ABSTRACT

Bacterial Dynamics at the Sediment-Water Interface
of a Stratified, Eutrophic Reservoir

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Sediment-water interfaces (SWIs) are loci of dynamic physical, chemical, and biological interactions in stratified, eutrophic reservoirs. Seasonal reservoir mixing and stratification affects SWI physicochemical processes as well as bacterial abundance, diversity, biomass, and metabolism. Because SWI bacteria transform chemicals and release nutrients that affect water quality and eutrophication, seasonal changes in these bacterial dynamics help define reservoir carbon and nutrient cycles and trophic interactions.

Four studies were conducted to assess SWI bacterial dynamics in Belton Reservoir, a eutrophic, monomictic impoundment. The first utilized [^{3}H]-L-serine to measure SWI bacterial activity and biomass production. Highest activity and production occurred during summer stratification under anoxic conditions. Lowest activity and production occurred under oxic conditions during autumnal overturn and winter mixing. The second study consisted of two parts, both utilizing Biolog EcoPlates to measure SWI carbon substrate utilization rates (CSURs). The first part tested the effectiveness and

interpretability of EcoPlates. Optimal use was dependent upon inoculum density, incubation temperature, and aerobic/anaerobic incubation techniques. The second part concluded that CSURs for carbohydrates were highest during onset of stratification and winter mixing, CSURs for amino acids were highest during winter mixing, and CSURs for carboxylic acids were highest during late season stratification. The third study analyzed quantities and sources of SWI carbon, nitrogen, and bulk organic matter (OM). OM concentration did not differ among seasons. Inorganic carbon and nitrogen differed seasonally. OM C/N ratios and stable isotopes (^{13}C and ^{15}N) were significantly different at the SWI of the shallowest depths, indicating that OM at this site was of allochthonous origin. The last study utilized automated ribosomal intergenic spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE) to elucidate total and sulfate-reducing (SRB) SWI bacterial diversity and similarity. Total SWI bacterial diversity did not significantly differ. During stratification, high similarity occurred among sites on individual dates. During mixing, high similarity occurred through time. Although SRB are functionally strict anaerobes, they exhibited higher richness during oxic rather than anoxic conditions.

Bacterial Dynamics at the Sediment-Water Interface
of a Stratified, Eutrophic Reservoir

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DEDICATION

To Dad,
You always said I could do this,
and as always, you were right

CHAPTER ONE

Introduction and Background

What are Sediment-Water Interfaces?

No established or common definition exists for sediment-water interfaces (SWIs) (Hulbert et al. 2002). Often the definition is a function of the biological, physicochemical, or geological study being conducted. Mortimer, who conducted the first studies on lake SWI chemical dynamics, described the SWI as a 'frontier between two very different domains' (Mortimer 1941, 1942, 1971). Other early SWI studies were conducted on marine systems and were primarily chemical investigations (Santschi et al. 1990). The common theme of these studies noted that SWIs were not just physical barriers between solid and liquid phases, but also sites of steep gradients in dissolved oxygen, pH, redox potentials, and inorganic and organic chemistry (Stumm 2004). As sampling methodology and resolution improved along with a better appreciation of microbial metabolism, it was revealed that bacteria were responsible for many SWI chemical transformations (Jones 1979; Novitsky 1983; Schallenberg and Kalff 1993).

Current literature classifies SWIs as viscous zones between overlying water and deposited sediment in aquatic ecosystems accompanied by steep changes in chemical gradients due to microbiological metabolic processes (Boudreau and Jørgensen 2001; Bloesch 2004). These microbiological processes are primarily bacterial, often involving degradation (oxidation) of organic carbon with concomitant reduction of an electron acceptor (Liikanen and Martikainen 2003). The reductions of various electron acceptors,

often multiple acceptors within millimeter gradients, render SWIs chemically unique. Reduced compounds, as various bound molecules of carbon, nitrogen, iron, sulfur, phosphorus, and trace metals, affect the water column nutrient dynamics. Often, these bacterially-mediated chemical releases substantially impact eutrophication and ecosystem water quality (Beutel 2003).

Current knowledge of SWI physical, chemical, and biological processes is overwhelmingly derived from marine investigations (Boudreau and Jørgensen 2001). Further, SWI microbial studies are often single time-point investigations that overlook seasonal ecosystem changes (e.g. stratification, temperature gradients, and weather events) or are based on mesocosm, rather than *in situ*, studies (Rosselló-Mora et al. 1999; Ding and Sun 2005). Thus seasonal changes in freshwater (especially lake and reservoir) SWI bacterial dynamics are lacking.

In this investigation, bacterial dynamics (i.e. abundance, activity, biomass production, carbon substrate utilization, and diversity) and corresponding physicochemical dynamics (dissolved oxygen, redox potential, temperature, pH, etc.) were measured at the SWI in a stratified, eutrophic reservoir throughout seasonal mixing and stratification cycles. Sources and fates of SWI organic matter were also studied. The results demonstrate that highly diverse and active SWI bacterial communities conduct many nutrient transformations that impact the sediment chemistry, and these bacterial processes are tremendously influenced by key reservoir seasonal mixing and stratification events. On a broader scope, this investigation bolsters the basic tenet of ecology—linking the living and the nonliving and how these interactions impact overall ecosystem processes.

Characteristics of Reservoir Sediment-Water Interfaces

Physical (Transport) Processes

Transport of substances to and from the SWI occurs via three processes: 1) diffusion of dissolved substances to and from sediments, 2) transport of particle-associated substances (i.e. burial) to the sediment surfaces, and 3) bioturbation (Santschi et al. 1990; Austen et al. 2002).

Dissolved nutrient and ion diffusion into sediments is a function of the sediment porosity and compactness. In clay-rich lake sediments, this depth may be only a few millimeters due to high compactness of the sediment matrix. Low water content is often characteristic of clay-rich sediments, often rendering diffusive processes relatively unimportant in SWI biogeochemical dynamics (Huettel et al. 2003). Above the sediment surface is the diffusive sublayer, consisting of the dissolved substances that freely diffuse into the sediments. This layer can vary from less than 0.1 mm to several mm based on friction velocity and bottom stress due to water mixing dynamics (Higashino and Stefan 2004).

Particle-associated transport is primarily through the deposition of particulate organic matter (POM). Depending on reservoir trophic status (inputs of autochthonous material) or surrounding landscape (inputs of allochthonous material), rates of POM deposition may vary from millimeters to several centimeters per year. Substantial resuspension of deposited organic matter may occur depending on reservoir mixing dynamics including internal seiches, internal breaking waves, and plunging flows (Gantzer and Stefan 2003). Sources of POM are often determined from their stable isotope (^{13}C and ^{15}N) profiles as well as C/N ratios (Meyers and Teranes 2001).

Bioturbation is the process of living organisms affecting SWI particle and diffusion dynamics. In reservoirs these processes are conducted exclusively by bacterial processes in anoxic SWIs (minimal bioturbation), while in oxic SWIs macroinvertebrates (e.g. oligochaetes, insects, mollusks) may substantially disturb the sediments. For example, chironomid larvae can migrate vertically through the sediment surface influencing the rate of particle and pore water exchange through the SWI (Forja and Gómez-Parra 1998).

Mixing Processes

SWI temperature, dissolved oxygen, and pH are partially dependent upon mixing of the water column above the sediments (Brune et al. 2000). In monomictic eutrophic reservoirs, thermal stratification is prevalent during summer. As warm and calm weather conditions prevail during late spring and early summer, the upper waters (epilimnion) become warmer than the deeper waters (hypolimnion), forming a large temperature gradient throughout the water column. These density differences prevent the water column from mixing. Intense bacterial activity in the hypolimnion along with lack of dissolved oxygen diffusion into the hypolimnion results in hypolimnetic anoxia. This anoxic layer blankets the sediments, affecting physical, chemical, and biological SWI processes (Horne and Goldman 1994; Wetzel 2001).

As weather conditions become cooler in the fall, along with rain and wind events, epilimnetic and hypolimnetic density differences become negligible, and the epilimnetic waters mix with the anoxic hypolimnetic waters. This process replenishes dissolved oxygen to the sediments, but ultimately decreases SWI temperature (Beutel 2003). In

addition the mixing events affect SWI physical transport processes and the stability of SWI bacterial communities.

Biogeochemical Processes and Redox Potential

Chemical dynamics at SWIs are mediated by bacterial metabolic processes. Not only are particulate and dissolved inorganic and organic compounds deposited at and diffuse through the SWI, but they are transformed, mineralized, and recycled by the bacteria (Rosselló-Mora et al. 1999). Many SWI bacteria are heterotrophs, requiring a source of organic carbon for their metabolism (i.e. oxidation). Organic carbon is oxidized by the bacteria while they utilize various electron acceptors in respiratory processes (Liikanen and Martikainen 2003). These electron acceptors are various ions of oxygen, nitrogen, manganese, iron, and sulfur (Kelly et al. 1988). In addition, silicon, hydrogen, phosphorus, and trace metal-containing compounds are required and utilized in SWI bacterial metabolic processes (Nealson 1997).

Electron acceptors are reduced by the SWI bacteria in a sequential order based on decreasing redox potential and decreasing energy yield. This order also follows a vertical depth gradient at the SWI. The vertical SWI redox gradient varies both spatially and temporally depending on selective pressures imposed by the reservoir's seasonal physicochemical changes (Santschi et al. 1990).

Carbon. Of the major elements consumed by SWI bacteria, carbon is present in excess relative to their need. Most of this carbon is present in an organic form. Approximately 50% of the dry organic matter at the SWI in freshwater ecosystems is composed of organic carbon (Bloesch 2004). Three reasons exist for the abundance of SWI organic carbon: 1) high epilimnetic primary productivity and subsequent sinking of

fixed carbon, 2) low respiration rates that decrease organic matter oxidation, and 3) inputs of allochthonous organic matter (Atlas and Bartha 1998).

Organic carbon is present at the SWI in two forms: 1) particulate organic matter (POM) from deposition of autochthonous and allochthonous sources; or decay and degradation of large polymeric substances, and 2) dissolved organic matter (DOM), usually in the porewater or at the diffusive boundary layer, often resulting from decay of, or excretion from, various organisms (Jonsson et al. 2001). Much DOM and POM is recalcitrant, unavailable to the bacteria as a substrate. The remaining (i.e. labile) OM is present as low molecular weight (LMW) and high molecular weight (HMW) substances (Wirtz 2003). Various consortia of heterotrophic bacteria degrade the labile OM, mineralizing the carbon to CO_2, or forming smaller organic molecules (e.g. amino acids, carbohydrates), which are further oxidized by other heterotrophic bacteria (Rosenstock et al. 2005). Labile OM varies often on a vertical scale, with surface sediment OM undergoing higher rates of oxidation than deeper sediments which are more impervious to degradation (Vreča 2003).

