerobic metabolic activity, or total respiration as a rate of oxygen uptake and carbon dioxide production. Insufficient oxygen and/or carbon may cause a decline in DHA. Because dissolved oxygen has increased to before-

construction conditions, it would suggest an increase in dehydrogenase activity. However, dehydrogenase is also dependent on a wide variety of environmental parameters. The lack of recovery of dehydrogenase activity may be explained by lack of sediments for microbial growth and metabolism, the decrease in pH, the increase in conductivity, the increase in precipitation, and the increase in water temperature.

The multivariate analysis of variance (MANOVA) determined that there was not a significant difference between sites. This would indicate that the reference site and the sampling sites all responded the same and perhaps climatic variables are at play such as drought, flood, and climate. There is also the possibility that microbial enzyme activities are not a sensitive indication for environmental conditions.

Overall, South Indian Creek has not fully recovered to levels before construction of Interstate 26. Some parameters seem to have increased to levels in proximity with before-construction, while others declined during the height of construction and never recovered. The interstate construction altered the landscape of the Appalachian Mountains and its effects on the water nearby are still evident. But because the interstate is not the only alteration of the land (failing septic tanks, agriculture, residential neighborhoods), the interstate may not be entirely responsible for the lack of recovery. Numerous environmental parameters are at play and microorganisms alter metabolism in order to proliferate.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The construction of Interstate 26 caused adverse impacts on the water quality of South Indian Creek. All biological parameters dropped during the height of construction activity. Present-day data analyses determined that some parameters did not recover to levels from before-construction. The sites in the study are all headwater streams in mountainous terrain with little buffering capacity. Because many of the sites lack deep-rooted vegetation for prevention of erosion, they are in direct contact with runoff from roads/Interstate 26, farms, and residential neighborhoods.

Microbial enzyme activities are sensitive to a variety of parameters and provide a glimpse of the ambient environmental conditions. Galactosidase activity, dehydrogenase activity, and acridine orange direct counts for sediment have not recovered to the same levels before interstate construction activity. Glucosidase activity, acid phosphatase activity, and acridine orange direct counts for water, however, have recovered to levels in proximity with before-construction conditions.

Because South Indian Creek is a flowing water body with numerous interweaving relationships with physical, chemical, and biological parameters, it is difficult to determine the effect of a single environmental measure in the field. Runoff from the interstate and other impervious surfaces may be responsible for the increase in water temperature. The highest total precipitation averages were in the year 2012/2013. The decrease in acridine orange direct counts in sediment suggests that perhaps the sediments have been scourged by heavy erosion and habitats are not available for the bacterial growth. Severe precipitation events can also cause

large amounts of toxins, nutrient, and/or organic matter to enter into the stream. Present-day pH levels have significantly decreased from before-construction, during-construction, and after-construction pH levels. Because of the increase in precipitation averages, acid precipitation or acid leaching from pyrite may explain the lower pH values. Future studies might determine if the previous pyrite encapsulation and other mitigation strategies are still effective for prevention of acid leaching into South Indian Creek.

As the ecological stoichiometric theory suggests, when the ratio of carbon: nitrogen: phosphorus changes, microbial metabolism and activity alter in response. This may have played an essential role in the response of the enzyme activities. For instance, alkaline phosphatase dropped substantially during the height of construction, which suggests an increased input of inorganic phosphate caused repression of the enzyme activity.

Future studies may include more sampling sites and dates to gain more information about the area and environmental dynamics involved. One might also attempt to determine the source of the pollution by measuring various heavy metals associated with the roads, phosphates and nitrates associated with agriculture, and detergents and personal care products associated with residential runoff.

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APPENDICES

Appendix A

Standard Operating Procedure for Detailed Field Sampling

1. APPLICATION:

The application of this SOP is to provide a standard procedure for details of water analyses and sampling in the field.

2. SUMMARY OF METHOD:

The procedures for the standard field analyses and sample collection at designated sample sites is outlined. Field measurements include pH, conductivity, dissolved oxygen, water and air temperatures.

3. SAFETY:

3.1 For depth measurements and width measurements, the person entering the water should wear a lifejacket. It is also advisable to have a person on the bank to wear a lifejacket in case the person in the water would require assistance.

3.2. Refer to EHSL safety manual for general laboratory safety procedures.

4. APPARATUS AND EQUIPMENT:

4.1 Whirl-Paks, 4 oz (120 mL) with and without sodium thiosulfate, and/or sterile plastic sample bottles (500 mL or 1 L)

4.2 Cooler with ice or freezer pack to maintain samples from the field

5. CALIBRATION PROCEDURES:

5.1 Each instrument will be calibrated before leaving the lab. The procedure for calibrating the field pH meter, the DO meter, and the conductivity meter are available in EHSL-SOP . All data will be recorded in a field logbook at the time of collection.

6. CHEMICALS AND REAGENTS:

6.1 Ensure that all glassware and other lab apparatus involved with this procedure are clean and nontoxic. Use only detergents that are designed for microbiological work.

7. PROCEDURES:

7.1 Dissolved Oxygen

9.1.1 The dissolved oxygen will be measured with the **YSI Model 57 Oxygen Meter**. Turn switch to Red line and adjust Red line dial until meter is in line with the red line on the scale. Turn switch to zero and turn zero dial until meter reaches zero. Make sure salinity dial is set on fresh (0). Immerse probe in water and read DO from 0-20 scale.

7.2 Conductivity

7.2.1 Switch to x100 If the reading is below 50 on the 0-500 range (5.0 on the 0-50 mS/m range), switch to x10. If the reading is still below 50 (5.0 mS/m), switch x1 scale. Read the meter scale and multiply the reading appropriately. The answer is expressed in micromhos/cm (mS/m). Measurements are not temperature compensated.

7.3 pH

7.3.1 pH will be determined using the model ESD pH 59. Measure pH after water temperature has been determined. To measure pH immerse probe in water and turn unit on. Adjust temperature knob to corresponding water temperature and then allow pH to stabilize and record reading. The pH meter should be recalibrated at station using a one point calibration (pH 7.00 buffer) because the standard knob may be moved during travel.

7.4 Temperature Measurements

7.4.1 Water and air temperature measurements will be determined at each sample site using an alcohol thermometer. The air temperature will be taken first then the water temperature.

7.5 Depth

7.5.1 Three depth measurements will be taken at the sample site using a meter stick. These measurements will be taken at approximately 1/4, 1/2, and 3/4 distances across the sample site.

7.6 Width

7.6.1 The width of the stream will be taken at the point of sample collection. Appropriate safety precautions should be observed.

8. REFERENCES:

8.1 APHA, AWWA, and WPCF. 1992. Standard Methods for the Examination of Water and Wastewater, 18th edition. A.E. Greenberg, L.S. Clesceri, and A.D. Eaton, Eds. American Public Health Association, Washington, D.C.

8.2 U.S. Environmental Protection Agency. 1999. National Primary Drinking Water Regulations. U.S. Code of Federal Regulations, 40 CFR, Part 141, Subpart C, Section 141.21, Washington, D.C.

8.3 U.S. Environmental Protection Agency. 1997. Manual for the Certification of Laboratories Analyzing Drinking Water. EPA 815-B-97-001. Office of Groundwater and Drinking Water, Cincinnati, OH.

Appendix B

Standard Operating Procedure for Ion Chromatograph for Total Phosphorus

1. Application:

The application of this SOP is to provide a standard operating procedure for setting up and operating the Ion Chromatography (IC) to determine the concentrations of either cations or anions in a given sample.

2. Summary of Methods:

Introduce samples into a mobile phase, usually a liquid or gas, so the sample can be separated into components. The sample is passed through a matrix which results in separation based on different migration of the ions. In order for the samples to pass through the matrix they must first be mixed with an eluent. The eluent is an aqueous solution consisting of ionic salts. The eluent serves to stabilize the sample ions, provide kinetic flow through system, and provide counter ions to compete with the sample ions for active site on the stationary phase. The matrix is contained inside a column that receives the mobile phase (sample and eluent) and allows the compounds to pass through at different speeds base upon their individual physiochemical characteristics.

3. Interferences:

Refer to Principles and Troubleshooting Techniques in Ion Chromatography,

Dionex Corporation, January 2002. Document No. 065035. p 28-54.

4. Sample Collection, Preservation and Handling:

Samples must be collected, labeled, and handled according to department procedure. Samples must be stored at 4°C and analyzed within 24 hours of collection.

5. Safety:

Refer to EHSL Safety Manual for general laboratory safety procedures.

6. Apparatus and Equipment:

6.1 Ion Chromatograph- An analytical system capable of performing IC separations using conductivity detection.

6.2 Ion Chromatography Autosampler- fully prepares samples for IC once loaded.

6.3 IC Autosampler Sample Trays- small trays that hold sample for IC preparation.

6.4 IC 5 ml sample vials.

6.5 Filter Caps for 5 ml vials.

6.6 Eppendorf pipet 100µl-1000µl.

6.7 Eppendorf pipet tips.

6.8 Erlenmeyer flask 2L- for eluent waste.

6.9 Eluent Reservoir 2L.

6.10 Data System- desktop P.C. with software capable of controlling IC and Autosampler.

7. CALIBRATION PROCEDURES:

7.1. Standards (see section 8) are made and ran with sample to ensure accurate measurements.

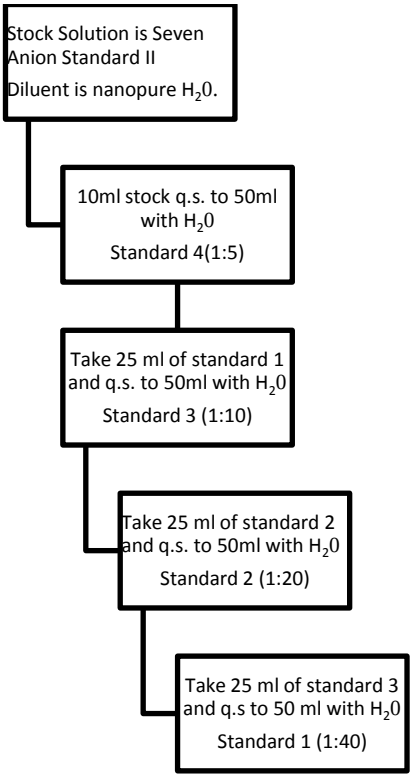
8. Chemicals and reagents:

8.1 Seven Anion Standard II (use if testing for anions only).

8.1.1 Use Seven Anion Standard II to make standards to run with each batch. To make standards follow the chart below with the diluent being nanopure H_20.

8.2 Eluent- Use eluent concentration bottle provided by manufacture and follow directions provided. The eluent used should be made from a .16 M sodium carbonate and .22 M sodium bicarbonate concentration.

Serial Dilution for Standards



9. Procedure:

9.1 Turn on computer, autosampler, and I.C. unit.

9.2 On the desktop of the computer is Chromeleon Icon double click with the mouse to start the program.

9.3 If samples site is already programmed into Chromeleon then skip steps 9.4 through step 9.12.3.

9.4 To program a new sample series click on the file tab, then click on new, and use the sequence (using wizard) option.

9.5 Click next to start the wizard. Then, click next again (Choosing a Timebase default setting should be used).

9.6 **Unknown Samples**

9.6.1 Select use template (default)

9.6.2 Under Template for Sample Name: Choose start blank.

9.6.3 Beside Number of vials choose the number correlating to unknown plus three (Q.C., Q.C. Spiked, and end blank).

9.6.4 Beside Injections per vial choose 3.

9.6.5 Beside Start position choose sample start number (count over to first sample and use that number, remember samples are done in triplicate and one vial is equal to three entries on the computer).

9.6.6 Beside Injection volume choose 20ml (default setting).

9.6.7 Click apply and then next to continue programming.

9.7 **Standard Samples**

9.7.1 Select use template (default setting)

9.7.2 Under Template for sample name, name the standards (names can be changed later.

9.7.3 Beside Number of vials choose number of standards that are made (usually 4) plus one for a start blank, so usually 5 total.

9.7.4 Beside injections per vial choose 3.

9.7.5 Beside start position choose 1.

9.7.6 Beside injection volume choose 20ml (default setting).

9.7.7 Click apply and next to continue.

9.8 **Methods and Reporting**

9.8.1 Click next (leave default settings)

9.9 **Saving the Sequence**

9.9.1 Under Name and Title, Name the sequence and title it.

9.9.2 Under Location, choose default settings.

9.9.3 Click finish and then done to complete sequence writing.

9.10 **Appling the Type and Name to Each Entry**

9.10.1 Under the Name Column on the sequence browser rename the first three entries to “Run in”.

9.10.2 Under the Type Column choose “blank” on No. 1,2,3.

9.10.3 Under the Name Column rename No. 4,5,& 6 “Blank”

9.10.4 Under the Type Column choose “blank” on 4,5,& 6.

9.10.5 Under the Name Column rename 7,8,& 9 “Std 1 (1:40)”.

9.10.6 Under the Type Column choose “standard” on 7,8,9.

9.10.7 Under the Name Column rename 10,11,12 “Std 2 (1:20)”.

9.10.8 Under the Type Column choose “standard” on 10,11,12.

9.10.9 Under the Name Column rename 13,14,15 “Std. 3 (1:10)”.

9.10.10 Under the Type Column choose “standard” on 13,14,15.

9.11.1 Under the Name Column rename 16,17,18 “Std 4 (1:10) ”.

9.11.2 Under the Type Column choose “standard” on 16,17,18.

9.11.3 Under the Name Column rename 19,20,21 “QC”.

9.11.4 Under the Type Column choose “validate” on 19,20,21.

9.11.5 Under the Name Column rename 22,23,24 “QC Spike” Note in the name which sample was used to spike the QC example: “QC Spike Buffalo 2”

9.11.6 Under the Type Column choose “spiked” for 22,23,24.

9.11.7 Under the Name Column rename 25,26,27 the first sample name and number. Example “Buf 1”

9.11.8 Under the Type Column choose “unknown” for 25,26,27.

9.11.8 Continue naming every three numbers with one sample and changing the type to unknown.

9.11.9 When all samples have been named, name the last six numbers as “End Blank”

9.12.1 On the end blanks change the type to blank.

9.12.2 On the last end blank, under the program column, change the program to shutdown.

9.12.3 At the top of the sequence page is a short list of the written program. Copy and past a shutdown program from a saved template to the last part of the sequence at the top of the page.

9.13 **Starting the Batch**

9.13.1 Fill the eluent bottle on top of the I.C with the liquid made in step 8.2. Then sonicate the eluent for a few seconds with a sonicator to degas. Then connect the bottle to the eluent tube on top of the I.C.

9.13.2 Place the empty labeled vials in the autosampler racks. Fill the 5ml vial with appropriate sample, standard, or Q.C. Fill the vials between the two markings on the racks.

9.13.3 Cap the vial with the filtercaps using the capping device.

9.13.4 Load the autosampler racks in the appropriate order in the autosampler according to the program that is being used.

9.13.5 Under the batch menu on the sequence browser select start.

9.13.6 When the start menu is displayed click the “ready check” option. Make sure all criteria are met under the ready check.