SWI organic carbon is oxidized by heterotrophic bacteria under aerobic and anaerobic conditions. A greater number of carbon transformations occur via aerobic respiration, including oxidation of large polymeric substances (Ding and Sun 2005). Via anaerobic respiration, heterotrophic bacteria reduce a variety of electron acceptors in a predictable order (e.g. nitrate, ferric iron, sulfate, carbon dioxide), however each of these reactions yields less energy than aerobic respiration (Nealson and Stahl 1997). Further, many SWI bacteria undergo fermentative, rather than respirative, metabolism which are independent of redox processes. In fermentation, an organic compound serves as the

terminal electron acceptor and yields less energy than aerobic and anaerobic respiration (Bastviken et al. 2001). In addition, end-products of fermentation are LMW-organic compounds such as small acids and alcohols, which can be further utilized by other SWI bacteria (Ding and Sun 2005).

Inorganic carbon (as CO_2) also plays a unique role in SWI bacterial dynamics. While CO_2 fixation in the well-lit epilimnion occurs primarily via autotrophic phyto- and bacterioplankton, SWI CO_2 fixation is primarily via methanogenic archaea, producing methane (Liikanen and Martikainen 2003). This CO_2 reduction occurs as the final step in redox-dependent reductions, after sulfates have been depleted as the terminal electron acceptor (Nealson and Stahl 1997). Unlike autotrophic CO_2 fixation that results in gross primary production (i.e. production of organic compounds), methanogenesis is strictly a chemolithotrophic process (Casper 1992).

Oxygen. Thermodynamically, oxygen (O_2) is the preferred electron acceptor by SWI heterotrophic bacteria. Not only is O_2 used by aerobic bacteria, but it is also preferentially utilized by facultative anaerobic bacteria over other, less energetically favorable electron acceptors (Ding and Sun 2005). Under aerobic conditions, redox potential is maintained from +600 to +450 mV (Nealson and Stahl 1997). Sources of O_2 to the SWI are from: 1) photosynthetic O_2 production in overlying waters, 2) infusion of dissolved O_2 into the water column from the atmosphere, as a function of water temperature, and 3) cycling in different mineral reservoirs such as nitrate, sulfate, and carbonate (Atlas and Bartha 1998).

In oligotrophic waters, low concentrations of organic matter does not place a demand on dissolved O_2, hence all bacterial respiration is aerobic. In eutrophic lakes,

dissolved O_2 is depleted on a seasonal basis, forming an anoxic hypolimnion due to bacterial O_2 consumption that occurs faster than O_2 replenishment from the aerobic epilimnion (Kelly et al. 1988). However, in both oligotrophic and eutrophic environments, the SWI is relatively impermeable to O_2 below a depth of several millimeters or centimeters, depending on the composition and consistency of the sediments (Brune et al. 2000). Thus dissolved O_2 penetration, and hence redox gradients, are much more steep and pronounced at the sediment surface than in the above water column (Santschi et al. 1990).

Nitrogen. Nitrogen exists in various particulate and dissolved organic forms in aquatic ecosystems, often as a component of amino acids in proteins (Danovaro et al. 1998). Upon dissolved oxygen depletion, nitrate (NO_3^-) becomes the preferred electron acceptor by heterotrophic SWI bacteria. This is accompanied by a sediment redox potential lower than +400 mV. Bacterial NO_3^- reduction occurs via a dissimilatory pathway producing either nitrite (NO_2^-) or nitrogen gas (N_2) while oxidizing organic carbon. NO_3^- reduction to NO_2^- is known as dissimilatory nitrate reduction, while reduction to N_2 (via an NO_2^- intermediate) is known as denitrification (Capone 2002). Many of the bacteria that reduce NO_3^- are facultative anaerobes, containing membrane bound NO_3^- and/or NO_2^- reductases that are inhibited by O_2 (Liikanen and Martikainen 2003). Hence, if O_2 is present it is preferentially reduced instead of NO_3^-. Highest rates of dissimilatory NO_3^- reduction and denitrification occur at the SWI during the onset of stratification as dissolved O_2 becomes depleted and redox potential decreases. Organisms that perform dissimilatory NO_3^- reduction often convert NO_2^- to free ammonium ions (NH_4^+) via ammonification (Sweerts et al. 1991).

NO_3^- is also assimilated into many bacteria via assimilatory NO_3^- reduction. NO_3^- is taken up by bacteria and reduced to ammonia (NH_3) or NH_4^+ which is then incorporated into amino acids (Nealson and Stahl 1997). Unlike dissimilatory NO_3^- reduction, assimilatory NO_3^- reduction is independent of O_2 and inhibited by NH_4^+ (Atlas and Bartha 1998). Also, many bacteria other than dissimilatory NO_3^- reducers and denitrifiers can assimilate NO_3^-. In addition, NH_4^+ can be directly assimilated into bacteria and higher trophic organisms to build amino acids and protein biomass (Wheeler and Kirchman 1986).

Upon return of oxic conditions to the SWI, various chemolithotrophic bacteria can oxidize NH_4^+ to NO_2^- or NO_3^- while assimilating CO_2 via a process called nitrification (Capone 2002). Nitrification is oxygen-dependent, therefore counterbalancing denitrification during weak oxic/anoxic gradients. However, nitrification processes are difficult to measure; therefore it is uncertain if this process can oxidize high quantities of NH_4^+ in sediments (Tomaszek and Czerwieniec 2003).

While free molecular nitrogen (N_2) is present in the water column, and presumably the sediments, fixation of N_2 into biomass is primarily conducted via cyanobacteria, a photosynthetic process. Some free living aerobic heterotrophs can fix N_2 (e.g. *Azotobacter*), but are believed to be quantitatively unimportant in SWI nitrogen cycles (Atlas and Bartha 1998).

Because NO_3^- reduction and denitrification are bacterial processes, biogeochemical nitrogen cycling rates depend on the presence, abundance, and activity of specific functional guilds of bacteria expressing the genes required for nitrogen transformation processes (Capone 2002; Taroncher-Oldenburg et al. 2003). Bacterial

genes responsible for nitrogen cycling are diverse and found in various metabolically defined bacterial groups. Denitrifying bacteria are not defined phylogenetically because denitrification genes are found in over 50 diverse genera (Braker et al. 1998; Hallin and Lindgren 1999). Instead, denitrifying bacterial diversity is defined through identification of base sequence differences in *nir* (NO_2^- reductase) genes. Nitrite reductase genes (*nirS* or *nirK*) are unique to, but ubiquitous in, denitrifying bacteria and distinguish denitrifiers from nitrate respirers (Braker et al. 1998; Hallin and Lindgren 1999).

Iron. Upon depletion of NO_3^- as an electron acceptor and redox potential decrease to +200 mV, ferric iron (Fe^{3+}) is utilized by SWI bacteria as an electron acceptor. This process of dissimilatory iron reduction forms ferrous iron (Fe^{2+}) which remains soluble under anoxic conditions (McMahon 1969). Dissimilatory iron reduction is inhibited by NO_3^- (Hyacinthe et al. 2006). Often Fe^{3+} is in the form of ferric oxyhydroxide (FeOOH) or iron phosphate ($FePO_4$), which becomes reduced by the bacteria while oxidizing small organic acids and alcohols. The resulting Fe^{2+} often becomes complexed to various compounds, forming siderite ($FeCO_3$) or iron sulfides (FeS_2); the latter causing black discoloration of sediments (Lovley and Phillips 1988). Assimilatory iron reduction is independent of NO_3^- concentration and redox potential because all bacteria require iron as a cofactor. Fe^{2+} assimilation thus occurs under aerobic or anaerobic conditions via secretion of siderophores that chelate iron to allow uptake (Mills 2002).

Iron oxidation occurs at SWIs when oxic conditions return to sediments. Fe^{2+} is unstable in the presence of oxygen and spontaneously oxidizes to Fe^{3+}. However, at low

pH Fe^{2+} is stable enough to be oxidized by various aerobic chemolithotrophic bacteria (Buffle et al. 1989).

Sulfur. At a redox potential of approximately 0 mV, O_2, NO_3^- and Fe^{3+} are depleted at the SWI, thus sulfate (SO_4^{2-}) is the preferred bacterial electron acceptor (Atlas and Bartha 1998). This process is known as dissimilatory SO_4^{2-} reduction, and is inhibited by O_2, NO_3^- and Fe^{3+}. SO_4^{2-} reducing bacteria are strict anaerobes that include various heterotrophs and chemolithotrophs that produce hydrogen sulfide (H_2S) (Hines et al. 2002). Because organic carbon is also oxidized by heterotrophic bacteria in more energetically favorable redox-dependent reactions, organic compounds are often depleted when redox potential conditions are favorable for SO_4^{2-} reduction, selecting for bacterial taxa that undergo chemolithotrophic metabolism (Karr et al.2005).

H_2S resulting from dissimilatory SO_4^{2-} reduction has a toxic effect on aquatic plants and animals and antimicrobial properties. H_2S has a characteristic 'rotten egg' smell that often causes taste, odor, and aesthetic problems in aquatic ecosystems. Often the H_2S combines with various metals in sediments, such as iron, to produce metal sulfides (Geets et al. 2006). These complexed sulfides often form black precipitates, causing sediments to appear solid black.

The key enzyme in dissimilatory SO_4^{2-} reduction is dissimilatory sulfite reductase, coded by the *dsrB* gene, ubiquitously found in all SO_4^{2-} reducing bacteria, which catalyzes the reduction of sulfite to sulfide (Minz et al. 1999). Bacteria containing the genes that code for the dissimilatory sulfite reductase enzyme are phylogenetically diverse and are found in many anaerobic bacteria and at least one species of Archaea (Dar et al. 2005).

Assimilatory SO_4^{2-} reduction is not inhibited by O_2, NO_3^-, or Fe^{3+}. However due to the toxic effects of H_2S, sulfur must be assimilated by bacteria in the form of SO_4^{2-}. SO_4^{2-} is then reduced intracellularly and is incorporated into sulfur-containing compounds such as cysteine or stored in cellular sulfur deposits (Hines et al. 2002).

Upon oxygen replenishment to the SWI, a variety of obligate aerobic chemolithotrophic and chemoautotrophic bacteria can oxidize H_2S to elemental sulfur, SO_4^{2-}, or sulfuric acid. The production of sulfuric acid can often drastically lower the pH of sediments, releasing phosphorus, contributing to eutrophication (Nealson and Stahl 1997).

Phosphorus. Unlike the previously mentioned elements, SWI phosphorus-containing molecules do not undergo redox-dependent changes, usually existing as a phosphate (PO_4^{3-}) molecule bound to an inorganic or organic molecule (Jones 2002). The assimilation of soluble reactive phosphorus (SRP) by bacteria is essential in the production of ATP, DNA, phospholipids, and polyphosphate storage products (Gächter and Meyer 1993). Because only a small percentage of phosphorus is biologically available, it is often the limiting nutrient for microbial and planktonic production in reservoirs (Boström et al. 1982). SWI phosphorus is provided by decaying epilimnetic phytoplankton blooms that sink to the sediment surface as well as external loading from point and non-point source pollution (Harrison et al. 1972; Jones 2002). However, much of this phosphorus is refractory and becomes permanently buried in the sediments (Gächter and Meyer 1993).

In oxic sediments, the largest inorganic and overall source of SWI PO_4^{3-} is sequestered as a complex with Fe^{3+}. As anoxic conditions develop, Fe^{3+} is reduced to

Fe^{2+} and the iron-phosphate complexes undergo dissolution (Gächter et al. 1988). Recent evidence suggests that PO_4^{3-} release is proportional to H_2S production in sediments, which implies that PO_4^{3-} release is dependent on redox potential (Golterman 2001). In addition, SWI shift to anoxia is often associated with lower bacterial metabolism and increased lysis of strict aerobic bacteria, resulting in higher mobilized phosphorus released into the water column. Thus SWI bacteria often serve as important sources, not just sinks, of phosphorus (Boström et al. 1988).

Summary of Research Objectives

The primary goal of this study was to assess seasonal differences in SWI bacterial composition, diversity, function, and ecological interactions in a seasonally stratified (monomictic) reservoir. While methodological approaches to elucidate these bacterial dynamics are no longer limited as historically the case, no single approach can address all objectives (Kirk et al. 2004). Therefore a suite of methods were used to conduct the various investigations.

The following is a brief summary of the objectives and procedures in this investigation, presented in this document as individual chapters:

Chapter Two presents a seasonal study, conducted quarterly, that measured SWI bacterial activity and biomass production. Preliminary investigations determined that the amino acid L-serine was readily utilized by SWI bacteria under various seasonal SWI physicochemical conditions. Therefore, radioassays using tritium-labeled L-serine were employed to measure total SWI bacterial uptake, used as a surrogate of activity. These uptake rates were converted to rates of bacterial biomass production and community

generation times to assess which seasonal mixing and stratification events (i.e. seasons) were related to the highest active SWI bacterial consortia.

In Chapters Three and Four, Biolog EcoPlates were utilized to determine the 'functional potential' of SWI bacterial consortia via their use of various organic carbon substrates. Biolog EcoPlates are microtiter plates containing a suite of individual organic carbon compounds in which SWI bacterial communities were inoculated and incubated. SWI bacterial utilization rates and patterns of these carbon substrates produced a multivariate data set that elucidated seasonal patterns of preferential substrate utilization, grouped by their functional class (e.g. amino acids, carbohydrates, carboxylic acids). Due to seasonal SWI anoxia, anaerobic inoculation and incubation methods were required. This anaerobic method as well as other EcoPlate modifications was novel, thus Chapter three is devoted to the methodological issues concerning Biolog EcoPlates. Chapter Four pertains to seasonal SWI bacterial carbon substrate utilization.

The investigation presented in Chapter Five was an analysis of seasonal differences in SWI organic matter sources, quantities, and nutrient stoichiometry. These organic matter dynamics were related to SWI bacterial abundance and biomass. Stable isotopes of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) were used to elucidate sources of SWI organic matter and determine if SWI organic matter is fractionated on a seasonal or spatial basis.

Lastly, Chapter Six reports the use of two current molecular biology methods to measure total SWI bacterial diversity, as well as presence and diversity of sulfate-reducing bacteria (SRB). Total DNA was extracted from SWI samples and amplified with: 1) primers specific for regions between the 16s rRNA and 23s rRNA gene (16s

rDNA and 23s rDNA, respectively) to amplify the total bacterial community or, 2) functional primers specific for SRB, located within the 16s rRNA gene. Automated ribosomal intergenic spacer analysis (ARISA) was used to analyze the total-community amplified DNA to obtain a measure of total community diversity among seasons, while denaturing gradient gel electrophoresis (DGGE) was used to measure the richness of SRB among various SWI sites and seasons.

General Methodology

Bacterial Abundance

Estimating bacterial abundance in water and sediment is commonly performed by filtering a formalin-preserved sample on a polycarbonate membrane filter followed by staining the bacteria with acridine orange (AO), or 4`6-diamidino-2-phenylindole (DAPI) fluorochrome. The filters are then magnified under either blue (AO) or UV (DAPI) light, in which bacterial cells glow either orange/red for the AO method, or white/blue for the DAPI method (Hobbie et al. 1977; Porter and Feig 1980). Sediment bacteria prove especially difficult to stain due to high amounts of detrital and other organic matter. Evidence has shown that in presence of clays and organic matter, DAPI provides superior staining and better contrast than AO (Kuwae and Hosokawa 1999). Thus DAPI was used in investigations requiring estimation of bacterial abundance, as mentioned in Chapters Two through Six.

Bacterial Production

Bacterial production is often measured indirectly via frequency of dividing cells, or through uptake of a radiolabeled substrate (Ducklow 2000). Traditional radiolabeled

substrates include tritium-labeled (^{3}H) or carbon-14 (^{14}C) thymidine or L-leucine. Uptake of thymidine is a measure of DNA replication, while L-leucine uptake measures rates of protein synthesis (Findlay 1993). Problems exist with using thymidine in anoxic waters and sediments, thus amino acids are preferred radiolabeled substrates for measuring in these environments, such as SWIs (Johnstone and Jones 1989).

Protein comprises a large and constant portion of most bacteria, making it a significant fraction of biomass production (Kirchman et al. 1985). Upon incubation with a radiolabeled amino acid, the sample is usually boiled in the presence of trichloroacetic acid to precipitate proteins. Thus uptake of the substrate into the total cell mass and the protein mass exclusively can be measured (Kirchman 2001). Conversion factors are used to convert uptake rates into grams of carbon produced per volume and unit of time (Kirchman 1993).

The ideal radiolabeled substrate (e.g. amino acid) for measuring bacterial production should be determined empirically. *A priori* interactions of the substrate with the environmental matrix cannot be predicted. In this investigation, it was determined that the amino acid L-serine was utilized under various SWI physicochemical conditions. Chapter 2 presents results of an investigation conducted with [^{3}H]-L-serine to measure SWI bacterial production.

Carbon Substrate Utilization

Organic carbon uptake and oxidation by bacteria contribute substantially to organic matter cycling in sediments (Bloesch 2004). A large portion of organic carbon is dissolved, and is thus easily oxidized by aerobic and anaerobic SWI bacteria (Bastviken et al. 2001). However, the types and classes of organic substrates utilized by bacteria

(e.g. amino acids, carbohydrates, and carboxylic acids) remain largely unknown. Studies involving carbon substrate utilization by sediment bacteria historically involved use of radiolabeled tracers (often specific for a single compound) or selective plating. A recent alternative to these methods is Biolog microtiter plates (i.e. GN, GP, and ECO) containing individual carbon substrates and a redox-sensitive tetrazolium dye indicator. Samples are inoculated into the plates and incubated, in which the amount of color development measured at OD_{590} is equal to the rate of substrate oxidation (Choi and Dobbs 1999; Mills and Garland 2002).

This investigation utilized Biolog EcoPlates to assess seasonal preference of SWI bacteria to various classes of substrates. Much debate has ensued about ecological interpretation of Biolog data, thus Chapter Three is devoted to interpretation issues involving utilization of Biolog EcoPlates for aerobic and anaerobic freshwater bacterial communities, while Chapter Four is an ecological study utilizing Biolog EcoPlates to assess seasonal differences in carbon substrate utilization by SWI bacteria.

Sediment Chemistry

While Chapter Four included data regarding rates and types of organic carbon utilization by SWI bacteria, Chapter Five focused on seasonal changes of *in situ* SWI carbon (i.e. organic and inorganic) quantities. In addition, sources of total carbon and nitrogen to the SWI were analyzed. These data were related to bacterial abundance and biomass to denote bacterial ability to degrade organic matter and fractionate various autochthonous and allochthonous organic matter inputs.

Organic Matter. SWI total organic matter reveals how much organic carbon that remains impervious to bacterial oxidation, assuming rates of bacterial oxidation are

greater than organic matter inputs. In addition, large seasonal differences in SWI organic matter may indicate increases or decreases in sinking autochthonous matter (i.e. decreased bacterial mineralization) or allochthonous inputs.

The most common method of organic matter analysis is the loss on ignition (LOI) method. Dried sediment is ignited at 550°C for one hour, burning off all sources of organic matter. The difference between the initial sediment dry weight and remaining residue (ash) after ignition is equal to organic matter concentration (Dean 1974). This organic matter includes carbon, which is approximately 50% of total organic matter. Much organic matter includes organically bound nitrogen and phosphorus compounds (Meyers and Teranes 2001).

Total Carbon and Nitrogen. Elemental analyzers are used to measure SWI total carbon and total nitrogen as well as inorganic carbon. Dried (unashed) sediments are analyzed for total carbon and total nitrogen using mass spectroscopy. In addition, ashed residue is analyzed for total carbon which is inorganic. The difference in carbon concentration between total and ashed samples is equal to organic carbon concentration.

Carbon to nitrogen ratios are derived from these data. C/N ratios serve as a proxy for determination of SWI organic matter sources (i.e. autochthonous or allochthonous), as well as types of allochthonous inputs (Meyers and Teranes 2001).

Stable Isotopes. SWI carbon and nitrogen contain distinct ratios of their stable isotope signatures (i.e. $^{13}C/^{12}C$ and $^{15}N/^{14}N$). Analyses of these signatures at the SWI serve as proxy for organic matter sources as well as changes in organic matter availability and usage by bacteria (Hoefs 2004). These ratios are well defined for a variety of organic sources, enabling tracking of allochthonous inputs to the SWI. Determination of carbon

and nitrogen isotopes from sediments is determined from continuous flow-isotope ratio mass spectrometers (CF-IRMS) after removal of all carbonates via acid extraction (Vreča 2003).

Molecular-Based Analyses

Attempts to provide an unbiased assessment of bacterial communities in their natural environments have been historically plagued by a lack of adequate instrumentation and methodology. Until recently, most ecological studies of bacteria involved the plating and growth of bacteria on selective culturing media (Ferrara-Guerrero et al. 1993; Kelly and Wood 1998; Kostka and Nealson 1998). Unfortunately, culturing methods remove bacteria from their original habitat and severely alter their *in situ* growth conditions. Additionally, selective culturing methods allow growth of only a small percentage of bacteria, while most bacteria do not grow due to complex and/or fastidious growth requirements (Torsvik et al. 1998; Zhang and Fang 2000).

This investigation needed to incorporate methods to 'fingerprint' SWI bacterial communities without the bias of culturing and plating techniques. Ideally, these fingerprinting methods should provide a measure of the number of different bacterial taxonomic units (i.e. species richness) as well as the proportion of each taxonomic unit relative to the entire community (i.e. species evenness). Collectively, richness and evenness define the diversity of the bacterial community, which should be measured in such a way to allow comparison among samples (i.e. species similarity) (Hewson and Fuhrman 2004).