9.13.7 Click on start to begin the batch.

9.13.8 When the batch is finished click on each individual number on the browser screen to view results.

9.13.9 Click on each peak that has not been automatically named to manually name it.

9.14.1 Print the results after a total trend line has been established by the computer.

10. **Calculations:**

All calculations are performed by the Chromeleon program.

11. **Documentation:**

The results are automatically saved until the program is used again. A hardcopy should be obtained after each batch is completed with a trend line of the final results.

12. **Quality Assurance and Quality Control:**

12.1 Q.C should meet criteria and any failing Q.C should be reported according to department policies.

12.2 Preventative maintenance should be performed and a batch should be ran at least weekly to maintain hardware of I.C. unit.

12.3 Blanks should be run with every sample batch. Blanks must be less than the lowest reported limit for samples analyzed. If blanks fail criteria repeat unit acceptable.

12.4 A replicate sample should be with every batch.

Appendix C

Standard Operating Procedure for Analyzing Total Organic Carbon

1. APPLICATION:

The application of this SOP is to provide a standard operating procedure for the Elementar LiquiTOC analyzer for determination of total organic carbon (TOC) concentration. Assays determined by analyzer include total carbon (TC), total inorganic carbon (TIC), dissolved organic carbon (DOC), non-dissolved organic carbon (NDOC), purgeable organic carbon (POC), and non-purgeable organic carbon (NPOC).

2. SUMMARY OF METHODS:

The Elementar LiquiTOC analyzer uses a combustion oxidation method where a water sample is injected into a high temperature furnace containing platinum or a cobalt catalyst. An acid (HCl) is added to the sample to convert inorganic carbon into CO2 gas that is stripped out of the liquid by a sparge carrier gas. The remaining inorganic carbon-free sample is oxidized and the CO2 generated from this process is directly related to the TOC. All carbon materials are oxidized to CO2 which is pushed into a non-dispersive infrared detector (NDIR) via a stream of nitrogen, which yields a measurement of total carbon. A lower temperature furnace measures the TIC while the higher temperature furnace measures the TC. The TOC can be calculated by subtracting the TIC values from the TC values.

3. INTERFERENCES:

Any contact with organic material may contaminate a sample. Avoid contaminated glassware, plastic containers, and rubber tubing. The acidification and purging process can cause a loss of volatile organic substances. Larger carbon containing molecules could fail to be extracted by the needle for injection. Interference with the detection system may occur due to the gases evolved from combustion such as water, halide compounds, and nitrogen oxides.

4. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

Preserve samples that cannot be examined immediately by holding at 4 C with minimal exposure to light and atmosphere. Rinse sampling bottles with sample water prior to filling. Unstable samples may be acidified with phosphoric or sulfuric acid to a pH < or = 2 though acid preservation invalidates any inorganic carbon determination on samples.

5. SAFETY:

Refer to EHSL Safety Manual for general laboratory safety procedures. High temperature combustion furnaces are allocated within the analyzer, it is vital to keep analyzer door closed during operation. The acidification process utilizes hydrochloric acid (HCl), which is a non-flammable but highly corrosive acid. Inhalation of vapor can cause serious injury, ingestion may be fatal, and the liquid can cause damage to the skin and eyes. Analyzing

aggressive chemicals, acids, solvents, explosives, or materials that can form explosive gases is explicitly forbidden.

6. APPARATUS AND EQUIPMENT:

6.1. Elementar LiquiTOC analyzer – high temperature combustion technique with Infrared detector.

6.2. Autosampler with syringe for liquid sample injection, magazine for sample vials has 53 via positions.

6.3. PC with interface; status display, control and evaluation unit with Windows operating system, installed with LiquiTOC program.

6.4. Air Pump for synthesized air.

6.5 Printer

7. CHEMICALS AND REAGENTS:

7.1 Hydrochloric acid (HCl), 0.8 % dilution.

7.2 Nanopure H2O.

7.3 TOC, (KHP) Standard 500 mg/L (Potassium Phthalate).

7.4. TIC, Na2CO3 Standard 500 mg/L.

7.5. Preparing Parent Solution for Standard:

7.5.1. For routine analysis, a 1: 20 dilution will be used.

7.5.2. 5 ml of TOC Standard 500 mg/L put in volumetric flask.

7.5.3. 5 ml of TIC Standard 500 mg/L is added to flask.

7.5.4. Bring solution to 100 ml volume with Nanopure water.

8. CALIBRATION PROCEDURES:

8.1. TIC and TOC can either be calibrated by means of a mixed standard or a single standard, the mixed standard is recommended.

8.2. A higher concentration from one standard solution should be prepared. This parent solution should be diluted accordingly.

8.3. Multiple point calibration from one standard solution, the user is able to perform calibration from one standard parent solution. In general, working with at least a 5 point calibration and a triple determination per calibration point is recommended.

9. PROCEDURE:

9.1. Start-up and initialization

9.1.1. Switch on computer and wait for entire booting process.

9.1.2. Turn on the autosampler and wait for reference run to be completed.

9.1.3. Turn on the main switch to the LiquiTOC analyzer (located on right side panel) and wait until the entire initialization is complete. This involves allowing the syringe to go through a reference run to find the end position.

9.1.4. Start the LiquiTOC software and wait until completely set up for running.

9.1.5. Turn on the air pump and if necessary, plug in the CO2 removal unit. Check the secondary valve on the CO2 removal unit to be certain it is open. The gas pressure can now be adjusted to 1.0 – 1.10 bars on the screen, or on the pressure regulator.

9.2. Perform leak check prior to each analysis run or once a week with the Leak Check Wizard.

The leak check wizard is located under Options, Diagnostics ► Leak Check. Be sure to follow all on screen prompts correctly.

9.2.1. Leak Check Wizard Step 1: The pressure of the system is released. This takes a certain time. The process is displayed in a bar graph.

9.2.2. Steps 2 & 3: In these steps you will be instructed to remove the respective side wall of the analyzer in order to close a certain gas pathway by means of a clamp. The wizard shows you how and where this must be done.

9.2.3. Step 4: The gas will be reopened, thereby building up pressure again. The program will wait until a certain time until the pressure situations have been stabilized. This will be displayed in a bar graph.

9.2.4. Step 5: This is the actual leak test. The program checks whether the flow surpasses a certain "zero" threshold inside a defined time span. If this is the case then the test will be considered "passed." Otherwise the test will be considered "failed." The flow curve as well as the test results will be displayed and can be printed. If the leak test has not been passed it can be started anew.

9.2.5. Step 6: The pressure of the system will be released. This takes a certain time. The process is displayed in a bar graph.

9.2.6. Steps 7 & 8: In these steps you will instructed to remove clamp and to replace the side wall. The wizard will show you how and where to this is to be done. After leaving the wizard the gas supply will be reopened and the leak proof system will be ready for operation after a short while.

9.3. Make sure that the Nanopure water and 0.8% HCl containers are filled completely before each analysis. The autosampler injection needle resting reservoir should also always be filled with Nanopure water.

9.4. Water analysis. System ready and on Stand-by. Set Mode (located under System) to TIC / NPOC / TC = TIC + NPOC.

9.4.1. Select calibration wizard for set up process, under Wizards.

Step1: Select next to begin calibration definition input.

Step 2: Samples are ran in triplicate, so number of measurements per sample will be 3. The number of Run-In samples will increase by the factor input from number of measurements per sample.

2 Run-In samples will be used (due to triplicate increments total number of Run-In samples will total 6. The number of Blank samples (also by increments from the number of measurements per sample) will be 3. The concentration range will be selected at 50 ppm.

Step 3: 5 calibration points are used for routine analysis. Since one parent solution of TOC/TIC standard was prepared, select "different volumes from unique parent solution."

Step 4: Concentration values of particular parent solution will be selected. TIC concentration of parent solution is 25 ppm, and NPOC concentration of parent solution is also 25 ppm.

Step 5: After data is entered correctly, selecting "finish" will automatically insert data specified by previous steps of method building.

9.4.2. Proceeding last standard data entry (STD 0.50) the next hole position should be named "QC" (in triplicate) followed by "Spike" hole position (also in triplicate).

9.4.3. Following "Spike" enter, in triplicate, data lines for each site.

Example:	No.	Name	Hole Position
	31	CarrollCreek01	*11*
	32	CarrollCreek01	*11*
	33	CarrollCreek01	*11*
	34	CarrollCreek02	*12*

9.4.4. Proceeding final sample site entry, add final Run-In sample, in triplicate. Save method by date in following folder: Local Disk [C:]

→LiquiTOC→Data→Creek or site name (create folder if new creek)→choose water or sediment accordingly and save.

9.5. Sample vial preparation

9.5.1. All sample vials should be rinsed with Nanopure water. Vials should be filled to top to ensure complete injection volumes, since each vial will be measured three times.

9.5.2. Vials for sample sites should be rinsed once with sample water before filling with sample water.

9.5.3. Vials used for Run-Ins and Blanks will be filled with Nanopure water.

9.5.4. Hole position following final Run-In hole position should have a vial filled with Nanopure water, this will be the injection needle's final holding position (do not include this vial in the method building).

9.5.4. All vials should be covered with supplied cover foil (located in TOC supply drawer).

9.6. Upon completion of method building and vials prepped and placed in appropriate hole positions, click symbol " □ " in toolbar for complete run or " □ / O " for single run.

10. **MAINTENANCE**

10.1. Elementar LiquiTOC Analyzer

10.1.1 Reactor tube removal. The reactor is a glass tube located behind the front door panel, and partially contained inside the furnace. To uninstall the reactor tube, remove the clamps located on the top and bottom and gently remove the tube from the top of the machine. The machine should be allowed to cool before removal of the tube.

10.1.2. Reactor tube cleaning. Once removed, the Platinum catalyst should be removed and stored in a labeled container. The reactor then can be soaked in an appropriate acid bath overnight, and then rinsed thoroughly with Nanopure water. Once allowed to dry completely the Platinum catalyst will be reinstalled to a given height of 70mm. Re-install the tube and attach the clamps.

10.1.3. HCl Absorption tube. This tube can be removed and filled with new copper chips as deemed necessary.

10.2. Prior to each run, both 0.8% HCL and Nanopure Water reservoirs should be filled accordingly. After each routine maintenance job has been completed the task should be logged in the maintenance bar on the main screen of the LiquiTOC program.

11. CALCULATIONS:

Subtract inorganic carbon from total carbon when TOC is determined by difference.

12. DOCUMENTATION:

Documentation will consist of hardcopy of data report from computer and disk.

13. QUALITY ASSURANCE AND QUALITY CONTROL:

13.1. Analyst will review at the bench level to assure all daily QC criteria have been met and will notify any failed QC to their supervisor.

13.2. Maintenance of test instruments and equipment are necessary to maintain accuracy. (see "Good Laboratory Practices")

13.3. Analyze a blank and a laboratory control sample prepared from a source of material other than the calibration standards, at a level similar to the analytical samples. Preferably prepare the laboratory control sample in a matrix similar to that of the samples. Alternatively, periodically make known additions to samples to ensure recovery from unknown matrices.

14. REFERENCE:

Method 5310, Total Organic Carbon (TOC).

Appendix D

Standard Operating Procedure for Heterotrophic Plate Count: Pour Plate Method

1. APPLICATION:

The application of this SOP is to provide a standard procedure for the pour plate method for the Heterotrophic Plate Count (formerly known as the Standard Plate Count) for estimating the number of live heterotrophic bacteria in drinking water.

2. SUMMARY OF METHOD:

Heterotrophic plate counts are used to estimate general bacterial contamination of drinking water and natural waters. These plate counts can also be used to measure changes during water treatment and distribution, and in swimming pools. Water samples are collected in Whirl-Paks or sterilized plastic bottles.

In the pour plate method, the undiluted and/or diluted sample is aseptically pipetted onto the bottom of a sterile petri dish before heat-tempered melted agar is added and carefully mixed. After agar solidification the plates are inverted and placed in a 35°±0.5°C incubator for 48±3 hours. Colonies must be counted manually using a darkfield colony counter such as the Quebec colony counter.

Water samples can also be assayed by spread plate or membrane filtration. The pour plate method must be used however, if enumerating heterotrophs in drinking water under 40CFR 141.74 (a)(3). If a variance has been granted for Total Coliform Rule's maximum contaminant level, then the spread plate or membrane filter methods can also be used (see EHSL-SOP#73 and SOP#74). It is important to note that this membrane filtration procedure is not the same as that used for total and fecal coliforms.

3. INTERFERENCES:

Efficiency of pour plate procedure may vary widely dependent on water quality. Actual pouring of the heated-tempered agar may cause initial heat shock to the sample microorganisms. Colony forming units (CFU's) may be more difficult to discern due to depth within the agar. These colonies are usually slower growing and smaller, making them difficult to transfer if required. Cross-contamination caused by inadequately sterilized media, instruments and working surfaces may lead to false-positive results.

4. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

NOTE: Aseptic handling and sampling procedures should be followed at all times to inhibit contamination of sample and prevent personal exposure to possible bacterial contamination.

4.1 Water samples can be collected in sterile plastic sample bottles or Whirl-Paks.

4.2 Collect samples in the following manner:

- 4.2.1 It is necessary to use the Whirl-Paks that contain the tablet of sodium thiosulfate with all samples that are not collected directly at the source. This enables the neutralization of potential chlorine in the water supply. Nonchlorinated sources may be collected in Whirl-Paks that do not contain the tablet or in sterile plastic bottles.
- 4.2.2 Before collecting the sample, label the Whirl-Pak or sterile sample bottle with project or owner identification (project or resident's name), site of collection (actual site ID or place at the house), date, and initials of person performing the collection.
- 4.2.3 Open the Whirl-Pak or sample bottle immediately before collection of the sample. Tearing off the top perforation and pulling the two white tabs (with sodium thiosulfate) or yellow tabs (without sodium thiosulfate) outward open the Whirl-Pak.
- 4.2.4 Allow the water sample to flow from the tap for at least two to three minutes before collecting the sample. Do the same for the sample bottle but allow the sample to completely fill the bottle.
- 4.2.5 Close the container immediately after collection. With Whirl-Paks, hold both ends of the long tabs and quickly rotate ("whirl") the bag around. The two ends of this tab are then twisted together to make a tight seal.
- 4.2.6 Collect a sample volume that is at least 2X more than the suggested volume needed for the assay (see Section 9.). This may require multiple Whirl-Paks or a larger sample bottle.
- 4.2.7 Immediately place the samples in a cooler with ice for transport back to the lab.

4.3 Samples should be processed in the lab as soon as possible. Do not exceed six hours between collection and actual assay.

5. SAFETY:

7.1 Maintain aseptic techniques at all times to prevent personal exposure to high concentrations of bacteria.

7.2 Wash down working surfaces with 95% ethanol before and after performing the filtrations and counts.
7.3 All disposable petri plates that have been inoculated with sample must be placed in properly marked biohazard bags.
7.4 Refer to EHSL safety manual for general laboratory safety procedures.