Historically Used Molecular Methods

Biochemical (e.g. FAME) and molecular (e.g. DNA, RNA, and protein) based methods have been used with varying degrees of success to measure bacterial community diversity and similarity without the problems of traditional culturing approaches. Unfortunately no single method is without drawbacks.

Signature Lipid Biomarker Analysis. Individual bacterial taxonomic units (*alias dictus* species) contain specific fatty acids within their cell walls. These fatty acids are extracted from sediments using a series of organic solvents and esterified, forming fatty acid methyl esters (FAMEs) which are analyzed via gas chromatography. The resulting chromatograms provide a fingerprint of the bacterial community. In addition, presence of some specific FAMEs serve as biochemical markers for various groups of bacteria (White et al. 1979; Vestal and White 1989; Findlay et al. 1990; White and Ringelberg 1998). While effective, this method lacks the sensitivity and resolution to completely profile a bacterial community. Many identical fatty acids are found in functionally diverse bacteria, while many rare and unusual bacteria have unknown fatty acid profiles. Therefore there is uncertainty in converting fatty acid profiles to bacterial community fingerprints (Findlay and Dobbs 1993).

Probe Hybridizations. Functional group-specific or phylogenetic probes are designed to hybridize to community DNA. Generally 16s rRNA genes are the target if taxonomy or phylogeny of a community is to be determined. Functional (group specific) probes are used if phenotypic detection of the community is desired. This includes Fluorescent *in situ* hybridization (FISH) in which fluorescent probes specific for various DNA sequences fluoresce when attached to DNA, which are then viewed with confocal

laser microscopy (Liu and Stahl 2002). Probe hybridization, while effective, generally requires an *a priori* knowledge of taxonomic or functional groups present in the bacterial community. For many investigations, little is known about the communities present; therefore many probes would overlook many important members of the total community.

(Terminal) Restriction Fragment-Length Polymorphisms (RFLPs and T-RFLPs). In this procedure, total community DNA is extracted from a sample, amplified via polymerase chain reaction (PCR) using domain or group-specific primers, and digested with restriction enzymes (e.g. *Eco*RI, *Bam*HI). Digested DNA is separated on agarose gels via electrophoresis, or if terminally labeled with a fluorescent dye, resolved on an automated electrophoresis system. The end result gives different sized DNA fragments, conferring a fingerprint of the bacterial community. While this procedure is a quick and effective way to screen for changes in a bacterial community, each amplicon can give multiple restriction fragments based on the type of restriction enzyme used. In addition, resulting fragments are a function of restriction sites and do not represent true operational taxonomic units, therefore this procedure cannot be used to generate true measures of richness or evenness (Liu and Stahl 2002).

Molecular-Based Analyses in this Investigation

Two recent molecular-based methods that have overcome many limitations of previous molecular-based analyses are automated ribosomal intergenic spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE). Both methods are vastly different, but complementary in the community diversity information that they provide. Both methods were incorporated into this study to measure seasonal changes in SWI bacterial diversity, as described in Chapter Six.

ARISA. Automated Ribosomal Intergenic Spacer Analysis (ARISA) is a relatively recent and effective way to fingerprint bacterial communities from environmental matrices. Each bacterial taxon contains a span of nucleotides between the 16s and 23 rRNA genes that differ in both length and sequence. This intergenic space is unique to each operational taxonomic unit (OTU) (i.e. species); therefore heterogeneity of these sequences can be used to differentiate among bacterial communities (Fisher and Triplett 1999).

DNA is extracted from sediments and amplified via PCR using primers that flank the intergenic space. The forward primer is fluorescently labeled, allowing the amplified DNA to be analyzed on an automated fragment analysis system. Via this process, electropherograms are produced with peaks that result from each amplified fragment. Each distinct peak indicates an individual bacterial operational taxonomic unit (OTU), while the area under the peak represents the relative amount of the OTU (Brown et al. 2005).

DGGE. DGGE is an electrophoretic process that separates PCR-amplified DNA sequences of identical lengths, but of different base pair sequences (Muyzer et al. 1993). First, DNA is extracted and purified from the sediment and specific sequences and/or genes are amplified via polymerase chain reaction (PCR) using domain-specific or functional group-specific primers that amplify a hypervariable region on the 16s rRNA gene (rDNA). PCR products are loaded into a vertical polyacrylamide gel containing a linearly increasing gradient of DNA denaturants, urea and formamide, held at a constant temperature. The DNA fragments migrate through the gel until a sufficient amount of denaturant transforms the helical DNA into a partially melted molecule, retarding its

movement through the gel. While each melted molecule (amplicon) is equal in its number of base pairs, these 'melting domains' differ for each DNA fragment that differs in base sequence (Muyzer and Smalla 1998). Because each bacterial taxonomic unit differs in this sequence, each unique fragment produced represents a bacterial taxonomic unit. These fragments appear as bands in the gel when stained with an appropriate dye. (Schäfer and Muyzer 2001; Heuer et al. 2001).

For this investigation, both ARISA and DGGE were used. ARISA provided fingerprints that measured total diversity (richness and evenness) of the SWI bacterial communities on a seasonal basis, while DGGE was used to measure diversity of sulfate-reducing bacterial (SRB) populations.

Study Location

Because most studies on SWIs have been conducted from marine systems or natural lakes, physicochemical characteristics of these SWIs typically included narrow ranges of dissolved oxygen and redox potentials. In this investigation, it was necessary to choose a reservoir in which the SWI experienced seasonal stratification, allowing for oxic/anoxic cycles and reduced redox potentials. A reservoir that meets these criteria is Belton Reservoir, located in Bell County in central Texas. Belton Reservoir was impounded in 1954 to serve as a municipal water source and flood control structure for the cities of Temple, Belton, and Killeen as well as Fort Hood (Rutherford 1998).

Belton Reservoir is monomictic and is considered eutrophic. However, these are generalized classifications due to the varying reservoir bathymetry and morphometry. The northern arm of the reservoir is defined by a shallow 20 mi riverine zone, formed from the Leon River (Figure 1.1). The Leon River serves as the primary inflow for

Belton Reservoir and is surrounded by both urban and rural land development, as well as unimproved grasslands and pastures, dairy and farming operations, and industrial operations (USACE 2002). The unique serpentine river flow naturally decreases the amount of organic matter input into the transition and lacustrine zones downstream. Hence the deeper open waters near the dam are considerably less eutrophic than the riverine zone (Lind 1984). The surrounding limestone cliffs form a deep reservoir basin near the dam that, unlike the shallow upstream zones, thermally stratifies in late spring and throughout summer. Due to the steep cliffs, very little emergent vegetation exists around the shoreline, as well as little submerged vegetation in this area of the lake. Details of lake area, volume, and other physical and chemical characteristics are presented in Chapters Two through Six.

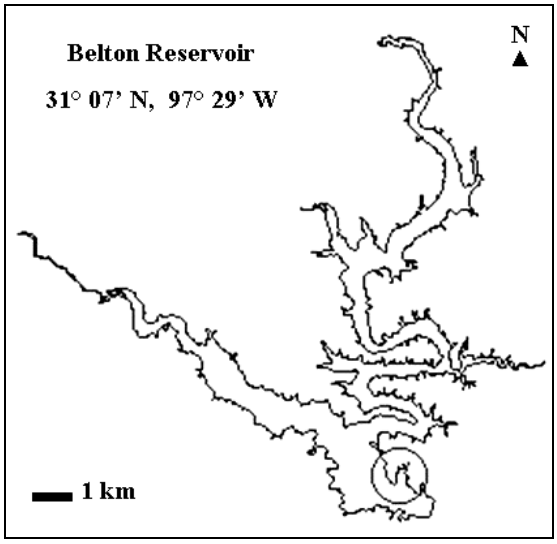

Figure 1.1: Belton Reservoir. The circled area near the dam was the sampling location.

The longitudinal axis of the lacustrine zone runs parallel to the dam, and is characterized by an increasing depth gradient from north to south. Hence the depth of the hypolimnion blanketing the sediment surface varies spatially. Because fall overturn is

often a gradual process, the shallower depths undergo mixing days or weeks before greater depths, providing a temporal component to stratification and mixing events. To capture both the spatial and temporal component of stratification and its effects on the SWI, five sites along a linear transect along the longitudinal depth gradient were chosen. These sites were not equally spaced, but instead chosen to represent the overall depth gradient of the lacustrine zone.

The following chapters refer to these sites as Sites A – E, from shallowest to deepest, respectively (Figure 1.2). Because the studies were conducted at different time scales, the mean depth of these sites varied depending on various seasons with greater or lesser rainfall and/or changing rates of water release from the dam. Site A, the most shallow site is deeper than the mean depth of the reservoir (10.7 m); however mean depth considers the depth of the extremely long and shallow riverine zone. In addition, several deep holes occur in the lacustrine zone, some as deep as 37 m, however most of the deepest sites near the dam are approximately 27 m, which corresponds to Site E.

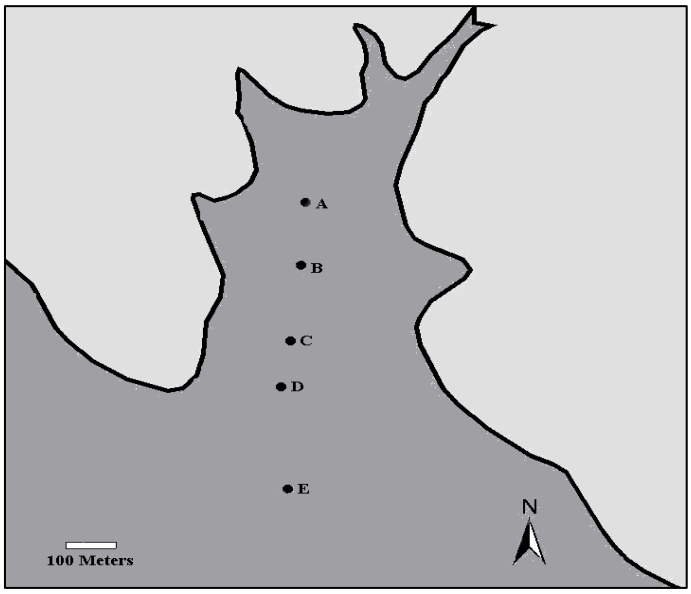

Figure 1.2: Close up map of Belton Reservoir sampling sites. Sites increase in depth from A through E.

Sediments from these sites were always similar in consistency, composed of fine clay. Most were very compact, allowing for little sediment porewater penetration. Another unique attribute was the lack of benthic macroinvertebrates (e.g. crustaceans, worms) within all SWI samples, both aerobic and anaerobic. Also, much discussion has arisen about the presence of perchlorate contamination within Belton Reservoir sediments from upstream industrial inputs, which are no longer being produced and released into the watershed. A 2002 study by the United States Army Corps of Engineers suggested further investigations be conducted on the effects of perchlorate levels in Belton Reservoir on toxicity to fishes, plants, and microbiota (USACE 2002), however as of 2006 no other studies have been performed on perchlorate levels of Belton Reservoir.