6. APPARATUS AND EQUIPMENT:

7.5 Whirl-Paks, 4 oz (120 mL) with and without sodium thiosulfate, and/or sterile plastic sample bottles (500 mL or 1 L)
7.6 Cooler with ice or freezer pack to maintain samples from the field
7.7 Graduated cylinders and Erlenmeyer flasks for preparing culture medium
7.8 Sterile pipettes with ability to measure 0.5 mL aliquots. Best obtained with an Eppendorf pipet. Also require 10 mL and 25 mL pipets.
7.9 Hot plate/stir plate with clean stir bars
7.10 Aluminum foil
7.11 Balance (accurate to 0.1 g)
7.12 Bunsen burner
7.13 Petri dishes (100 mm diameter x 15 mm height)
7.14 Incubator, 35 ± 0.5°C
7.15 Waterbath, 44 – 46°C
7.16 Quebec colony counter or other darkfield colony counter

7. CALIBRATION PROCEDURES:

7.1 The temperatures of the incubator and waterbath must be verified to ensure they are within the proper temperature range.

8. CHEMICALS AND REAGENTS:

8.1 R2A agar and plate count agar (tryptone glucose extract agar) can be used for the HPC. R2A agar has been shown to give higher plate counts. Make sure to stay with one media if data comparison is necessary. The R2A agar must be used if the drinking water is in any water that has been granted a variance from the Total Coliform Rule's maximum contaminant level.

8.2 R2A Agar

8.2.1 Dissolve 18.2 g of R2A media (Difco) in 1 L of dH_2O. Mix on a stir/heat plate and check the pH to make sure it is 7.2 ± 0.2. If necessary, adjust with solid K_2HPO_4 or KH_2PO_4.

8.2.2 Turn on the heater, add 15 g of agar, and bring to a quick boil. Make sure the flask is covered with aluminum foil after adding the agar.
8.2.3 Autoclave this boiled agar mixture at 121°C for 15 minutes.
8.2.4 This medium can be used immediately after heat-tempering (see 9.2). If necessary, it may be stored at 4°C in screw-capped bottles or tubes for up to six months, or in petri dishes for up to one week.

8.3 Plate Count Agar (tryptone-glucose yeast agar)
8.3.1 Suspend 23.5 g of the Plate Count Agar (Gibco) in 1 L of dH_2O and mix well on a stir/heat plate. Cover the flask with aluminum foil.
8.3.2 Heat to boiling then promptly remove from hot plate.
8.3.3 Autoclave this boiled media at 121°C for 15 minutes.
8.3.4 As noted above (8.2.4), this medium can be used immediately after heat-tempering or may be stored at 4°C for up to six months. If already poured in petri plates then it should be used within two weeks.
8.4 Ensure that all glassware and other lab apparatus involved with this procedure are clean and nontoxic. Use only detergents that are designed for microbiological work.
8.5 It is important that the working surface is clear and wiped down with 70% ethanol before adding the media to the petri dishes.

9. PROCEDURE:

9.1 Sample Dilution:
9.1.1. Prepare the area for this procedure by cleaning the counter tops with 70% ethanol or an antibacterial cleaner.
9.1.2. Before transferring the required volume of sample, mix the contents of the container or sample bag by quickly inverting or lightly swirling the contents.
9.1.3. With drinking water, an undiluted sample will usually work. If necessary, prepare dilutions as shown below.

SAMPLE →→→→ →→→→→→ **10^{-1}** →→→→→→→→→→ **10^{-2}**

(0.5 mL q.s. 5.0 mL w/dH_2O) (0.5 mL q.s. 5.0 mL w/dH_2O)

↓ ↓ ↓

dispense

on plate: 1.0 mL 1.0 mL 1.0 mL

9.2. Pouring and Incubation:

9.2.1. If agar has solidified then it is necessary to gently melt the agar medium in boiling water in a partially closed container. It would be best if the agar was first distributed in smaller aliquots (100 to 200 mL) during media preparation to aid in the melting process and to help prevent contamination during actual pouring.

9.2.2. Allow the melted agar to heat-stabilize in a 44-46°C waterbath. This melted agar should not be held for longer then three hours. This agar medium should only be melted one time.

9.2.3. Aseptically pipet 1.0 mL of undiluted and/or diluted sample (see 9.1) onto the bottom of a sterile 100 mm x 15 mm petri dish.

9.2.4. Partially remove the foil cover on the media flask and aseptically add approximately 15 mL of the heat-tempered agar medium to each petri dish. It is best to use a larger pipet and aliquot the media to a number of plates to help avoid contamination.

9.2.5. Quickly place the lid back on the plate and gently swirl to allow mixing of the sample and medium.

9.2.6. Allow the plates to solidify on a level surface then invert and incubate at 35 ± 0.5°C for 48 ± 3 hours. Do not stack more than four plates and give adequate space to allow uniform incubation temperature.

9.3. Counting:

9.3.1. Count all visible colony-forming units (CFU) using a darkfield colony counter such as a Quebec colony counter. Count plates having 30 to 300 CFU's to calculate the best concentration. It is acceptable to count less then 30 colonies on plates inoculated with 1.0 mL of undiluted sample.

9.3.2. If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies. Report this as estimated CFU per mL.

9.3.3. If plates from all dilutions of any sample have no colonies, report the count as <1 per mL. If a larger volume was used then report as <1 per sample volume used.

10. CALCULATIONS

10.1. Calculation of heterotrophic plate count:

Compute the heterotroph concentration using the plate that has the most distinctive individual colonies that fall within the 30 to 300-colony range.

Obtain an average number CFU when performing in duplicate or triplicate (see Section 9.3).

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{volume sample plated (mL)}}$$

11. DOCUMENTATION:

11.1 Record as 'HPC = # CFU per mL'. Also, report the method used, the incubation temperature and time, and the medium.
Example: 'pour plate method, 35°C, 48 hrs, R2A agar'.

12. QUALITY ASSURANCE AND QUALITY CONTROL:

12.1 A blank control plate for each batch of agar will be checked for sterility. Data will be rejected if this plate is contaminated.

13. REFERENCES:

13.1 APHA, AWWA, and WPCF. 1992. Standard Methods for the Examination of Water and Wastewater, 18th edition. A.E. Greenberg, L.S. Clesceri, and A.D. Eaton, Eds. American Public Health Association, Washington, D.C.

13.2 U.S. Environmental Protection Agency. 1999. National Primary Drinking Water Regulations. U.S. Code of Federal Regulations, 40 CFR, Part 141, Subpart C, Section 141.21, Washington, D.C.

13.3 U.S. Environmental Protection Agency. 1997. Manual for the Certification of Laboratories Analyzing Drinking Water. EPA 815-B-97-001. Office of Groundwater and Drinking Water, Cincinnati, OH.

Appendix E

Standard Operating Procedure for Acridine Orange Direct Counts

1. APPLICATION:

The application of this Standard Operating Procedure is to provide a standard procedure for Acridine Orange Direct Count.

2. SUMMARY OF THE METHOD:

AODC is a procedure that allows one to count the number of bacterial cells in a sediment sample. The AO stain is a nucleic acid stain that is useful for determining cell cycles. The stain interacts with DNA. DNA intercalated fluoresces green while RNA electrostatically bound AO fluoresces red.

Sediment Samples are collected using Whirl-Paks. The sediment samples are measured out and put into a 50 ml centrifuge tube. PBS + Tween 80 is added and then the sample is centrifuged. The sample must be allowed to sit for at least 3 hours, overnight is preferable (if overnight place in cooler).

The supernatant is then removed and added to a sterile 15ml centrifuge tube. AODC satin is added and vortexed. This is allowed to sit at room temp for 2 minutes.

The samples are then filtered using a .2 micron pore filter. The filtered is removed from the vacuum and mounted on a microscope slide and the cover slip is sealed. If the slide is to be store it should be kept at 4 C.

3. INTERFERENCES:

3.1 When taking the sediment sample be sure to minimize the number of small pebbles and rocks and other things that will not break down in the centrifuge tube.

3.2 When prepping the slide be sure there are no air bubbles under the coverslip.

3.3 Be as aseptic as possible.

4. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

4.1 Samples are collected using the following method:

4.1.1 Samples are collected in Whirl-Paks, before collecting the sample label the Whirl-Pak with project or owner identification, site of collection, date, and initials of the person performing the collection.

4.1.2 Open the Whirl-Pak immediately before collecting the sediment sample. Tear off the top perforation and opening it via the two tabs on the sides.

4.1.3 Fill the Whirl-Pak with approximately 5 grams of sediment and immediately close the bag after collection by holding the long yellow tabs on the side and whirling it around.

4.1.4 The samples are then immediately put into a cooler with ice and brought back to the lab.

5. SAFETY:

5.1 Be as aseptic as possible.

5.2 Wear goggles while using the vacuum.

5.3 Wash filtering apparatus with 70% ETOH and dH2O.

5.5 All samples must be disposed of in biohazard containers.

5.6 Refer to the EHSL safety manual for general Lab safety procedures.

6. APPARATUS AND EQUIPMENT:

6.1 Whirl-Paks

6.2 Cooler

6.3 Graduated Cylinder

6.4 50 and 15 ml centrifuge tubes

6.5 10 and 1 ml sterile pipettes
6.6 Membrane filters, 0.2 micron polycarbonate filters
6.7 Balance
6.8 Tweezers

6.9 Microscope slides, coverslips and clear nail polish
6.10 Petri dishes

7. CALIBRATION PROCEDURES:

7.1 The samples must be at room temperature when read under the microscope.

8. CHEMICALS AND REAGENTS:

0.1% Acridine Orange (AO) Stain

Dissolve 0.1 g of AO in 100 mL of dH_2O. Filter sterilize through a 0.2 µm filter into a sterile glass bottle. Store at 4°C.

Irgalan Black (IB) Stain

Best to use prestained polycarbonate membrane filters, 0.2 µm, 25 mm, black (Proetics, Cat# 11021). If these filters are not available then make stain by dissolving 0.2 g of IB in 100 mL of 2% acetic acid. Store at 4°C.

0.2 M NaH_2PO_4

Dissolve 24.0 g of NaH_2PO_4 (or 27.6 g of $NaH_2PO_4 \cdot H_2O$) in approximately 900 mL of dH_2O. Stir well and q.s. to 1 L. Store at room temperature.

0.2 M Na_2HPO_4

Dissolve 28.4 g of Na_2HPO_4 (or 53.6 g of $Na_2HPO_4 \cdot 7H_2O$) in approximately 900 mL of dH_2O. Stir well and q.s. to 1 L. Store at room temperature.

PBS + Tween 80, pH 7.2

Add 140 mL of 0.2 M NaH_2PO_4 and 360 mL of 0.2 M Na_2HPO_4 to approximately 500 mL of dH_2O. Stir well and add 10 mL of polyoxyethylene (20) sorbitan monoleate (Tween 80). Adjust pH to 7.2 with concentrated HCl or NaOH and q.s. to 1 L. Autoclave at 121°C for 60 minutes. Store at 4°C.

9. PROCEDURES:

Sediment Sample

1. Add 30 mL of sterile PBS + Tween 80 into a sterile 50 mL centrifuge tube. Record this buffer volume on the benchsheet.

2. Place 0.3 g of sediment into the tube and record this mass on the benchsheet. Make sure no large rocks or other solids that will not break up in a vortexer are included. It is important that all matter is able to be broken down.
3. Vortex on high for one minute. After votexing, ensure the mixture is completely homogenous. If clumping is noticed, vortex until the clumps break down.
4. Allow the solids to completely settle, leaving a relatively clear supernatant. This must be done for at least three hours. Often overnight is best.
5. Add 0.5 mL of this suspension into a 15 mL sterile centrifuge tube containing 5 mL of sterile dH_2O and 500 µL of AO stain.
6. Vortex sample for 30 seconds and allow the mixture to remain at room temperature for 2 minutes.

Slide Prep and Enumeration

1. Place a pre-stained 25 mm, 0.2 µm pore polycarbonate nucleopore filter on a sterile filtering apparatus and pour the stain suspension through this filter. Rinse the filter at least three times with dH_2O after filtration.
2. After removing the filter, rinse the filter apparatus with 70% ethanol and dH_2O.
3. Place the damp filter on a drop of immersion oil on a slide, then cover with another drop of immersion oil and a coverslip. If the slide is to be stored, then seal with clear nail polish and store at 4°C.
4. Examine the mounted filter at 1000X using epi-fluorescent microscopy. Make sure the slide is at room temperature before viewing.
5. Count 10 fields and record the number of fluorescing green, yellow or red bacteria.

10. CALCULATIONS:

Cells/g of sediment = **A * B * C**

Cells/mL of water = **D * B**

Where, **A** = avg # bacterial cells for 10 counts / buffer-diluted sediment volume filtered (mL),

B = area filtered (mm^2) / area viewed (mm^2),

C = buffer volume (mL) / sediment mass (g)

D = avg # bacterial cells for 10 counts / water sample volume filtered (mL)

11. REFERENCES:

http://www.med.umich.edu/flowcytometry/PDF%20files/aostain.pdf

Appendix F

Standard Operating Procedure for Phosphatase Activity

1. APPLICATION:

The application of this SOP is to provide a standard procedure for the determination of phosphatases in sediment or water samples.

2. SUMMARY OF THE METHOD:

For phosphatase testing of samples 4 tubes are used for testing acidity and four tubes are used for used for alkalinity. One tube of each set is needed for a blank and 3 are used for sample duplicates. Next, 5 mL of TRIS buffer pH 8.6 is added to the alkaline blanks and 4 mL of the buffer is added to the sample tubes. For the acid phosphatase samples, 5 mL of TRIS buffer pH 4.8 is added to the blank tubes and 4 mL of the same buffer is added directly to the sample tubes. Next, 1 gram of sediment or 10 mL of water is added to all tubes (acid + alkaline). The next step involves adding 1 mL of TRIS buffer with phosphatase substrate pH 7.6. to the sample tubes of both sets. Tubes are then vortexed and incubated overnight. Absorbance is read the following day at 418 nm.

3. INTERFERENCES:

3.1) Avoid adding substrate to sample blanks.

3.2) Correct amounts of substrates, buffers, and samples should be used.

3.3) Make sure that samples are properly vortexed and well mixed.

3.4) Avoid large pebbles and shells when weighing out samples.

3.5) Allow for ample incubation time after vortexing.

3.6) Only the clearer top portion of the sample should be analyzed. Avoid pipetting from the bottom of the test tube.

3.7) When using the spectrophotometer make sure that the blank and sample cells are clean, dry, and free of external scratches.

3.8) If any sample has an absorbance of > 0.6, a dilution (1:4,1:10) must be prepared for the entire 3 sample +1 blank series.

4. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

4.1 For sediments, samples are collected by spooning into a sterile Whirl-pak or equivalent polyethylene sampling bag.

4.2 Samples should be transported in a cooler and kept at 4°C until needed.

5. SAFETY:

5.1 Aseptic lab practices should be followed at all times.

5.2 Refer to EHSL safety manual for general laboratory safety procedures.

5.3 All glassware should be properly sanitized or autoclaved.

6. APPARATUS AND EQUIPMENT:

6.1 Micropipette + disposable tips
6.2 Small test tubes and caps
6.3 Balance or digital scales
6.4 Incubator
6.5 Vortex apparatus
6.6 Spectrophotometer
6.7 Cuvettes
6.8 pH Meter

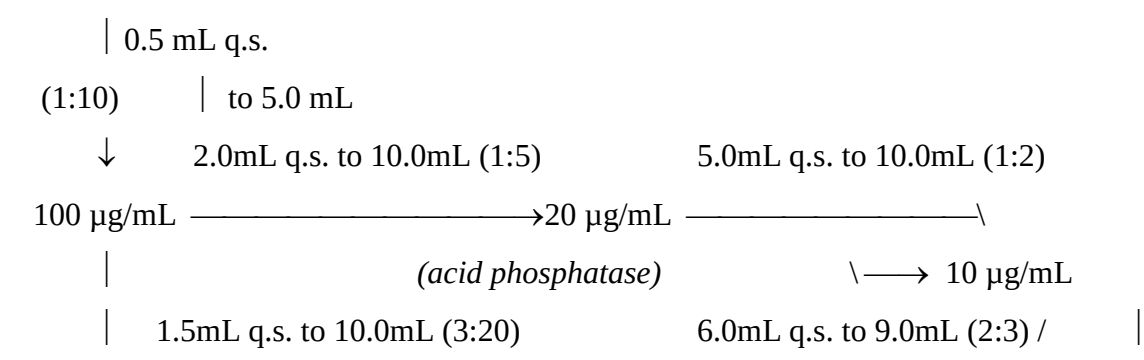
7. CALIBRATION PROCEDURES:

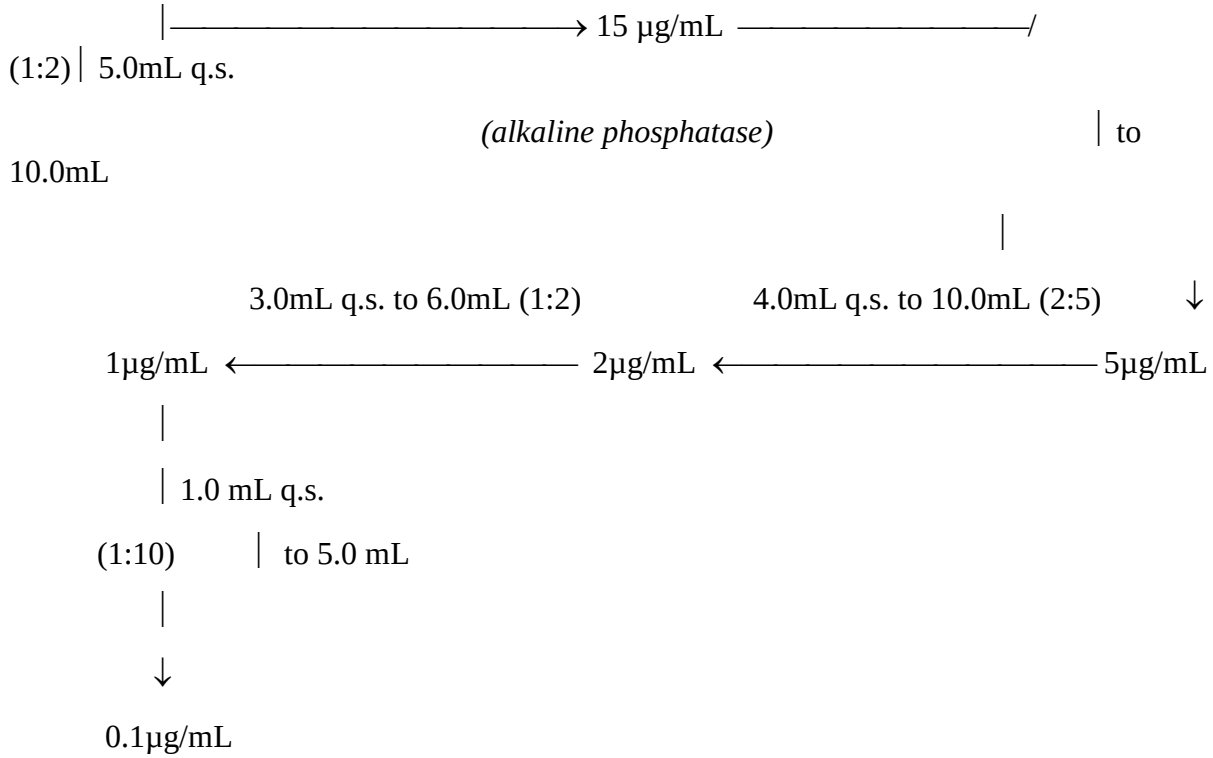
Prepare a standard curve using p-nitrophenol.

Stock Nitrophenol Standards, 1000 µg/mL

It is necessary to make up a separate standard for the alkaline phosphatase and the acid phosphatase. Dissolve 10 mg of nitrophenol in 10 mL of alkaline dilution buffer or acid dilution buffer. Prepare each standard as outlined in the below.

Nitrophenol Standards





8. CHEMICALS AND REAGENTS:

1 M TRIS Buffer, pH 8.6 (for alkaline phosphatase)

Dissolve 6.06 g of TRIZMA Base and 1.92 g of TRIZMA HCl in 500 mL of dH_2O. Adjust the pH to 8.6 with concentrated HCl or NaOH. Filter sterilize through 0.2 µm membrane filter into a sterile 500 mL flask.

1 M TRIS Buffer, pH 4.8 (for acid phosphatase)

Dissolve 0.60 g of TRIZMA Base and 15.76 g of TRIZMA HCl in 500 mL of dH_2O. Adjust the pH to 4.8 with concentrated HCl or NaOH. Filter sterilize through 0.2 µm membrane filter into a sterile 500 mL flask.

1 M TRIS Buffer with phosphatase substrate, pH 7.6

Dissolve 1.21 g TRIZMA Base and 1.21 g of TRIZMA HCl in approximately 90 mL dH_2O. Adjust the pH to 7.6 with concentrated HCl or NaOH. Add 0.1 g of phosphatase

substrate and stir until dissolved. Q.s. to 100 mL and filter sterilize through a 0.2 µm filter. Store at 4°C in a sterile container.

Alkaline Dilution Buffer

Combine 100 mL of 1M TRIS buffer, pH 8.6, with 25 mL TRIS buffer with phosphatase substrate, pH 7.6, in a sterile container. Store at 4°C.

Acid Dilution Buffer

Combine 100 mL of 1M TRIS buffer, pH 4.8, with 25 mL TRIS buffer with phosphatase substrate, pH 7.6, in a sterile container. Store at 4°C.

9. <u>PROCEDURES:</u>

9.1 For each sample arrange 4 small sterile glass test tubes for the alkaline phosphatase and 4 small sterile glass tubes for the acid phosphatase. One tube is for the blank and three tubes are for the sample duplicates. One tube for the negative control is also required for the entire set of samples for each analysis.

9.2 Prepare the dilution tubes before the actual analysis. The buffers are stable and can be prepared and added to the tubes days before the actual analyses.

Alkaline phosphatase:

Blank: 5 mL of TRIS buffer, pH 8.6

Samples and negative control: 4 mL of TRIS buffer, pH 8.6

Acid phosphatase:

Blank: 5 mL of TRIS buffer, pH 4.8

Samples and negative control: 4 mL of TRIS buffer, pH 4.8

9.3 On the day of sampling, add 1 g of sediment or 10 mL of water to each of the prepared dilution tubes. Do not add sample to the negative controls. Vortex to mix samples.

9.4 Now add 1 mL of the TRIS buffer with the phosphatase substrate to each of the sample tubes and negative control. Do not add the substrate to the blanks.

9.5 Vortex each test tube for at least 30 seconds. The sample:buffer mixture must be homogenous.

9.6 Incubate in the dark for 18 hours at 30°C.

9.7 Read absorbance at 418 nm using the blank for each sample to zero the spectrophotometer. If any sample has an absorbance >0.7 it will be necessary to dilute with dH_2O.

9.8 Calculate the concentrations of the samples based on the standard curve (see Section 7)

Appendix G

Standard Operating Procedure for Dehydrogenase Activity

1. Application:

The application of this SOP is to provide a standard procedure for the determination of Dehydrogenase activity (DHA).

2. Summary of the Method:

For the DHA testing of sediment samples four sterile test tubes are used. One tube is for the blank and three are for samples. For the blank tubes 2 mL of 0.1M phosphate buffer with a pH of 7.6 added to each tube. For the sample tubes, 1 mL of the phosphate buffer is added. Next, 1 gram of sediment is weighed out or 10 mL of water is added to each tube (samples + blanks). 1 mL of an INT solution is then added to the sample tubes and all tubes are vortexed. All tubes are then allowed to incubate for 45 minutes in complete darkness. A 1 mL aliquot of each tube is then filtered and the filters are then dried and dissolved with DMSO. The tubes then incubate overnight and results are read via spectrophotometer the next day.

3. Interferences:

3.1) Correct amounts of buffers, samples and substrates should be used.

3.2) INT is light sensitive and therefore begins to degrade in the presence of light. Storage containers should be wrapped in aluminum foil or other reflective material to minimize exposure to light.

3.3) Only add substrate to the three sample tubes. Doing so to the blanks will invalidate the blank.

3.4) When vortexing make sure the sample is thoroughly mixed together.

3.5) When weighing samples of sediment, try to avoid large pebbles, shells, etc.

3.6) Allow for ample incubation time after vortexing. This allows the sediment time to settle and become less turbid.

3.7) Only the clearer top portion of the sample should be analyzed. Avoid pipetting from the bottom of the test tube.

3.8) Practice sterile techniques when performing vacuum filtration.

3.9) Be sure that filter paper is placed in the bottom of a large test tube before adding

DMSO. This allows for a quicker dissolution.

3.10) When using spectrophotometer make sure that the blank and sample cells are clean, dry, and free of external scratches.

3.11) If any sample has an absorbance of > 0.6, a dilution (1:4, 1:10) must be prepared with DMSO for the entire 3 sample series and blank.

4. Sample Collection, Preservation and Handling:

4.1) For sediments, samples are collected by spooning into a sterile Whirlpak or an equivalent polyethylene sampling bag.

4.2) Samples should be transported in a cooler and kept at 4 degrees Celsius until needed.

5. Safety:

5.1) Aseptic Laboratory practices should be employed at all times.

5.2) Refer to EHSL safety manual for general laboratory safety procedures.

5.3) Use caution when working with DMSO. Nitrile gloves should be worn along with standard safety attire.

5.4) DMSO is carcinogenic and when absorbed cutaneously, inhaled, or ingested. Refer to MSDS sheets located in room #8 for more information on DMSO.

5.5) All glassware used should be properly sanitized or autoclaved.

6. Apparatus and Equipment:

6.1) Micropipette and disposable tips

6.2) Test tubes and caps

6.3) Balance or digital scales

6.4) Incubator

6.5) Vortex apparatus

6.6) Spectrophotometer (Sectronic Genesys 5) located in room #3

6.7) curvettes

6.8) pH meter

6.9) vacuum apparatus

6.10) sterile 0.45 micron membrane filters (cellulosic, white, plain, 25mm)

6.11) Vented chemical hood with operational fan and lights

6.12) aluminum foil

7. Calibration Procedure:

8. Chemicals and Reagents:

8.1) INT Solution: [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl 2H-tetrazolium chloride]. In reduced light conditions, use a glass rod to mix 0.5 g of INT with approximately 0.5 mL of 100% ethanol in a 150 mL beaker until a yellow paste is achieved. Next, add distilled water to approximately 90 mL and stir on a stir plate protected from light for 30 minutes. Bring volume to 100 mL in a volumetric flask with distilled water. Sterilize by passing through a sterile 0.2 micron membrane filter. Store this solution in a refrigerator at 4 degrees Celsius in a sterile container wrapped in aluminum foil (a pair of 50 mL centrifuge tubes works well). This supplies enough INT for 40-45 tubes.

8.2) 0.1 M Phosphate Buffer, pH 7.6 : Dissolve 1.56 g of NaH_2PO_4 (or 1.79 g of $NaH_2PO_4 * H_2O$) and 12.35 g of Na_2HPO_4 (or 23.30 g of $Na_2HPO_4 *7 H_2O$) in 1 Liter of distilled water. Adjust the pH to 7.6 with concentrated HCL or NaOH. Autoclave for 60 minutes and store at 4 degrees Celsius in refrigerator.

8.3) Dimethyl Sulfoxide (DMSO)

9. Procedure:

9.1) For each sample arrange 4 sterile glass test tubes. One Tube for the blank and three tubes for the sample duplicates. It should be noted that the smaller sized test tubes are most desirable for this purpose.

9.2) Add 2 mL phosphate buffer to each blank and 1 mL of phosphate buffer to each sample tube. This step can be completed ahead of time in order to expedite the process.

9.3) Add 1 g of sediment to each tube.

9.4) For each 3 sample tubes add 1 mL INT.

9.5) Vortex each test tube for 30 seconds.

9.6) Next, incubate all tubes for 45 minutes at 30 degrees Celsius in a complete darkness.

9.7)Filter a 1 mL aliquot of each blank and sample through separate 0.45 micron membrane filters. It is important for the tubes to remain unmixed during the process.

9.8) After vacuum filtration is complete, place each filter on a piece of aluminum foil to dry for 3 minutes at 103 degrees Celsius.

9.9) Working under a ventilated hood, place each filter into the bottom of a clean dry sterile test tube. Large tubes must be used for this purpose.

9.10) Carefully add 5 mL of DMSO to each tube. Cap the tube and vortex until it dissolves. It should be noted to wear nitrile gloves when working with DMSO.

9.11) Incubate overnight at room temperature in the dark.

9.12) The next day absorbance is read at 460 nm using the blank for each sample to zero the spectrophotometer. If any sample has an absorbance of > 0.6 it will be

necessary to dilute it with DMSO.

10. Calculations:

Mg/g or mg/mL = A x B x C x D

Where: A= absorbance

B= X coefficient obtained by regression form standard curve

C= dilution factor, if > 1

D= conversion factor

Conversion factor for sediment is 5 because filter was dissolved in 5 mL DMSO.

11. Documentation:

Documentation will consist of bench sheets with accompanying computer printout.

12. QA/QC:

12.1) Aseptic Techniques are practiced at all times

Appendix H

Standard Operating Procedure for Glucosidase Activity

1. CHEMICALS AND REAGENTS:

Phosphate Buffer, pH 7.6, with glucosidase indicator

Dissolve 0.156 g of NaH_2PO_4 (or 0.179 g of $NaH_2PO_4 \cdot H_2O$) and 1.235 g of Na_2HPO_4 (or 2.330 g of $Na_2HPO_4 \cdot 7H_2O$) in approximately 90 mL of dH_2O. Adjust the pH to 7.6 with concentrated HCl or NaOH. Add 0.151 g of 4-nitrophenyl-B-D-glucopyranoside and stir until dissolved. Q.s. to 100 mL and filter-sterilize through a 0.2 µm filter. Store in a sterile container at 4°C. Discard when solution becomes yellow.

Phosphate Buffer, pH 9.0

Dissolve 1.84 g of Na_2HPO_4 (or 3.47 g of $Na_2HPO_4 \cdot 7H_2O$) in 1 L of dH_2O. Adjust pH to 9.0 with concentrated HCl or NaOH. Autoclave for 60 minutes. Store at 4°C.