CHAPTER TWO

Increased Sediment-Water Interface Bacterial [^{3}H]-L-Serine Uptake and Biomass Production in a Eutrophic Reservoir during Summer Stratification

Introduction

Sediment-water interfaces (SWIs) of lakes and reservoirs offer sites of intense organic matter degradation and deposition (Butorin 1989; Dean 1999; Heinen and McManus 2004). SWI carbon cycling occurs via microbial oxidation of dissolved and particulate organic carbon (DOC and POC) and incorporation of DOC into bacterial biomass via secondary production (Schallenberg and Kalff 1993; King 2002). This linking of DOC to heterotrophic bacterial production defines the microbial loop (Wetzel 2001). While the microbial loop is traditionally defined in terms of planktonic microbial dynamics, sediment bacteria may play an important role in the microbial loop and ecosystem eutrophication processes (O'Loughlin and Chin 2004).

In thermally stratified reservoirs, bacterial metabolism often depletes dissolved oxygen below the metalimnion resulting in an anoxic hypolimnion and SWI. Hypolimnetic and SWI bacterial consortia respond to this oxygen depletion through the use of alternate and less energetically favorable electron acceptors (Sweerts et al. 1991; Liikanen and Martikainen 2003). Because many SWI bacteria are not facultative in their respiratory functions, the bacterial community must shift their composition and metabolism in response to anoxia (Kelly et al. 1988; Rosselló-Mora et al. 1999). However, shifts in SWI bacterial activities and biomass production throughout seasonal transitions of anoxia and mixing in reservoirs are poorly understood.

Bacterial activity and production in aquatic and sediment environments are commonly measured via uptake of radiolabeled substrates such as ^{3}H or ^{14}C labeled L-leucine or thymidine as a measure of bacterial protein synthesis or DNA synthesis, respectively (Bell 1993; Kirchman 1993; Ducklow 2000; Chin-Leo 2002). These substrates are well accepted for bacterial production studies; however advantages of using these isotopes are often based on theoretical rather than empirical data. Ideally, the radiolabeled substrate used for bacterial uptake should be metabolized under all environmental conditions (e.g. dissolved oxygen, temperature, redox potential) imposed in the study. For example, exogenous thymidine cannot be taken up by a variety of sulfate reducing bacteria, chemolithotrophs, and methanogens—bacteria that are commonly found in anoxic environments, such as SWIs (Johnstone and Jones 1989; Gilmour et al. 1990).

[^{3}H]-L-serine (Ser) was utilized to assess seasonal changes in SWI bacterial activity and production. While Ser is not commonly used to measure bacterial uptake rates and activity, this amino acid was chosen based on preliminary studies involving various unlabeled amino acid, carbohydrate, and carboxylic acid uptakes rates by SWI bacteria. Of these substrates, Ser exhibited high uptake by SWI bacteria under various physicochemical conditions as well as having a high direct correlation with bacterial abundance. These data provided empirical evidence that Ser could be used reliably as a radiolabeled substrate to measure SWI bacterial uptake rates and estimate biomass production without bias due to physical and chemical changes during stratification and mixing.

Using Ser, this investigation sought to understand seasonal changes in bacterial activity and production at the SWI of a seasonally stratified eutrophic reservoir and relate seasonal SWI physicochemical variables to variations in SWI bacterial activity and production. Providing a measure of seasonal SWI bacterial production is necessary if we are to adequately understand microbial loop processes and food web dynamics in lake and reservoir ecosystems.

Materials and Methods

Study Site and Sampling Protocol

Belton Reservoir, a deep, subtropical eutrophic reservoir located in central Texas, served as the study site. Belton Reservoir is monomictic, undergoing thermal stratification in late spring and maintaining an anoxic hypolimnion until overturn and thermal mixing in mid-autumn (Christian et al. 2002; Christian and Lind 2006). The reservoir basin has a maximum depth of 37 m, surface area of 49.8 km^2, and a total volume of 5.45 x 10^8 m^3. Secchi visibility ranges from 1.2 m to 2 m.

Five sample sites along a 1 km linear transect representative of the reservoir depth gradient were sampled quarterly. Each consecutive site increased in depth, offering sites that underwent thermal stratification in a sequential order. These differed in their physicochemical variables on a spatial and temporal basis (Table 2.1), with the deepest site (Site E, mean depth 25.9 m) becoming anoxic (dissolved oxygen < 0.2 mg l^{-1}) and the shallowest site (Site A, mean depth 13.4 m) becoming hypoxic (dissolved oxygen < 3 mg l^{-1}) during summer stratification. SWI temperature (°C), dissolved oxygen (mg l^{-1}), pH,

specific conductivity (mS cm^{-1}), and redox potential (mV) were measured at each site on a seasonal basis using a YSI 600 QS Data Sonde.

Table 2.1: Physicochemical characteristics and bacterial abundance at the five SWI sampling sites (A-E) of increasing depth measured seasonally over one year. Variation in depth at each site through time is due to fluctuating reservoir water levels.

Date	Site	Depth (m)	Temperature (°C)	Dissolved Oxygen (mg l^{-1})	pH	Redox (mV)	Specific Conductance (mS cm^{-1})	Bacteria x 10^6 ml^{-1}
14-Oct-04	A	13.8	24.6	7.4	7.1	431	0.80	1.06
14-Oct-04	B	16.8	24.6	7.2	6.9	405	0.81	1.46
14-Oct-04	C	20.2	24.6	5.3	6.9	411	0.80	2.25
14-Oct-04	D	22.2	24.6	6.8	6.9	415	0.80	2.15
14-Oct-04	E	26.2	24.5	6.6	6.5	341	0.81	3.11
3-Feb-05	A	14.5	10.9	9.5	7.2	398	0.91	1.51
3-Feb-05	B	16.4	10.9	9.4	7.1	405	0.91	1.29
3-Feb-05	C	20.0	10.9	9.7	7.2	393	0.91	1.41
3-Feb-05	D	23.4	11.2	9.1	7.1	454	0.92	1.27
3-Feb-05	E	26.2	11.1	9.4	7.4	459	0.90	1.5
1-Jun-05	A	13.3	20.2	2.9	7.0	380	1.11	2.62
1-Jun-05	B	16.4	18.5	2.8	7.0	398	1.12	1.62
1-Jun-05	C	20.0	16.7	2.1	6.9	393	1.12	1.51
1-Jun-05	D	22.8	16.5	1.7	7.0	391	1.11	1.11
1-Jun-05	E	26.1	16.4	1.1	7.0	420	1.11	2.26
21-Oct-05	A	13.0	24.0	4.6	7.0	243	0.93	1.25
21-Oct-05	B	15.9	23.5	0.3	6.8	309	0.93	1.18
21-Oct-05	C	19.3	22.9	0.2	6.6	228	0.95	1.05
21-Oct-05	D	22.5	20.8	0.3	6.7	175	1.12	2.06
21-Oct-05	E	25.0	19.9	0.2	6.6	159	1.16	1.06

Samples were collected at times corresponding to the onset of autumnal overturn (Oct 2004), winter mixis (Feb 2005), onset of summer stratification (Jun 2005), and late-season stratification (Oct 2005), respectively. Samples were retrieved by a 3.2 l horizontal Alpha water sampler, positioned at the sediment surface. This allowed sampling of the benthic boundary layer, defined as the water layer near the sediment surface that contains a steep gradient in physicochemical variables due to the sediment

itself (Boudreau and Jørgensen 2001). The sampler was rinsed with demineralized water between sample hauls to minimize sample cross-contamination. Duplicate samples from each site, consisting of water and sediment particles, were pooled together in 300 ml dark BOD bottles and immediately capped to prevent traces of oxygen contamination. Bottles were placed in Styrofoam containers containing water collected at sampling depth to maintain in situ temperature. Samples were returned to the laboratory for processing within 3 hours of collection.

Determination of L-Serine as Optimum Substrate

A multiple-season preliminary investigation was conducted to empirically determine the optimum substrate for measuring SWI bacterial activity and production. Biolog EcoPlates (n = 12) containing 31 distinct carbon substrates in microtitre plate-form were inoculated with SWI bacteria and measured for color development (rate of substrate oxidation) per the method of Christian and Lind (2006). Among the substrates (including 6 amino acids, 10 carbohydrates, 9 carboxylic acids, and 6 other compounds), Ser best fit the criteria necessary for successful use as a radiolabeled tracer in this study: (1) the rate of Ser utilization was directly correlated to bacterial abundance, (2) the rate of utilization was independent of large variations in environmental conditions (e.g. temperature, dissolved oxygen, redox potential), and (3) Ser is commercially available as a radiolabeled tracer (Figure 2.1).

Determination of Optimum Radiolabeled L-Serine Uptake

A preliminary study was conducted on a pooled SWI bacterial sample retrieved from the five sampling sites to determine the saturating total Ser uptake concentration.

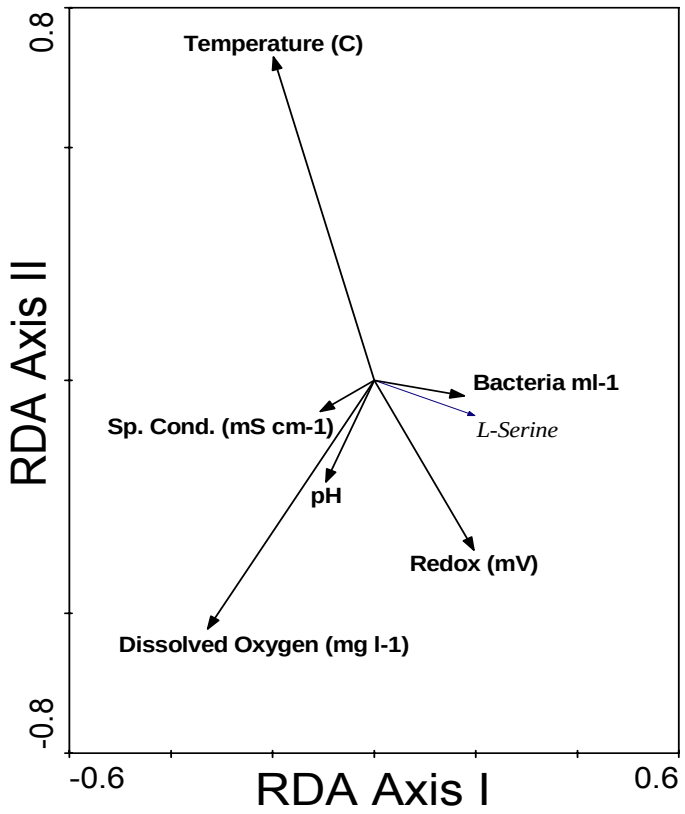

Figure 2.1: Redundancy Analysis (RDA) biplot with RDA axes I and II indicating loadings for environmental variables and SWI bacterial L-serine uptake from Biolog EcoPlate assays. Data was from a preliminary multi-seasonal study conducted on the Lake Belton SWI. L-serine was used by SWI bacteria independently of dissolved oxygen and pH, almost independently of temperature and dissolved oxygen, and positively correlated to bacterial abundance as indicated by perpendicular arrows (completely independent) or parallel arrows (positively correlated).

This determined the minimum Ser concentration required, as well as the concentration required to minimize isotope dilution. Incubations consisting of 25 nM G-[^{3}H]-L-serine with increasing amounts of unlabeled Ser giving total Ser concentrations of 25 nM, 50 nM, 100 nM, 250 nM, and 500 nM (final incubation concentrations of 5 nM, 10 nM, 20 nM, 50 nM, and 100 nM) were tested at 20 min, 40 min, and 60 min in a factorial design (Figure 2.2). Samples were filtered and counted using the methods described below for Ser_{tot}. Optimum concentration and time of incubation was determined to be 50 nM (final concentration 10 nM) for 20 min.

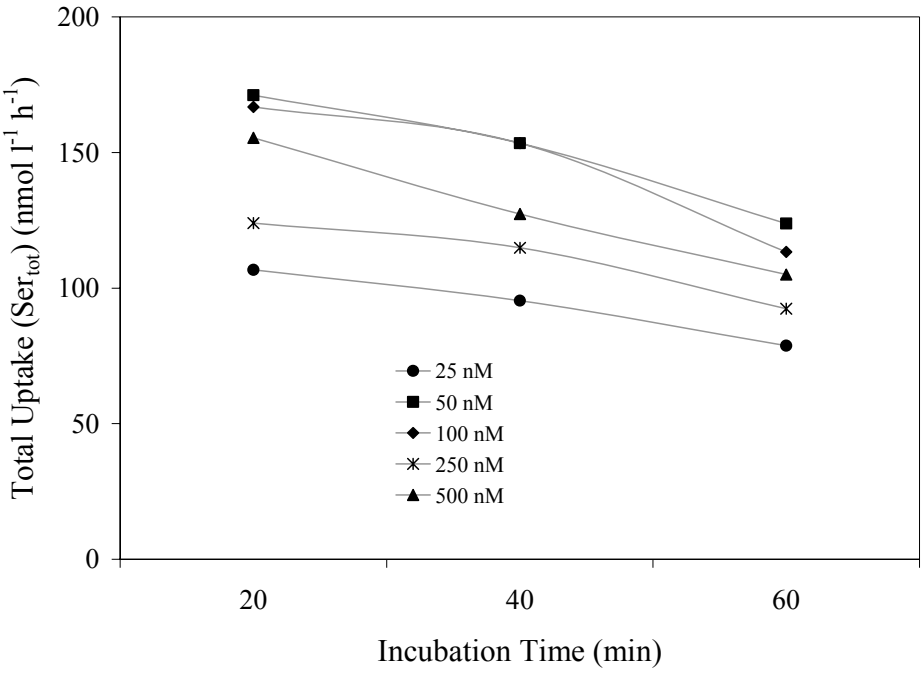

Figure 2.2: Ser_{tot} at various incubation times and concentrations from a pooled aerobic SWI bacterial sample taken from five SWI sites. 25 nM G-[^{3}H]-L-serine was used with increasing concentrations of unlabeled L-serine for concentrations of 25 nM, 50 nM, 100 nM, 250 nM, and 500 nM.

L-Serine Incubations

Three ml sterile plastic syringes fitted with 16-guage needles were prepared with 0.5 mL of 50 nM L-serine solution, specific activity 15.5 Ci mol^{-1}. For anaerobic incubations, the syringes and L-serine solution were first purged with nitrogen gas to remove traces of oxygen. Two ml of sample was drawn into the syringe for a final Ser concentration of 10 nM. Incubations were conducted by placing the inoculated syringes in plastic bags, purging the bags with nitrogen if samples were anaerobic, and immersing the bags in water adjusted to collection depth temperature. After 20 min, incubations were stopped by adding 0.5 ml of formalin (final concentration 2 percent) to the syringe. Zero-time killed controls were performed by adding formalin to syringes immediately after sample inoculation. All sample and control incubations were performed in

quadruplicate which resulted in a coefficient of variation among sample activities of less than 10 percent. Samples were held in the syringes at 4°C until radioassays were performed.

Total L-Serine Uptake

To measure total bacterial uptake of Ser (Ser_{tot}), one-half (1.5 ml) of each preserved sample was filtered through a 0.45 µm cellulose nitrate filter followed by rinsing the filter three times with bacteria-free (0.2 µm-filtered) water. Each filter was dried overnight and added to 1 ml of ethyl acetate in a 20 ml plastic scintillation vial. After allowing the filters to dissolve overnight, 9 ml of scintillation cocktail (Ultima Gold LLT) was added to the scintillation vial and vortexed. Vials were radioassayed on a Beckman LS 6500 liquid scintillation counter at a counting precision of 5 percent error. Samples were corrected for quench and counts per minute (CPMs) were converted to disintegrations per minute (DPMs) using an external quench curve composed from a series of commercially purchased quenched tritium standards.

L-Serine Uptake in Protein

To account for Ser exclusively in the protein fraction (Ser_{pro}), the other half of the preserved sample (1.5 ml) was added to a 2 ml microcentrifuge tube containing a 0.5 ml solution of 20% (w/v) NaCl and (v/v) trichloroacetic acid (TCA) (final concentration of TCA/NaCl 5%). The microcentrifuge tubes were heated at 80°C for 30 min to precipitate proteins, followed by centrifugation at 12,000 x g for 10 min. The supernatant was discarded, and 1.5 ml of 80 % ethanol added. The tubes were centrifuged again at 12,000 x g for 10 min and the supernatant discarded. This ethanol wash step was performed

three times. One and a half ml of scintillation cocktail was added to the centrifuge tubes and vortexed. The centrifuge tubes were placed in 20 ml scintillation vials and radioassayed as above.

Bacterial Enumeration

Aliquots of each retrieved sample were preserved with formalin (2% final concentration) for bacterial enumeration. Bacteria were stained with DAPI fluorochrome at a final concentration of 5 μg ml^{-1}. After staining, the samples were filtered through 0.2 μm blackened Nuclepore filters, placed on glass slides with a coverslip, and viewed under UV light (330 nm – 380 nm) at 1500 x magnification. Ten fields, corresponding to at least 300 bacteria were counted. Total bacteria per ml were calculated from the total area of the filter counted and amount of sample filtered (Porter and Feig 1980). Enumeration of bacteria attached to sediment particles were multiplied by 2 to correct for masking of bacteria by the sediment particle.

Statistical Analyses

All summary statistics were analyzed using Microsoft Excel 2003. Other statistical analyses were performed using JMP 5.0 (SAS Institute) or CANOCO 4.5 (Microcomputer Power) (ter Braak and Šmilauer 2002; Lepš and Šmilauer 2003). Graphical analyses were performed using Microsoft Excel or CanoDraw 3.0 for Windows, packaged with the CANOCO program.

Results

Ser_{tot} and bacterial abundance at each SWI site for all sampling seasons is shown in Figure 2.3. A two-way repeated measures analysis of covariance (ANCOVA)

analyzed for differences in Ser_{tot} by site and season. ANCOVA removed the covariable effect of bacterial abundance upon Ser_{tot}. Significant differences were observed in Ser_{tot} among seasons all sites inclusive ($F_{3,60} = 31.41$, $p < 0.00001$) and no differences among sites all seasons inclusive ($F_{4,60} = 1.09$, $p = 0.37$). Tukey's HSD test (Kirk 1999) revealed significantly higher Ser_{tot} at onset of stratification (Jun 2004) than during onset of autumnal overturn (Oct 2004), winter mixing (Feb 2005) and late season stratification (Oct 2005). Significantly higher Ser_{tot} was also observed during late season stratification than winter mixing (Table 2.2). No difference in Ser_{tot} among sites inclusive of all seasons suggests that similar selective pressures occur upon the SWI bacteria at each site.

Table 2.2: A posteriori differences of Ser_{tot} among dates. Values indicate Tukey's Honestly Significant Difference (HSD) test values. Single asterisks indicate significant differences at an $\alpha = 0.05$. Double asterisks indicate differences at an $\alpha = 0.01$.

Date	14-Oct-04	3-Feb-05	1-Jun-05	21-Oct-05
14-Oct-04		1.32	8.83**	-3.66
3-Feb-05	1.32		10.20**	4.98*
1-Jun-05	8.83**	10.20**		5.17*
21-Oct-05	-3.66	4.98*	5.17*	

No significant differences existed among seasonal bacterial abundances (One-way ANOVA, $F_{3,16} = 1.86$, $p = 0.177$). However a significant positive correlation was observed between bacterial abundance and Ser_{tot} during onset of stratification (Jun 2004) ($r^2 = 0.92$, $p < 0.01$). No significant correlations were observed between bacterial abundance and Ser_{tot} during winter mixing (Feb 2005) ($r^2 = 0.05$, $p = 0.72$), onset of autumnal overturn (Oct 2004) ($r^2 = 0.02$, $p = 0.77$), and late-season stratification (Oct 2005) ($r^2 = 0.06$, $p = 0.68$).