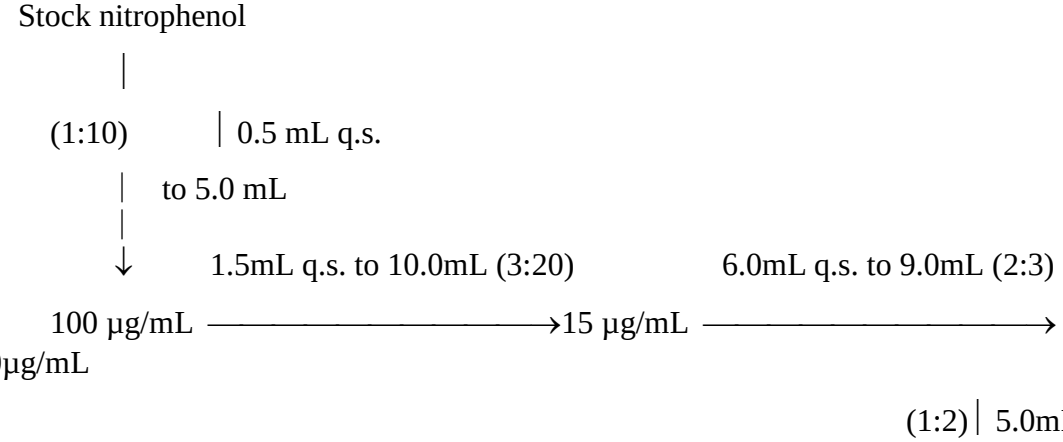
Phosphate Dilution Buffer for Glucosidase

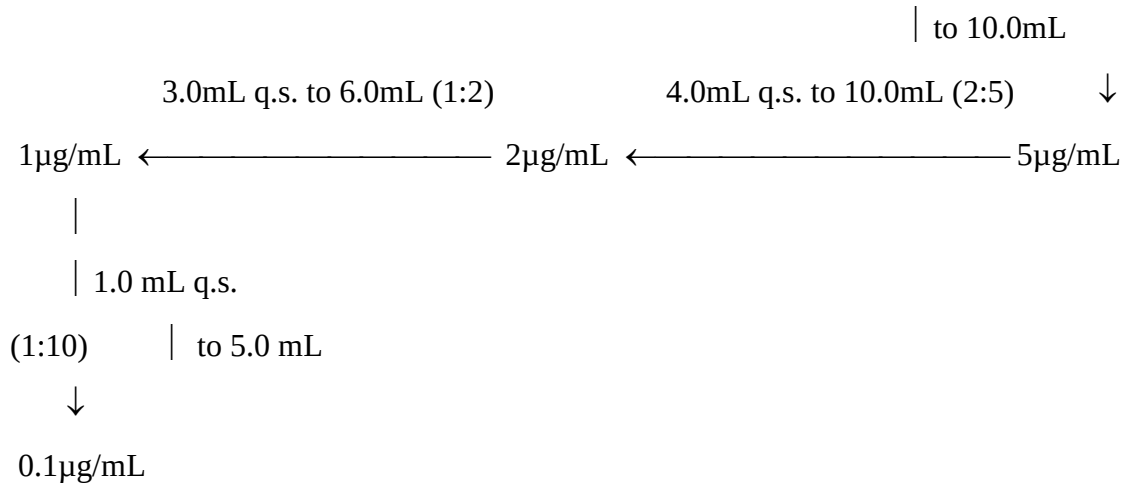
In a sterile container combine 100 mL of phosphate buffer, pH 9.0, with 25 mL phosphate buffer, pH 7.6, with glucosidase indicator. Store at 4°C.

Stock Nitrophenol Standard, 1000 µg/mL

Dissolve 10 mg of nitrophenol in 10 mL of phosphate dilution buffer. Mix well and store at 4°C.

Nitrophenol Standards





NOTE: Diluent is the phosphate dilution buffer for glucosidase

2. <u>PROCEDURES:</u>

2.1 For each sample arrange 4 sterile glass test tubes. One tube is for the blank and three tubes are for the sample duplicates. One tube is also required for the entire set of samples for the negative control.

2.2 Add 1 g of sediment to each of the tubes. Do not add sample to the negative control.

2.3 Blank: Add 5 mL phosphate buffer, pH 9.0.

2.4 Samples and negative control: Add 4 mL phosphate buffer, pH 9.0, + 1 mL phosphate buffer with glucosidase indicator.

2.5 Vortex for 30 seconds.

2.6 Incubate all tubes in the dark for 18 hours at 30°C.

2.7 Read absorbance at 418 nm using the blank for each sample to zero the spectrophotometer. If any sample has an absorbance >0.6 it will be necessary to dilute it with dH_2O.

Appendix I

Standard Operating Procedure for Galactosidase Activity

CHEMICALS AND REAGENTS:

Phosphate Buffer, pH 7.6, with galactosidase indicator

Dissolve 0.156 g of NaH_2PO_4 (or 0.179 g of $NaH_2PO_4 \cdot H_2O$) and 1.235 g of Na_2HPO_4 (or 2.330 g of $Na_2HPO_4 \cdot 7H_2O$) in approximately 90 mL of dH_2O. Adjust the pH to 7.6 with concentrated HCl or NaOH. Add 0.151 g of p-nitrophenyl-B-D-galactopyranoside and stir until dissolved. Q.s. to 100 mL and filter-sterilize through a 0.2 µm filter. Store in a sterile container at 4°C. Discard when solution becomes yellow.

Phosphate Buffer, pH 9.0

Dissolve 1.84 g of Na_2HPO_4 (or 3.47 g of $Na_2HPO_4 \cdot 7H_2O$) in 1 L of dH_2O. Adjust pH to 9.0 with concentrated HCl or NaOH. Autoclave for 60 minutes. Store at 4°C.

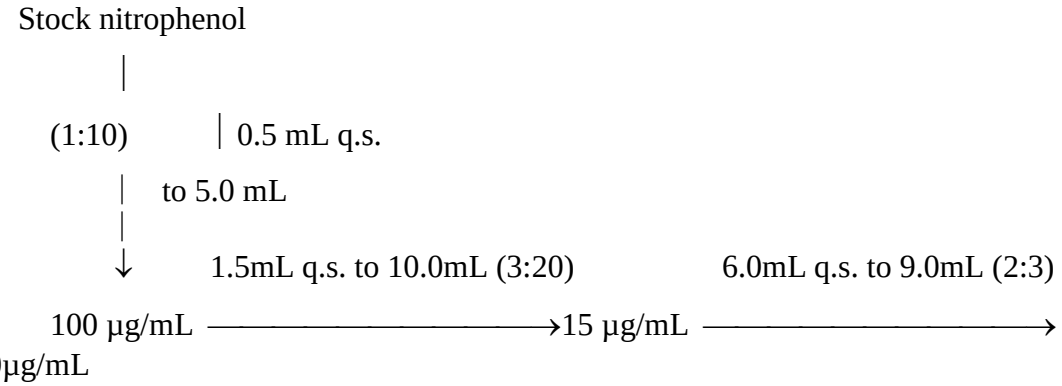
Phosphate Dilution Buffer for Galactosidase

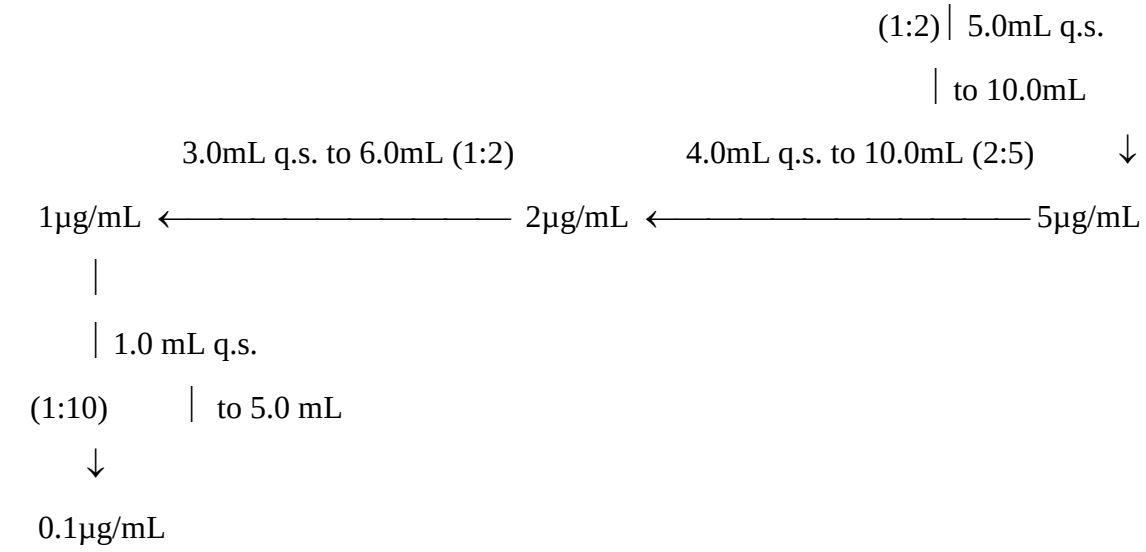
In a sterile container combine 100 mL of phosphate buffer, pH 9.0, with 25 mL phosphate buffer, pH 7.6, with galactosidase indicator. Store at 4°C.

Stock Nitrophenol Standard, 1000 µg/mL

Dissolve 10 mg of nitrophenol in 10 mL of phosphate dilution buffer. Mix well and store at 4°C.

Nitrophenol Standards





NOTE: Diluent is the phosphate dilution buffer for galacotsidase.

PROCEDURE:

1. For each sample arrange 4 sterile glass test tubes. One tube is for the blank and three tubes are for the sample duplicates. One tube is also required for the entire set of samples for the negative control.
2. Add 1 g of sediment of 10 mL of water to each of the tubes. Do not add sample to the negative control.
3. Blank: Add 5 mL phosphate buffer, pH 9.0.
   Samples and negative control: Add 4 mL phosphate buffer, pH 9.0, + 1 mL

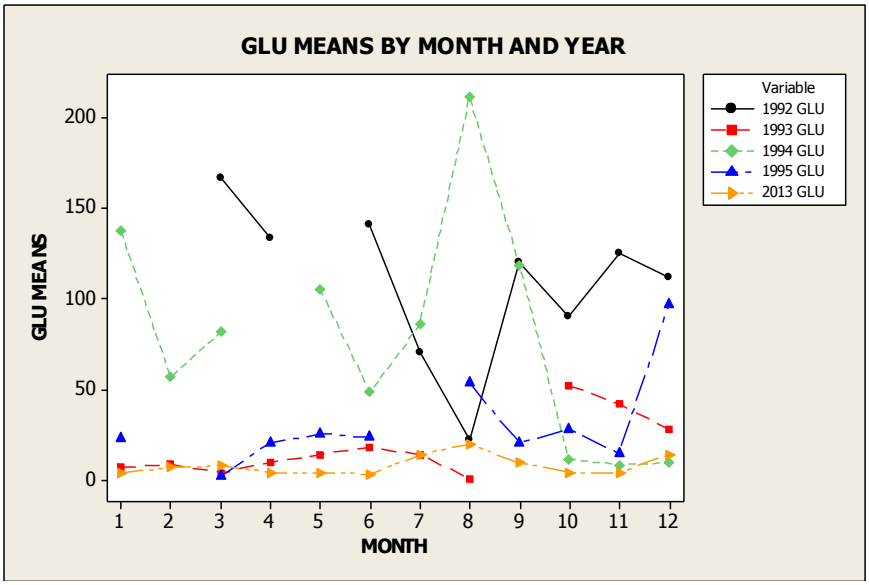
   phosphate buffer with galactosidase indicator.

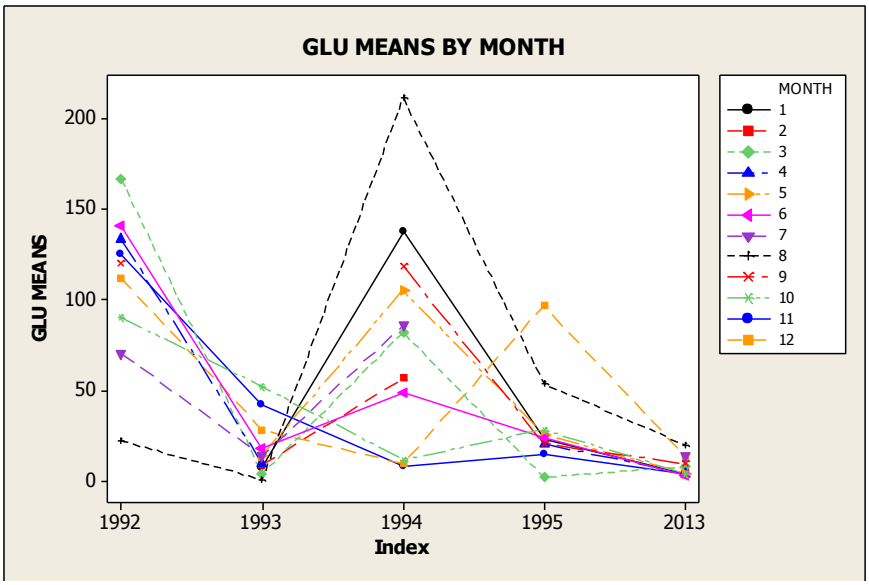
4. Vortex for 30 seconds.
5. Incubate all tubes in the dark for 18 hours at 30°C.
6. Read absorbance at 418 nm using the blank for each sample to zero the spectrophotometer. If any sample has an absorbance >0.6 it will be necessary to dilute it with dH_2O.

Appendix J

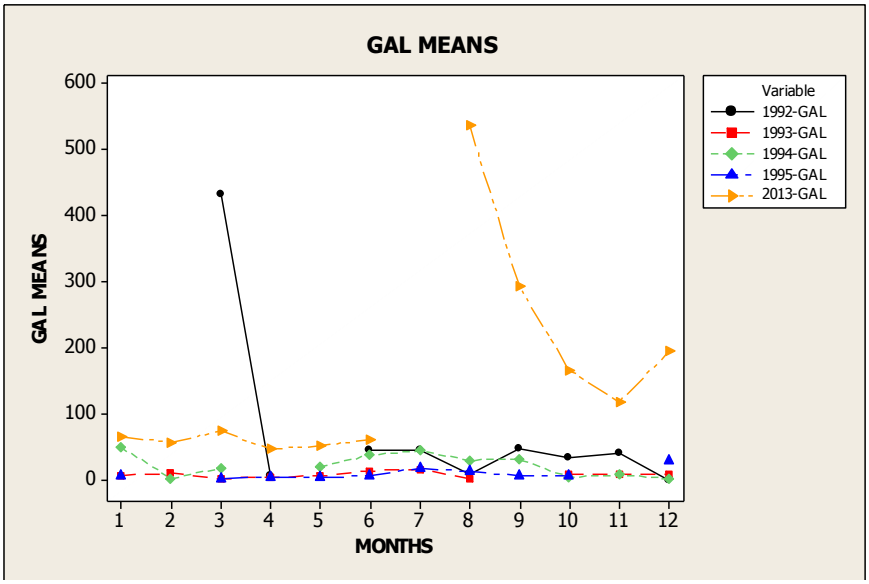
Monthly Variability Results

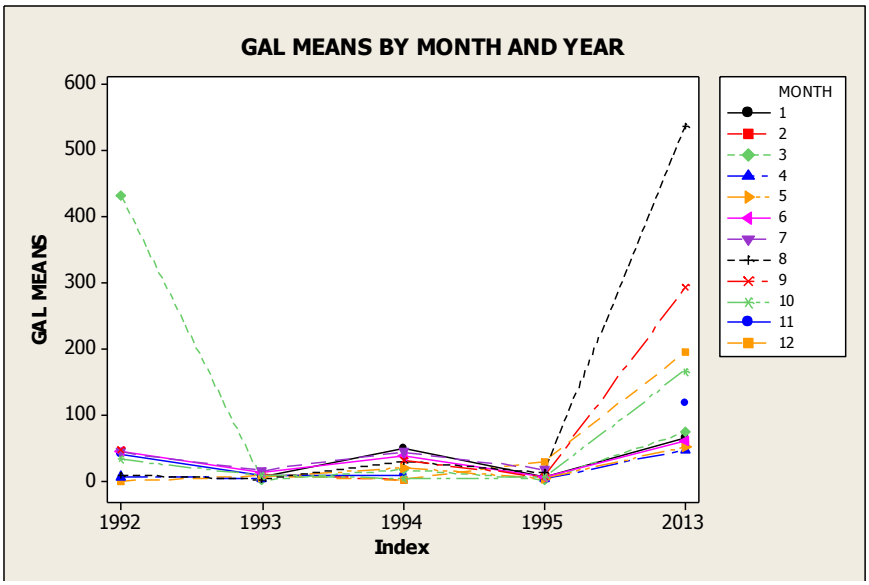
Between-month and between-season variation was evaluated for years 1992-1995 and 2012-2013. Years 1991 and 1996 were not included because there were not enough monthly data points. Data was graphed to qualitatively show differences between months and years. This is compared to recovery data to determine if differences are linked to seasonal variation or to another factor.