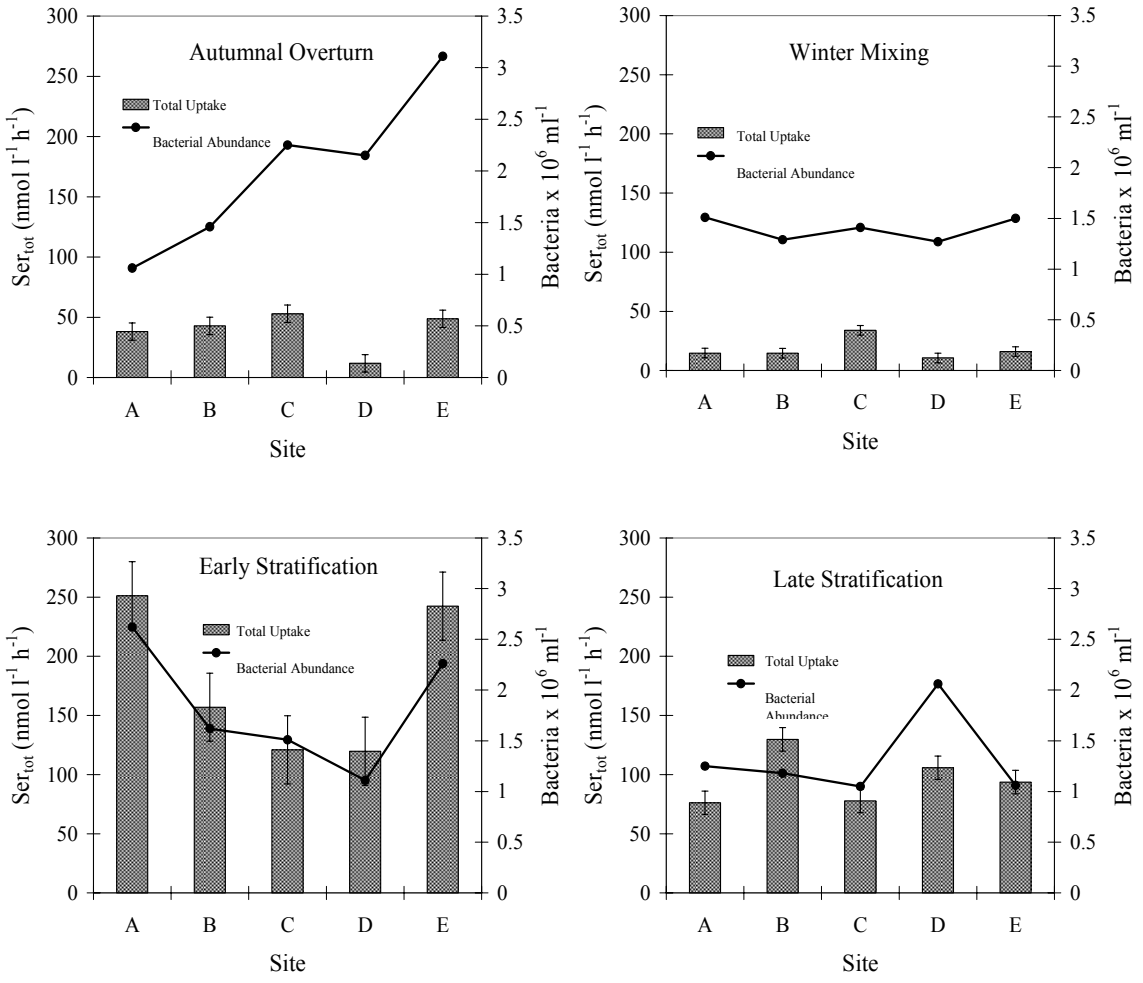

Figure 2.3: Total serine uptake (Ser_{tot}) and corresponding bacterial abundance among SWI sites (A – E) of increasing depth for each sampling date: autumnal overturn (Oct 2004), winter mixing (Feb 2005), early stratification (Jun 2005), and late stratification (Oct 2005). Error bars for Ser_{tot} indicate standard error of the mean.

Principal components regression (PCR) was used to correlate seasonal physicochemical (explanatory) variables in Table 2.1 with Ser_{tot} and generate a model that predicts the physicochemical conditions that result in higher Ser_{tot}. PCR is a form of multiple linear regression that removes colinearity among the explanatory variables, resulting in more robust correlations with the dependent variable (Ser_{tot}). Before PCR was performed, each physicochemical variable was z-transformed followed by principal components analysis (PCA) on the transformed data set (i.e. correlation matrix). Each

resulting principal component (PC) is independent of, and orthogonal to, each other and are linear combinations of all explanatory variables (Gotelli and Ellison 2004).

The first four PCs explained 99.1 % of the total variation in the physicochemical data from Table 2.1. According to the broken-stick criterion, only the first two PCs explained fractions of variability greater than the predicted null model (i.e. 85.2 % total variability), therefore only these two PC axes were retained for interpretation (Lepš and Šmilauer 2003). Within each of these two significant PC axes, the broken-stick criterion was used to assess significant loadings for each standardized physicochemical variable (Peres-Neto et al. 2003). PC I exhibits significant loadings for higher temperature and specific conductance; and lower dissolved oxygen, pH, and redox potential. PC II exhibits significant loadings for higher temperature and dissolved oxygen; and lower pH and specific conductance (Table 2.3). The PCR was developed by regressing the individual PC scores of the two significant components against all Ser_{tot} values. A direct relationship exists between PC I and Ser_{tot}, while an inverse relationship exists between PC II and Ser_{tot} (Table 2.4). The resulting PCR ($r^2 = 0.46$, $p = 0.006$) was:

$$Ser_{tot} = 10.3(\text{PC I}) - 14.7(\text{PC II}) + 82.9 \quad (2.1)$$

Table 2.3: Axis loadings of the first four principal components (PCs), their eigenvalues, and cumulative percentage of variation explained by the axes for the physicochemical variables. X_i = z-transformed scores for SWI temperature (X_1), dissolved oxygen (X_2), pH (X_3), redox potential (X_4), and specific conductance (X_5), respectively.

PC Axis	Axis Loadings	Eigenvalue	Cumulative Var. Exp. (%)
I	$0.798(X_1) - 1.186(X_2) - 1.126(X_3) - 1.111(X_4) + 0.674(X_5)$	0.573	57.3
II	$1.327(X_1) + 0.553(X_2) - 0.631(X_3) - 0.036(X_4) - 1.592(X_5)$	0.279	85.2
III	$1.074(X_1) - 0.966(X_2) + 0.458(X_3) + 1.591(X_4) + 0.414(X_5)$	0.093	94.5
IV	$- 0.795(X_1) + 0.16(X_2) - 1.759(X_3) + 1.111(X_4) + 0.116(X_5)$	0.046	99.1

Table 2.4: The coefficients of the principal component regression (PCR) model derived from the significant principal components of the physicochemical data in table 3. The standard error, Student's t-ratio, and p-value for each coefficient are shown.

Variable	Coefficient	Std Error	|t Ratio|	p-value
Intercept	82.9	12.4	6.7	< 0.0001
PCA I	10.3	3.4	3.00	0.008
PCA II	-14.7	5.6	2.56	0.02

In addition to Ser_{tot}, incorporation of Ser into bacterial protein (Ser_{pro}) was also measured. A significant positive linear correlation exists between Ser_{tot} and Ser_{pro} (r^2 = 0.6, $p < 0.0001$, n = 20). The ratio of Ser_{tot} to Ser_{pro} did not change significantly among seasons ($F_{3,16}$ = 0.78, p = 0.53). The linear model associated with the correlation, when forced through the origin, indicated that Ser_{pro} was approximately 40 percent of Ser_{tot} (Figure 2.4).

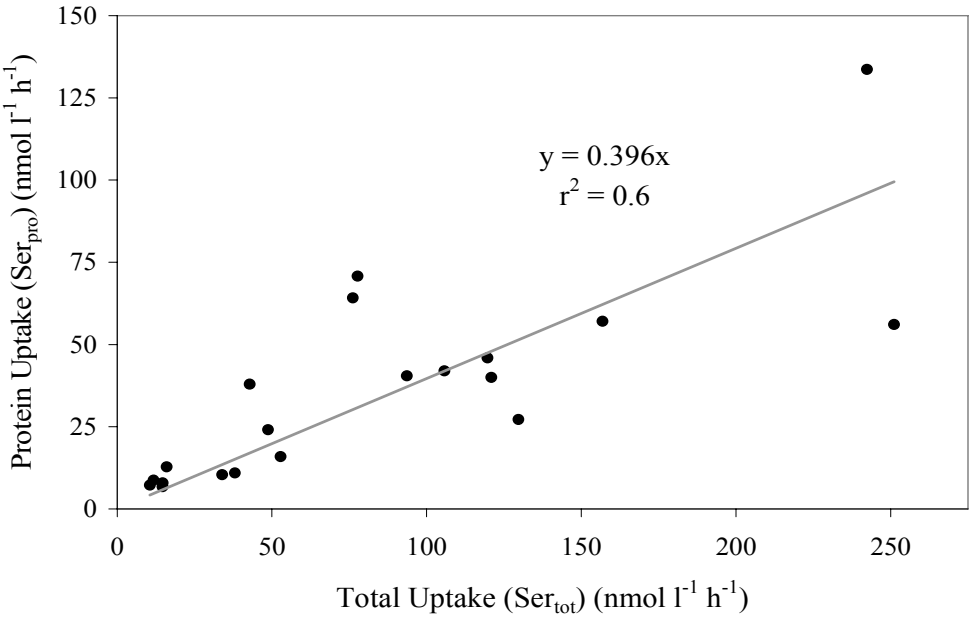

Figure 2.4: Correlation biplot of total serine uptake (Ser_{tot}) vs. serine uptake in protein (Ser_{pro}), all sampling seasons inclusive. Linear regression equation is forced through the origin.

To convert Ser uptake into bacterial biomass production (expressed as grams of carbon) a theoretical conversion factor of 5.5 mole-percentage of Ser in bacterial protein was used (Reeck 1973; Reeck and Fisher 1973). Therefore bacterial biomass production (BBP):

$$BBP\ (gC\ l^{-1}\ h^{-1}) = Ser_{pro} \times (\text{molecular weight of L-Serine} \div \text{mol \% Ser in protein}) \times \text{cell carbon per protein} \times \text{isotope dilution} \quad (2.2)$$

Where Ser_{pro} is in units of nmol l^{-1} h^{-1}, molecular weight of L-serine = 105.1, mole percentage L-serine in protein = 0.055 (5.5%), cell carbon per protein = 0.86, and isotope dilution is 1, assuming that added Ser is in excess of intracellular Ser pools.

Equation 1 is modified from the equation of Kirchman (1993, 2001) which utilizes L-leucine uptake. Mole percentage of Ser in bacterial protein was derived from the source Kirchman (1985) used to derive the percentage of L-leucine in protein (Reeck 1973). The cell carbon per protein conversion is based on Simon and Azam (1989).

Because cell abundance was measured in addition to Ser_{tot} and Ser_{pro}, total growth rate (μ) and generation time (h) was calculated using the theoretical conversion factor of 20 fg of carbon per cell (Lee and Fuhrman 1987; Ducklow 2000) where:

$$\text{Growth Rate } (\mu) = BBP \times (cells^{-1} \times 20\ fgC\ cell^{-1})^{-1} \quad (2.3)$$

and

$$\text{Generation Time (h)} = 0.693\mu^{-1} \quad (2.4)$$

Generation time is an estimate of the doubling time, or time required to generate the entire bacterial population. This equation is inclusive to the entire bacterial consortia, therefore is a ‘global’ doubling time which includes non-growing (dormant) populations and populations that are fast and slow growing.

Significant differences were observed for biomass production ($F_{3,16}$ = 7.3, p = 0.003) and generation time estimates ($F_{3,16}$ = 12.7, p = 0.0002) among seasons (Table 2.5). Tukey's HSD test indicated significantly greater production and shorter generation times ($p < 0.05$) during onset of summer stratification (Jun 2005) than during onset of overturn (Oct 2004) and during winter mixing (Feb 2005). Significantly greater production and shorter generation time ($p < 0.05$) was also observed during late-season stratification (Oct 2005) than during winter mixing as well as during onset of overturn.

Table 2.5: Bacterial biomass production and generation time for the SWI bacterial consortia at each site for fall overturn (Oct 2004), winter mixing (Feb 2005), onset of summer stratification (Jun 2005), and late season stratification (Oct 2005).