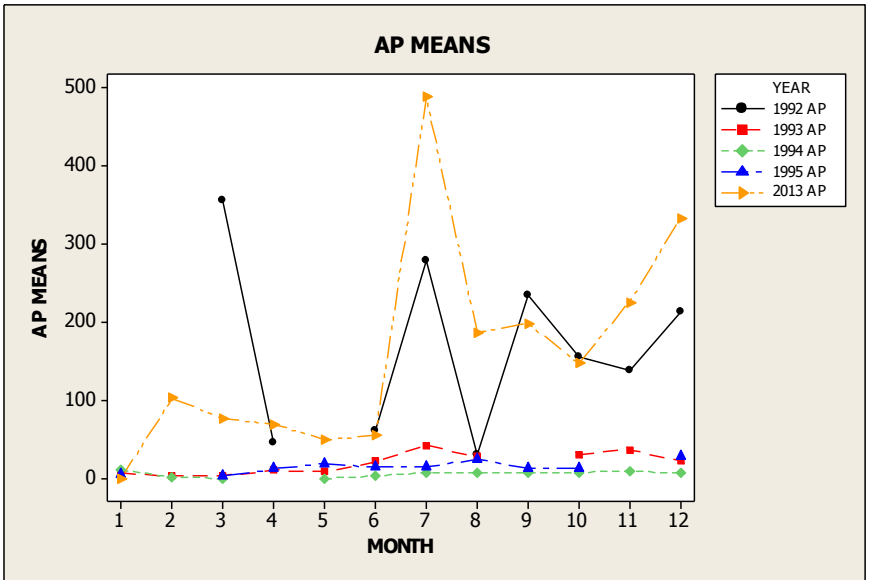


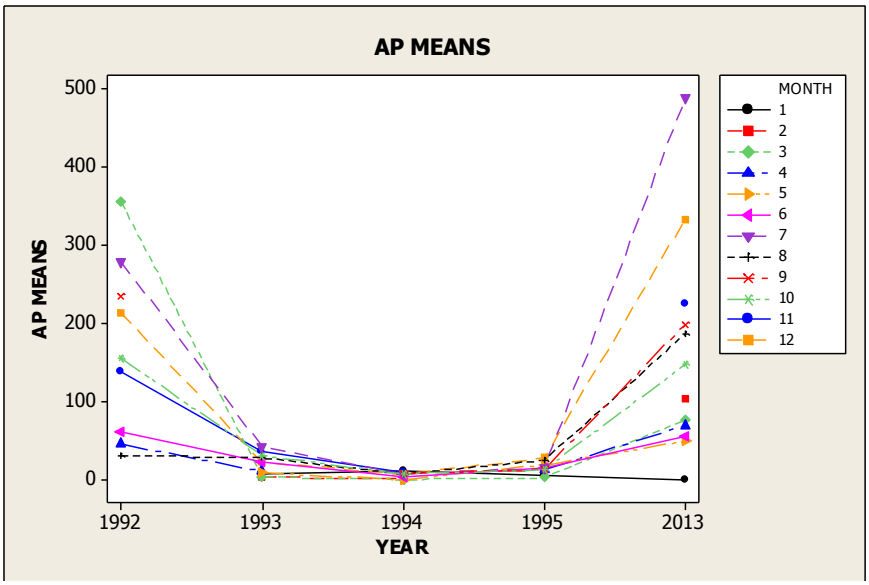
There were significant differences between 1992 and 1993 GLU (p=0.001), between 1992 and 1995 GLU (p=0.008), between 1992 and 2013 GLU (p=0.000), between 1994 and 2013 GLU (p=0.003), and between 1995 and 2013 GLU. No significant differences for GLU were found between 1992 and 1994, 1993 and 2013, 1993 and 1995, 1994 and 1995, and 1993 and 1994.



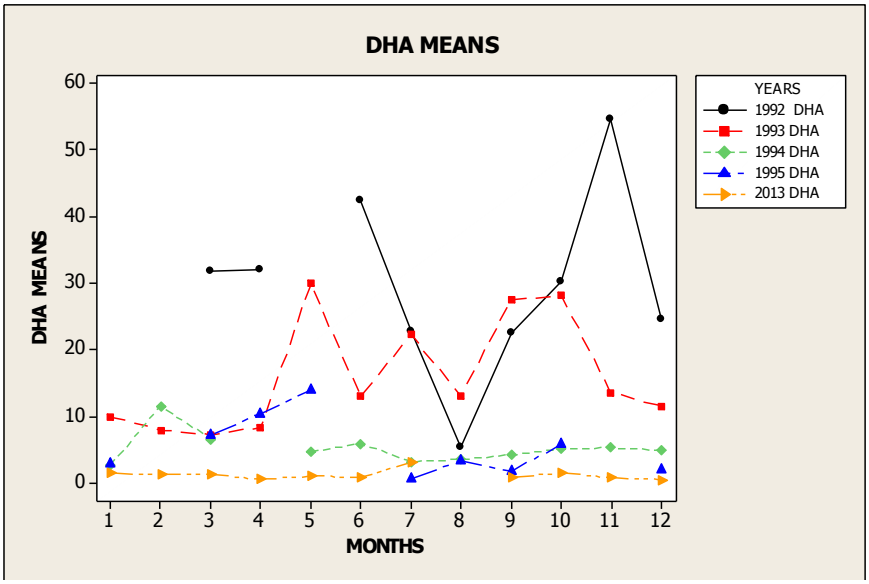


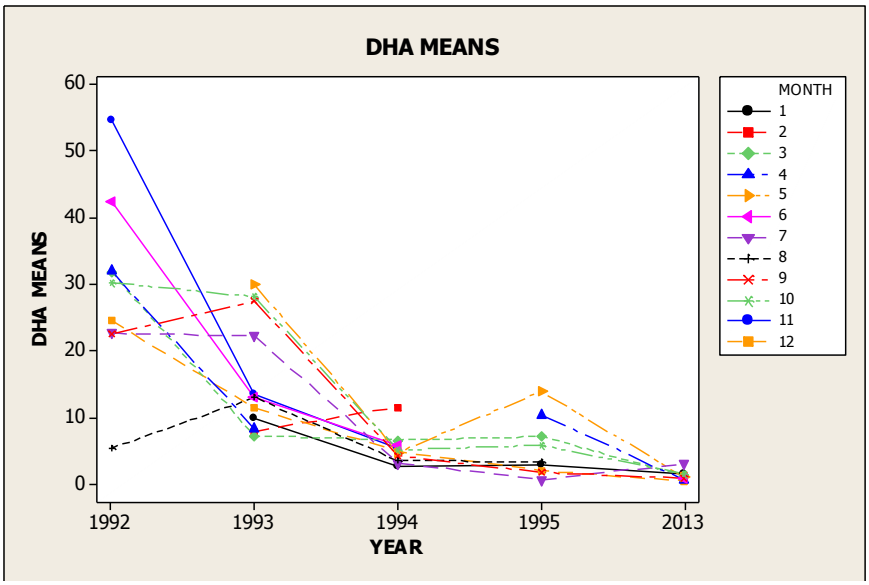
July 2013 GAL outlier was taken out. There were significant differences between 1993 and 2013 GAL (p= 0.023), between 1994 and 2013 GAL (p=0.016), and between 1995 and 2013 GAL (0.019). No significant differences were found between 1992 and 1993 GAL, 1992 and 1994 GAL, 1992 and 1995 GAL, 1992 and 2013 GAL, and 1993 and 1994 GAL.





Significant differences were found between 1992 and 1993 AP (0.014), 1992 and 1994 AP (0.003), 1992 and 1995 AP (0.009), 1993 and 2013 AP (0.007), 1994 and 2013 AP (0.003), and 1995 and 2013 AP. No significant differences were found between 1994 and 1995 AP and 1993 and 1995 AP.

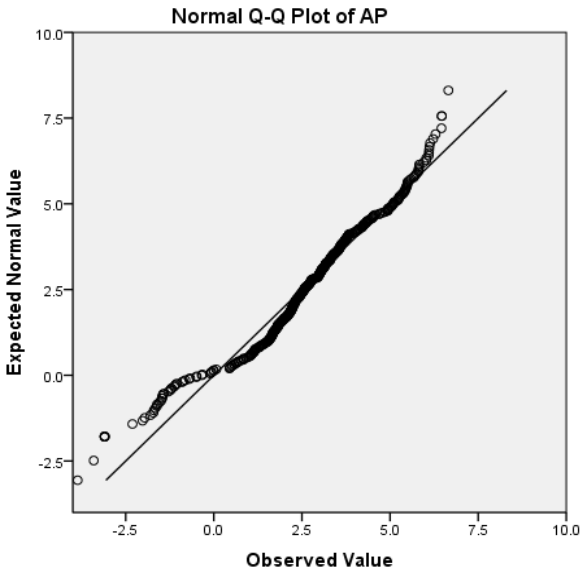




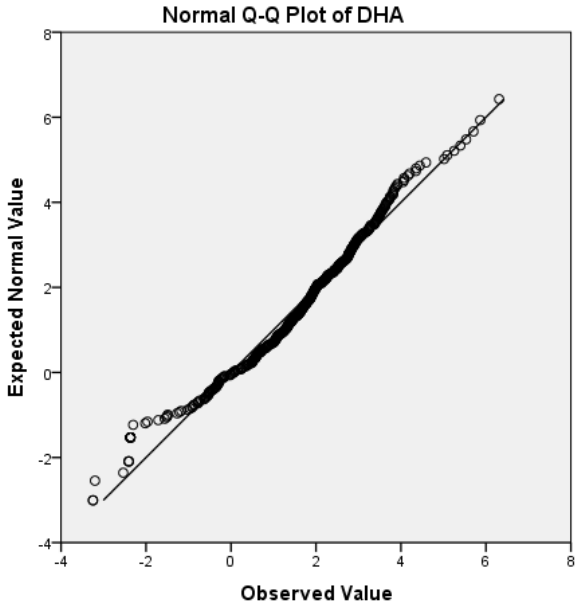
Significant differences lie between 1992 and 1994 DHA (p=0.002), 1992 and 1995 DHA (0.001), 1992 and 2013 DHA (0.000), 1993 and 2013 DHA (0.000), 1994 and 2013 DHA (0.001), 1993 and 1994 DHA (0.003), and 1993 and 1995 DHA (0.006). No significant differences were found between 1992 and 1993 DHA, 1995 and 2013 DHA, and 1994 and 1995 DHA.

Appendix K

Transformation Plots of Microbial Enzyme Activities using Natural Logarithm



Transforms: natural logarithm



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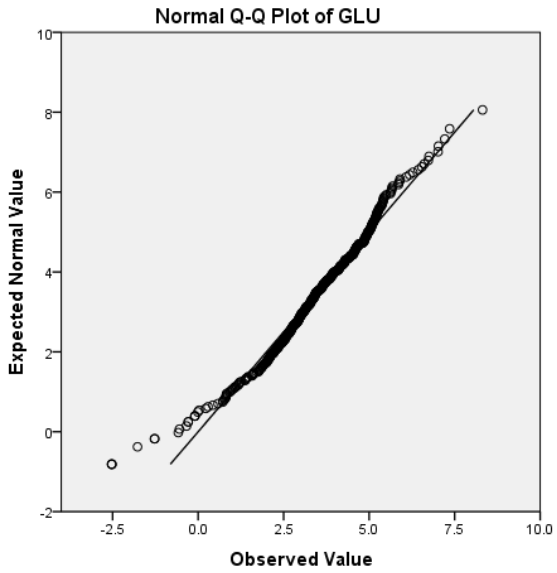

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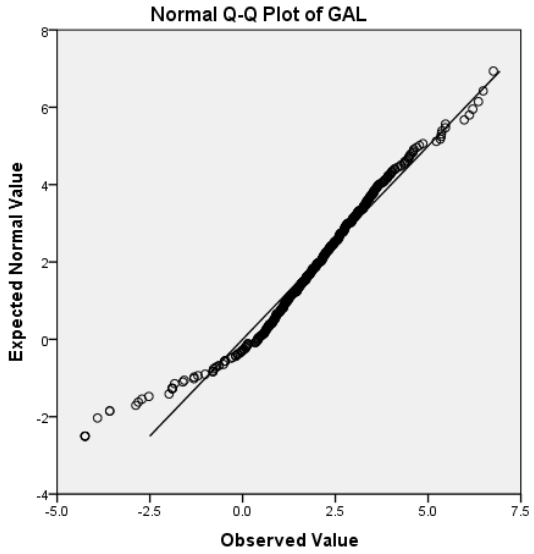
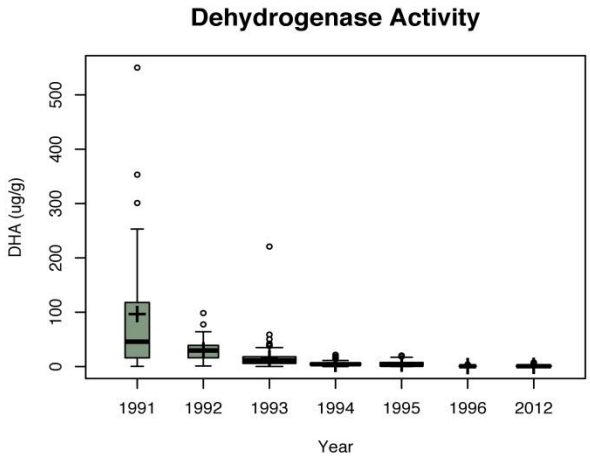
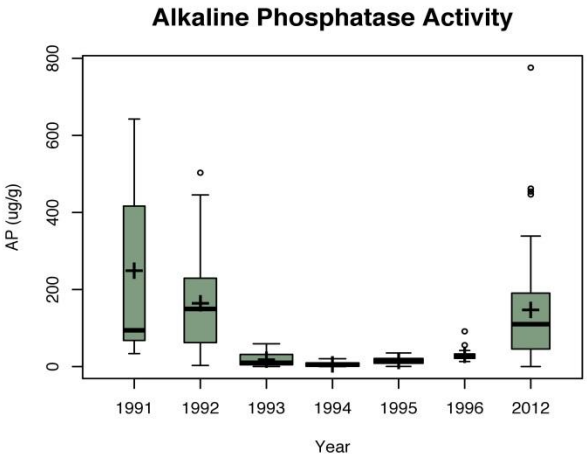


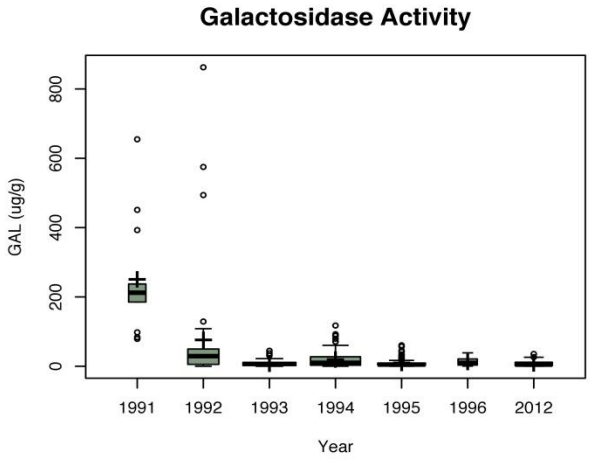
Transforms: natural logarithm

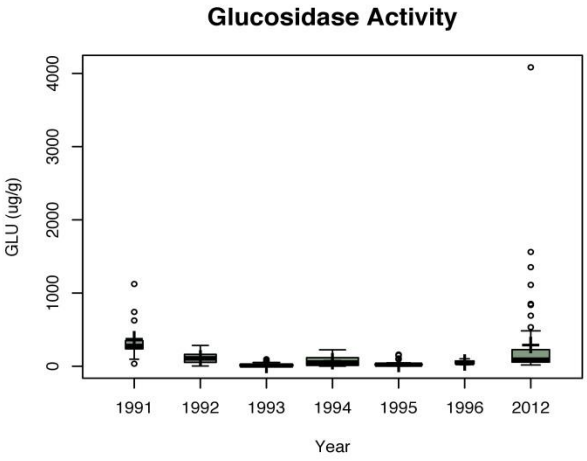
APPENDIX L

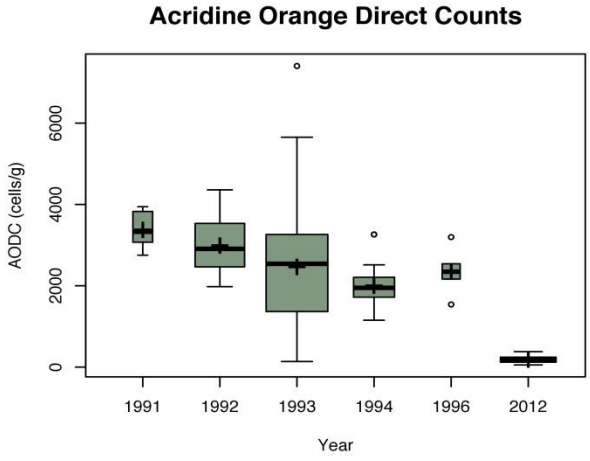
Box Plots of Microbial Enzyme Activities and Acridine Orange Direct Counts

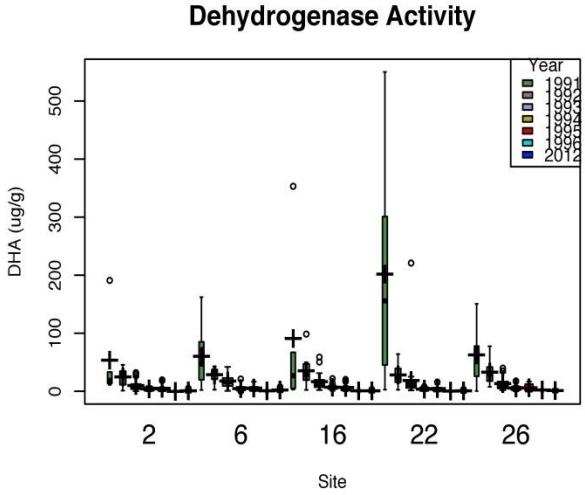


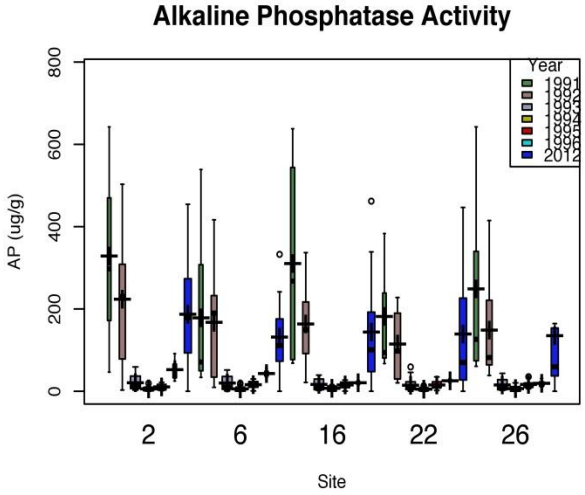


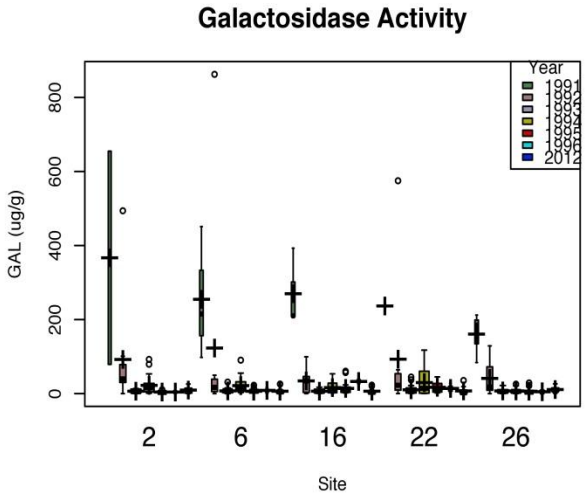


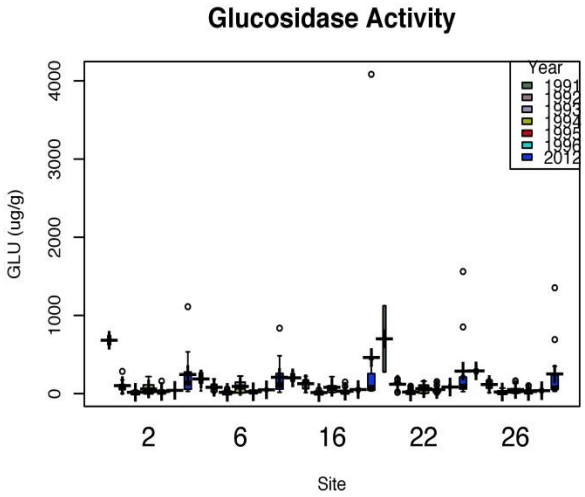


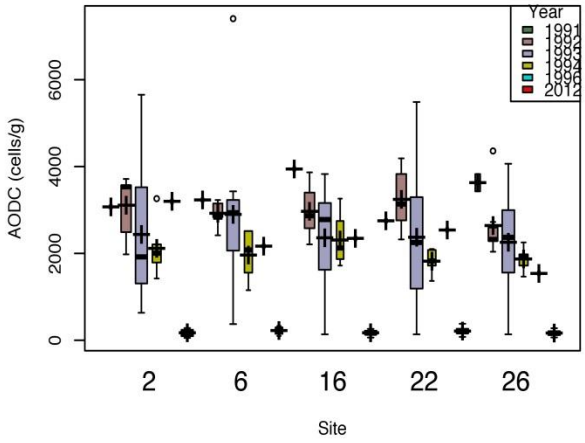


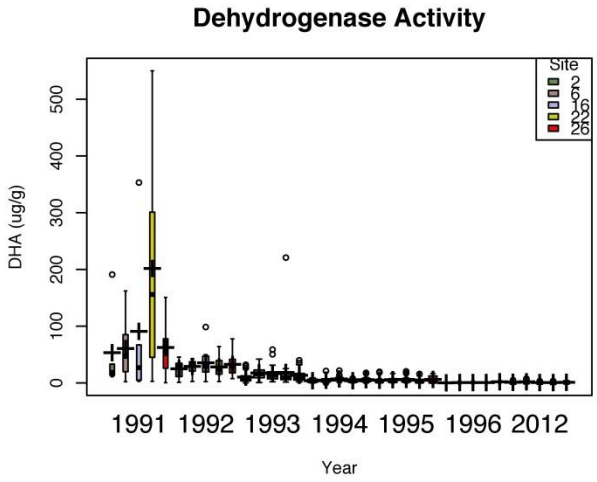

Dehydrogenase Activity
DHA (ug/g)
0
100
200
300
400
500
2
6
16
22
26
Site
Year
1991
2012

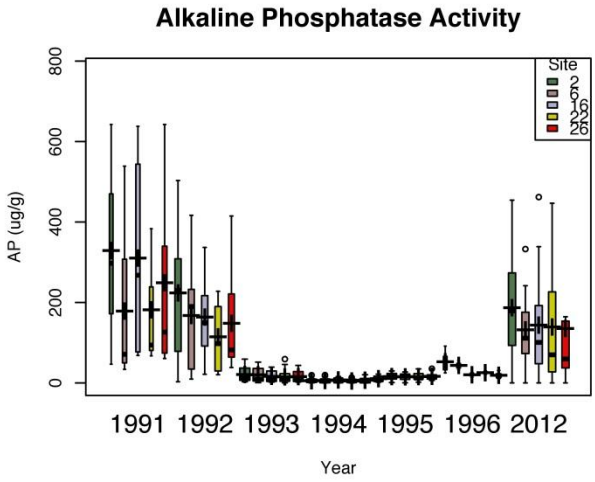

Alkaline Phosphatase Activity
AP (ug/g)
0
200
400
600
800
2
6
16
22
26
Site
Year
1991
2012

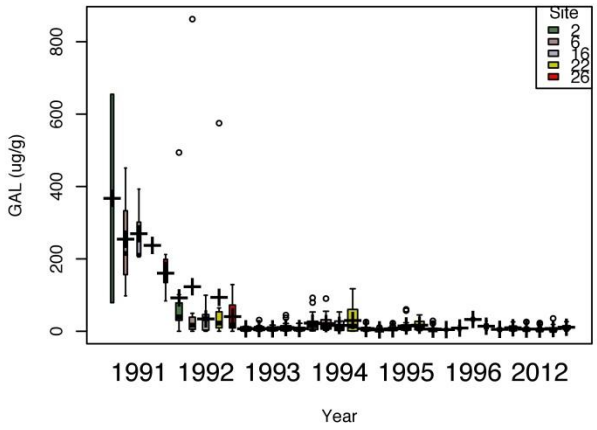

Galactosidase Activity
GAL (ug/g)
0
200
400
600
800
2
6
16
22
26
Site
Year

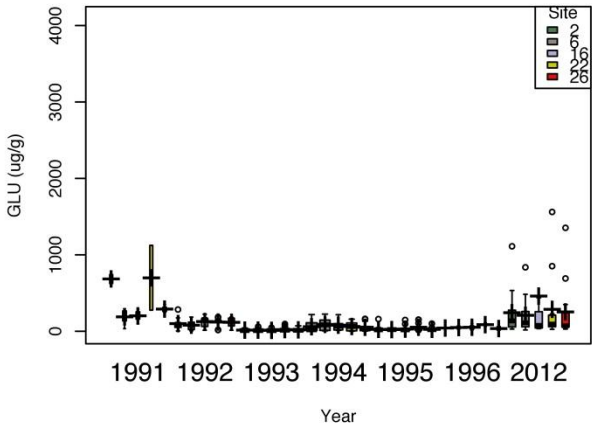

Glucosidase Activity
GLU (ug/g)
0
1000
2000
3000
4000
2
6
16
22
26
Site
Year

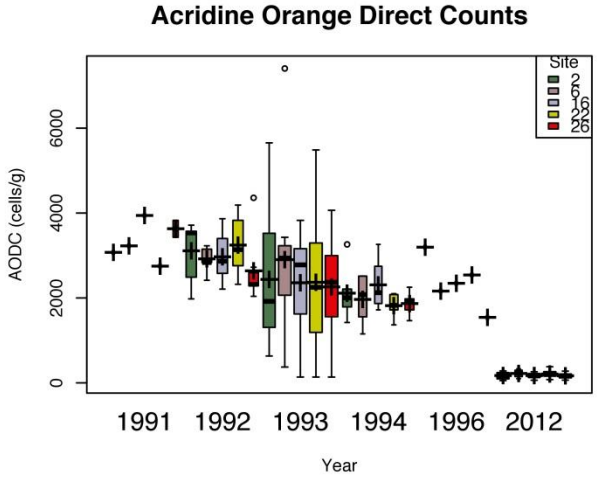

Acridine Orange Direct Counts
AODC (cells/g)
0
2000
4000
6000
2
6
16
22
26
Site
Year


Dehydrogenase Activity
DHA (ug/g)
0
100
200
300
400
500
Site
2
6
16
22
26
1991
1992
1993
1994
1995
1996
2012
Year


Alkaline Phosphatase Activity
AP (ug/g)
0
200
400
600
800
Site
2
6
16
22
26
1991
1992
1993
1994
1995
1996
2012
Year


Galactosidase Activity
GAL (ug/g)
0
200
400
600
800
Site
2
6
16
22
26
1991
1992
1993
1994
1995
1996
2012
Year


Glucosidase Activity
GLU (ug/g)
0
1000
2000
3000
4000
Site
2
6
16
22
26
1991
1992
1993
1994
1995
1996
2012
Year


Acridine Orange Direct Counts
AODC (cells/g)
0
2000
4000
6000
Site
2
6
16
22
26
1991
1992
1993
1994
1996
2012
Year

Appendix M

Data for Analysis

GALACTOSIDASE

YEARS

Site	1991 GAL	1992 GAL	1993 GAL	1994 GAL	1995 GAL	1996 GAL	12/13 GAL
2	227.4405	12.8638	3.5584	12.4113	3.358	2.746	3.153
6	211.7342	10.3965	3.2646	13.3203	5.4800	7.6781	3.010
16	257.2582	10.3038	4.6203	9.2506	8.8326	32.3595	3.2853
22	236.9247	17.1108	5.1312	7.9220	10.1383	8.6843	3.0951
26	148.8700	10.4167	4.3398	3.3439	4.7171	3.3826	8.2203

GALACTOSIDASE

TIME PERIODS

Site	91/92 GAL	93/94 GAL	95/96 GAL	12/13 GAL
2	120.1522	7.9849	3.0520	3.153
6	111.0654	8.2925	6.5791	3.010
16	133.781	6.9355	20.5961	3.2853
22	127.0178	6.5266	9.4113	3.0951
26	79.6434	3.8419	4.0499	8.2203

ALKALINE PHOSPHATASE

YEARS

Site	1991 AP	1992 AP	1993 AP	1994 AP	1995 AP	1996 AP	12/13 AP
2	207.1890	126.3617	13.9990	3.5943	7.9077	45.2321	83.1784
6	97.8256	97.4609	11.3176	2.2892	12.1209	42.3949	64.8975
16	202.4569	124.3685	10.7354	2.9405	10.8335	19.9544	59.1562
22	134.4632	81.9164	6.5578	2.4705	12.4701	25.4375	46.9785
26	165.4834	109.6685	9.7237	3.2390	14.6470	18.0745	45.5563

ALKALINE PHOSPHATASE

TIME PERIODS

Site	91/92 AP	93/94 AP	95/96 AP	12/13 AP
2	166.77535	8.79665	26.5699	83.1784
6	97.64325	6.8034	27.2579	64.8975
16	163.4127	6.83795	15.39395	59.1562
22	108.1898	4.51415	18.9538	46.9785
26	137.57595	6.48135	16.36075	45.5563

GLUCOSIDASE

YEARS

Site	1991 GLU	1992 GLU	1993 GLU	1994 GLU	1995 GLU	1996 GLU	12/13 GLU
2	681.2514	65.1179	8.1628	27.2102	17.2027	41.7831	134.7955
6	134.4183	51.6967	6.8015	49.9527	19.2285	45.5186	118.7216
16	183.2903	96.1292	6.4436	51.8367	18.4294	42.7485	120.7928
22	557.7857	93.0069	11.1161	27.3657	39.4910	85.1476	128.2725
26	287.2613	92.9032	9.5636	28.9566	23.7780	35.2511	116.8611

GLUCOSIDASE

TIME PERIODS

Site	91/92 GLU	93/94 GLU	95/96 GLU	12/13 GLU
2	373.18465	17.6865	29.4929	134.7955
6	93.0575	28.3771	32.37355	118.7216
16	139.70975	29.14015	30.58895	120.7928
22	325.3963	19.2409	62.3193	128.2725
26	190.08225	19.2601	29.51455	116.8611

DEHYDROGENASE

YEARS

Site	1991 DHA	1992 DHA	1993 DHA	1994 DHA	1995 DHA	1996 DHA	12/13 DHA
2	28.1258	16.8350	6.0910	3.444	2.7312	0.0388	0.4349
6	32.97325	22.8972	12.8516	3.3775	2.7333	0.3097	0.7553
16	24.5647	24.8867	12.6320	5.4641	3.1968	1.1236	0.5891
22	82.0258	20.6082	9.1368	3.1865	2.1370	0.5209	0.3481
26	23.3860	25.9411	9.5399	4.8829	3.3341	1.7906	0.7508

DEHYDROGENASE

TIME PERIODS

Site	91/92 DHA	93/94 DHA	95/96 DHA	12/13 DHA
2	22.4804	4.7675	1.385	0.4349
6	27.935225	8.11455	1.5215	0.7553
16	24.7257	9.04805	2.1602	0.5891
22	51.317	6.16165	1.32895	0.3481

26	24.66355	7.2114	2.56235	0.7508

ACRIDINE ORANGE DIRECT COUNTS- WATER

TIME PERIODS

Site	91/92 AODCW	93/94 AODCW	12/13 AODCW
2	283.4098	173.8359	287.5653101
6	298.6531	216.9711	296.3496479
16	367.2949	231.1687	233.427778
22	409.0781	209.3332	268.1643396
26	314.4611	158.9925	238.7865365

ACRIDINE ORANGE DIRECT COUNTS- WATER

YEARS

Site	1991 AODCW	1992 AODCW	1993 AODCW	1994 AODCW	1996 ADOCW	12/13 AODCW
2	307.9692684	258.8503253	178.654638	169.017118	232.758	287.5653101
6	284.2914658	313.0146517	225.0120252	208.930153	301.871	296.3496479
16	428.3754369	306.2144625	268.309954	194.0275191	230.442	233.427778
22	346.0788595	472.0773537	213.0524675	205.6138716	217.0222	268.1643396
26	350.724144	278.1980676	169.1471812	148.837803	194.41596	238.7865365

TOTAL MASS- AODCW

Site	1994	12/13
2	13234038.93	3163218.3
6	26835895.8	5334292.8
16	215952607.5	131886707
22	13282657.94	7776764.7
26	304373301	72591096

ACRIDINE ORANGE DIRECT COUNTS- SEDIMENT

TIME PERIODS

Site	91/92 AODCS	93/94 AODCS	12/13 AODCS
2	3054.395	2038.266	149.0676158
6	3069.334	2152.098	203.1815674
16	3433.063	1975.079	161.0578531
22	2968.453	1775.186	190.0758145
26	3092.431	1786.838	151.7489139

ACRIDINE ORANGE DIRECT COUNTS- SEDIMENT

YEARS

Site	1991 AODCS	1992 AODCS	1993 AODCS	1994 AODCS	1996 AODCS	12/13 AODCS
2	3071.4116	3037.378236	2031.162638	2045.368675	3197.1018	149.0676158
6	3229.233	2909.435298	2422.365933	1881.830025	2164.61977	203.1815674
16	3944.194	2921.931064	1708.435525	2241.722898	2344.904605	161.0578531
22	2751.771	3185.135149	1753.145122	1797.22664	2540.2048	190.0758145
26	3622.790992	2562.071599	1719.863145	1853.813226	1540.712	151.7489139

TOTAL MASS- AODCS

Site	1994	12/13
2	160152392.7	1639743.6
6	242756070	3657268.21
16	2495037699	90997685.25
22	116100864.2	5512198.2
26	3791047585	46131665.6

AIR TEMPERATURE- degrees Celsius

TIME PERIODS

Site	91/92 AT	93/94 AT	95 AT	12/13 AT
2	12.80	16.20	13.10	9.41
6	15.90	15.95	10.30	10.69
16	18.90	18.75	10.50	11.00
22	18.65	19.80	11.80	11.79
26	17.00	12.00	11.80	10.19

AIR TEMPERATURE- degrees Celsius

YEARS

Site	1991 AT	1992 AT	1993 AT	1994 AT	1995 AT	12/13 AT
2	12.4	13.2	15.6	16.8	13.1	9.41
6	18.1	13.7	15.5	16.4	10.3	10.69
16	21.4	16.4	18.1	19.4	10.5	11
22	20.9	16.4	19.8	19.8	11.8	11.79
26	17.2	16.8	19.2	20.3	11.8	10.19

WATER TEMPERATURE- degrees Celsius

TIME PERIODS

Site	91/92 WT	93/94 WT	95 WT	12/13 WT
2	9.65	10.25	8.20	9.43
6	12.35	12.00	7.60	10.28
16	13.70	13.50	7.30	11.10
22	16.15	15.20	9.20	11.92
26	12.00	12.20	7.80	10.86

WATER TEMPERATURE- degrees Celsius

YEARS

Site	1991 WT	1992 WT	1993 WT	1994 WT	1995 WT	12/13 WT
2	8.8	10.5	10.3	10.2	8.2	9.43
6	13.7	11	12.4	11.6	7.6	10.28
16	15.3	12.1	13.4	13.6	7.3	11.10
22	16.7	15.6	15.1	15.3	9.2	11.92
26	13.2	10.8	11.8	12.6	7.8	10.86

pH

TIME PERODS

Site	91/92 pH	93/94 pH	95 pH	12/13 pH
2	7.40	7.41	7.56	6.69
6	7.27	7.51	7.56	6.62
16	7.30	7.44	7.72	6.27
22	7.73	7.80	7.64	6.36
26	7.38	7.18	7.79	6.25

pH

YEARS

Site	1991 pH	1992 pH	1993 pH	1994 pH	1995 pH	12/13 pH
2	7.42	7.38	7.42	7.4	7.56	6.69
6	7.32	7.21	7.44	7.58	7.56	6.62
16	7.31	7.28	7.38	7.49	7.72	6.27
22	8.15	7.31	7.9	7.7	7.64	6.36
26	7.49	7.27	7.29	7.07	7.79	6.25

DISSOLVED OXYGEN- mg/L

TIME PERIODS

Site	91/92 DO	93/94 DO	95 DO	12/13 DO
2	10.99	8.79	7.16	10.31

6	9.94	8.31	7.52	9.29
16	9.92	8.48	7.44	9.94
22	9.71	8.34	7.58	8.84
26	10.22	8.62	6.56	9.83

DISSOLVED OXYGEN- mg/L

YEARS

Site	1991 DO	1992 DO	1993 DO	1994 DO	1995 DO	12/13 DO
2	10.94	11.04	10.47	7.11	7.16	10.31
6	9.11	10.77	9.35	7.27	7.52	9.29
16	8.79	11.04	9.51	7.45	7.44	9.94
22	9.06	10.36	9.19	7.49	7.58	8.84
26	9.49	10.94	10.01	7.22	6.56	9.83

CONDUCTIVITY-TIME PERIODS- in µs/cm

Site	91/92 COND	93/94 COND	95 COND	12/13 COND
2	144.65	312.1	390.9	426.33
6	90.1	241.75	187	225.78
16	52.65	130.1	90	95.14
22	130.9	196.2	196.8	317.72
26	24.1	30.85	55.1	14.875

CONDUCTIVITY- YEARS- in µs/cm

Site	91 COND	92 COND	93 COND	94 COND	95 COND	12/13 COND
2	136.6	150.7	249.5	374.7	390.9	426.33
6	92	88.2	225.2	258.3	187	225.78
16	56.4	48.9	127.4	132.8	90	95.14
22	120.3	141.5	164.3	228.1	196.8	317.72
26	36.2	12	27	34.7	55.1	14.88

DEPTH-TIME PERIODS (in centimeters)

Site	91/92 DEPTH	93/94 DEPTH	95 DEPTH	12/13 DEPTH
2	4.965	5.395	6.28	3.45
6	10.005	11.345	15.32	3.82
16	18.065	22.97	29.28	30.12
22	10.51	11.445	20.93	8.94
26	32.085	31.395	33.39	25.82

DEPTH- YEARS- (in centimeters)

Site	91 DEPTH	92 DEPTH	93 DEPTH	94 DEPTH	95 DEPTH	12/13 DEPTH
2	4.57	5.36	4.23	6.56	6.28	3.45
6	5.83	14.18	7.72	14.97	15.32	3.82
16	16.86	19.27	19.92	26.02	29.28	30.12
22	14	7.02	6.97	15.92	20.93	8.94
26	28.83	35.34	30.64	32.15	33.39	25.82

DEPTH- YEARS- (in meters)

Site	91 DEPTH	92 DEPTH	93 DEPTH	94 DEPTH	95 DEPTH	12/13 DEPTH
2	0.0457	0.0536	0.0423	0.0656	0.0628	0.0345
6	0.0583	0.1418	0.0772	0.1497	0.1532	0.0382
16	0.1686	0.1927	0.1992	0.2602	0.2928	0.3012
22	0.14	0.0702	0.0697	0.1592	0.2093	0.0894
26	0.2883	0.3534	0.3064	0.3215	0.3339	0.2582

WIDTH-TIME PERIODS- (in meters)

Site	91/92 WIDTH	93/94 WIDTH	95 WIDTH	12/13 WIDTH
2	0.98	1.055	1.04	1.26
6	1.38	1.795	2.19	1.25
16	5.5	4.89	5.32	5.04
22	2.275	2.04	1.72	1.94
26	5.02	4	6.63	6.88

DEPTH- YEARS- (in meters)

Site	91 DEPTH	92 DEPTH	93 DEPTH	94 DEPTH	95 DEPTH	12/13 DEPTH
2	0.0457	0.0536	0.0423	0.0656	0.0628	0.0345
6	0.0583	0.1418	0.0772	0.1497	0.1532	0.0382
16	0.1686	0.1927	0.1992	0.2602	0.2928	0.3012
22	0.14	0.0702	0.0697	0.1592	0.2093	0.0894
26	0.2883	0.3534	0.3064	0.3215	0.3339	0.2582

WIDTH- YEARS (in meters)

Site	91 WIDTH	92 WIDTH	93 WIDTH	94 WIDTH	95 WIDTH	12/13 WIDTH
2	0.99	0.97	0.89	1.22	1.04	1.26
6	1.07	1.69	1.71	1.88	2.19	1.25
16	5.33	5.67	4.42	5.36	5.32	5.04
22	2.12	2.43	2.05	2.03	1.72	1.94

26	4.00	6.04	3.97	4.03	6.63	6.88

FLOW- YEARS- meters/sec

Site	1994 FLOW	1995 FLOW	12/13 FLOW
2	0.978	0.49	0.248
6	0.46	0.17	0.386
16	0.798	1.643	0.372
22	0.2	0.1	0.168
26	1.578	3.002	0.171

DISCHARGE- YEARS = DEPTH x WIDTH x FLOW = CMS or Cubic meter/Second

Site	1994 DISCHARGE	1995 DISCHARGE	12/13 DISCHARGE
2	0.0783	0.032	0.011
6	0.129	0.057	0.018
16	1.113	2.559	0.565
22	0.0646	0.036	0.029
26	2.045	6.747	.304

STANDARD PLATE COUNT

Site	Year: 2012-2013
2	1937.9
6	2705.4
16	2242.9
22	7954.5
26	864.91

TOTAL MASS- SPC- CFU/sec

Site	12/13
2	16340960.9
6	38337903
16	1087136049
22	89503604.8
26	211123531.2

NOAA TEMPERATURES

Year	Degrees Celsius
1991	14.1
1992	12.9

1993	13.4
1994	13.3
1995	13.2
1996	12.8
12/13	12.8

NOAA PRECIPITATION

Year	**Total inches**
1991	47.29
1992	55.7
1993	41.2
1994	50.29
1995	53.67
1996	48.06
12/13	58.97

Appendix N

Water Quality Monitoring Program Developed by Scheuerman et al., 1995 Parameters

Temperature
pH
Dissolved oxygen
Conductivity
Flow
Acidity
Total solids
Total dissolved solids
Total settleable solids
Magnesium
Manganese
Calcium
Turbidity
Aluminum
Zinc
Copper
Iron
Sulfate
Chloride
Alkalinity
Hardness
Aquatic macroinvertebrates

VITA

CLARA MCCLURE

Personal Data:	Date of Birth: December 30, 1986 Place of Birth: Nashville, Tennessee Marital Status: Single
Education:	B.A. Psychology University of Tennessee, Knoxville, Tennessee 2009
	M.S. Environmental Health East Tennessee State University, Johnson City, Tennessee, 2013
Professional Experience:	Research Assistant II at Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee
	Instructor, Bioanalysis, Department of Environmental Health, ETSU, Fall, 2012
	Instructor, Environmental Analysis, Department of Environmental Health, ETSU, Spring, 2012
	Graduate Assistant, East Tennessee State University, 2010-2013
Awards:	Graduate School Research Grant, East Tennessee State University for "Long-term Recovery of South Indian Creek following Interstate Construction"