14-Oct-04			3-Feb-05		
Site	Biomass Production (x 10^{-5} gC l^{-1} h^{-1})	Generation Time (h)	Site	Biomass Production (x 10^{-5} gC l^{-1} h^{-1})	Generation Time (h)
A	1.8	8.2	A	1.3	16.1
B	6.2	3.3	B	1.1	16.1
C	2.6	11.9	C	1.7	11.4
D	1.4	20.8	D	1.2	14.8
E	4.0	10.9	E	2.1	9.8
1-Jun-05			21-Oct-05		
Site	Biomass Production (x 10^{-5} gC l^{-1} h^{-1})	Generation Time (h)	Site	Biomass Production (x 10^{-5} gC l^{-1} h^{-1})	Generation Time (h)
A	9.2	3.9	A	10.6	1.6
B	9.4	2.4	B	4.5	3.7
C	6.6	3.2	C	11.6	1.2
D	7.6	2.0	D	6.9	4.1
E	22.0	1.4	E	6.7	2.2

Discussion

While the SWI zone is often arbitrarily defined (Novitsky 1983), it is commonly recognized as a transition zone from fluid to solid matrix accompanied by a change in redox potential (Danovaro et al. 1998). Further, Boudreau and Jørgensen defined this zone as a 'benthic boundary layer' recognized as the location of chemical and energy exchange between the water column and sediment bed marked by high biological activity (2001). Changes in SWI redox potential are bacterially mediated by consumption and production of organic matter while utilizing a variety of electron acceptors. Because these organic matter cycles and changes in redox potential affect the quantity and quality of nutrients, the trophic status, and overall water quality of the lake ecosystem, rates of SWI bacterial activity and production are important in understanding microbial loop processes.

Tritium labeled L-serine (Ser) uptake was measured for SWI bacterial consortia using measures of Ser_{tot} and Ser_{pro}. Because few studies have used radiolabeled Ser (e.g. Murrell et al. 1999), it was unknown what percentage of Ser was incorporated into cellular protein, and what percentage was converted into other compounds. Our data indicate that for short term uptake (i.e. 20 min) Ser_{pro} is approximately 40 % of Ser_{tot}. Several reasons may account for the fate of introduced Ser not incorporated into protein. First, methanotrophic and methylotrophic bacteria, commonly found at oxic/anoxic zones such as the SWI, utilize Ser via a serine pathway to assimilate single-carbon compounds into cell material via formation of an acetyl-CoA intermediate (Madigan et al. 1997). Second, Ser may be incorporated into the bacterial cell wall as an interbridge component in the peptidoglycan layer (White 1999). Third, flagella-containing bacteria display a cell

surface receptor (Tsr) that binds Ser, which serves as a chemoattractant to allow functioning of the flagellar motor (Grebe and Stock 1998).

Because Ser may be incorporated into a variety of bacterial structures and pathways, Ser_{tot} was chosen to compare differences in seasonal SWI bacterial uptake rather than Ser_{pro}. When removing the covariable effect of bacterial abundance, highest Ser_{tot} was observed at the onset of summer stratification (Jun 2005) (119 nmol l^{-1} h^{-1} – 251 nmol l^{-1} h^{-1}) followed by late season stratification (Oct 2005) (76 nmol l^{-1} h^{-1} – 129 nmol l^{-1} h^{-1}). Lowest Ser_{tot} was observed during winter mixis (Feb 2005) (14 nmol l^{-1} h^{-1} – 33 nmol l^{-1} h^{-1}) and onset of autumnal overturn (Oct 2004) (11 nmol l^{-1} h^{-1} – 52 nmol l^{-1} h^{-1}) (Figure 2.3(A) – 2.3(D)).

Bacterial abundance did not change significantly among seasons; however bacterial abundance was coupled with high Ser_{tot} during the onset of stratification (Jun 2005) (Figure 2.3C). With the exception of Site D, this relationship also held for late-season stratification (Oct 2005) (Figure 2.3D). The relationship did not exist during overturn (Oct 2004) and winter mixis (Feb 2005) (Figure 2.3A and 2.3B). This coupling indicates that during onset and late-season stratification, individual bacteria have a higher specific Ser_{tot} than individual bacteria during onset of overturn and winter mixis. Higher specific cell uptake rates during summer months, as well as evidence that 60 percent of Ser is incorporated into protein suggest SWI bacteria during summer stratification increase in biomass more so than bacteria during overturn and mixing.

Physicochemical variables related to seasonal stratification and mixing were highly correlated (e.g. temperature and dissolved oxygen). Principal components (PCs) were derived to statistically account for these correlations and relate the physicochemical

data to Ser_{tot} (Table 2.3). When the significant principal components were incorporated into a regression (PCR), the resulting model predicted higher Ser_{tot} under SWI conditions of higher temperature, lower dissolved oxygen, lower pH, lower redox and higher specific conductance, consistent with late-season stratification. The model also predicted higher Ser_{tot} under SWI conditions of lower temperature, lower dissolved oxygen, higher pH, and higher specific conductance, consistent with the onset of stratification (i.e. late spring, early summer) (Table 4).

Solely as a function of temperature, greater SWI bacterial Ser_{tot} would be expected under higher temperatures due to increased function of metabolic enzymes (i.e. the Q_{10} principle) (Atlas and Bartha 1998). Indeed, lowest Ser_{tot} was observed under coldest conditions (Figure 2.3B), however highest activity was observed under temperatures that were neither highest nor lowest during the course of this study. In most cases, under high dissolved oxygen, Ser_{tot} was lower than during periods of oxygen depletion. Possible reasons for this trend could be due to multiple electron acceptors available to bacteria under cold, anoxic conditions (Kelly et al. 1988). During onset of and during prolonged anoxia, redox potential decreases, resulting in a greater variety of electron acceptors available to bacterial consortia not present under oxic conditions. While facultative anaerobes would be active under oxic and anoxic conditions and a variety of redox potentials, the remaining activity during anoxia would be due to strict anaerobes. This suggests that strict anaerobes collectively may exhibit higher activity than strict aerobes. This also supports evidence from an earlier study on Belton Reservoir that demonstrated greater individual bacterial volumes and biomass in anoxic

hypolimnia (near the SWI) relative to the upper mixing and aerobic water column (Christian et al. 2002).

Other physical factors may also account for seasonal differences in SWI bacterial activity. During summer stratification, water movement at the SWI is negligible, with fluxes between sediment and water occurring primarily through bioturbation, sinking of organic matter from decaying and degraded phytoplankton, and reduced nutrients entering the water column (Gantzer and Stefan 2003). Hence long-term stable bacterial populations may form at the SWI without being flushed out or removed. In contrast, during periods of overturn due to cooling and physical mixing, increased SWI water movement and increased flux of sediment and nutrients may flush the bacteria out of the SWI, resulting in frequent changes in SWI bacterial community composition and unstable community structure. This contradicts other studies that have shown that faster water movement during mixing brings in more bacterial substrates resulting in faster bacterial substrate utilization (Boudreau and Jørgensen 2001; Gantzer and Stefan 2003).

Because Ser has not been commonly used as a measure of bacterial activity, conversion factors for bacterial production are not well established. We have used the value of 5.5 percent of total bacterial protein composed of L-serine. This value was established by Reeck (1973) and Reeck and Fisher (1973) and is based on the amino acid composition of 69 different purified bacterial proteins. Kirchman (1985) derived the percent of L-leucine in protein based on this same source, which remains well accepted. This conversion is theoretical however, and the true percentage of L-serine in protein may differ for natural bacterial assemblages. Recently a conversion factor of 4.2 percent L-serine in bacterial protein was derived from an analysis of freshwater sediment bacteria

(Buesing and Marxsen 2005). However, given the range of our measured uptake rates, our values of bacterial production are comparable to that of L-leucine in a variety of aquatic and sediment environments (Servais 1995; Tuominen 1995; Buesing and Marxsen 2005).

Lastly, we estimated generation times of the SWI bacteria based on the equation of Ducklow (2000) and estimates of cellular carbon (Lee and Fuhrman 1987). While much debate has ensued about the amount of carbon per bacterium, 20 fg $cell^{-1}$ established by Lee and Fuhrman is widely accepted. Shorter generation times were observed during stratification and before autumnal overturn, implying higher rates of reproduction, consistent with balanced growth. While there may be tremendous variability in reproduction rates of individual populations, the values calculated represent a total community average. Higher rates of reproduction are consistent with the onset of and late-season stratification. During this time, calm SWI physical conditions prevail, therefore bacteria can devote more energy into reproduction, rather than survival.

Conclusions

Our study has revealed that summer stratification and related physicochemical dynamics drives SWI bacterial activity and biomass production at greater rates than thermal mixing processes. Results show that SWI bacterial uptake rates of L-serine - used as a surrogate of bacterial activity and biomass production - are greater during the onset of summer stratification and during late season stratification. These rates are independent of bacterial abundance, but during stratification bacterial abundance is coupled with high Ser_{tot}, suggesting higher per-cell activity, consistent with higher rates of cell reproduction. Several physicochemical dynamics that change during stratification

and overturn, including SWI temperature, dissolved oxygen and redox potential are associated with and modeled to variations in Ser_{tot}. We conclude SWI bacterial metabolism is more important in this reservoir's carbon cycling during summer months than during winter months, and therefore contributes an important role in nutrient dynamics and eutrophication processes often attributed mostly to planktonic, and often autotrophic, microorganisms.

Acknowledgments

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CHAPTER THREE

Key Issues Concerning Biolog Use for Aerobic and Anaerobic Freshwater Bacterial Community-Level Physiological Profiling

Introduction

Originally designed to rapidly identify cultured bacterial isolates, microbial ecologists have tailored Biolog MicroPlates™ (e.g. GN and GP MicroPlates) to generate bacterial community-level physiological profiles (CLPPs) from environmental matrices such as soil, sewage sludge, and water. Applications and limitations of Biolog MicroPlates for terrestrial bacterial community profiling are well documented (Smalla et al. 1998; Balser et al. 2002; Classen et al. 2003; Insam and Goberna 2004). Aquatic (freshwater and marine) bacterial communities have been profiled to a lesser extent (Choi and Dobbs 1999; Grover and Chrzanowski 2000; Schultz and Ducklow 2000; Tam et al. 2003; Sala et al. 2005), consequently several methodological and analytical CLPP issues involving aquatic bacterial assessment have not been adequately addressed.

Biolog MicroPlates contain 96 microtiter wells, each containing a distinct organic carbon substrate and a redox-sensitive tetrazolium dye indicator. When plates are inoculated with a bacterial community and incubated, purple formazan salt forms in each well proportional to the extent of bacterial community substrate oxidation. Each well is scored for substrate use or disuse or read for its optical density at 590 nm (OD_{590}). The resulting carbon substrate utilization pattern (CSUP) is used to develop the CLPP (Garland and Mills 1991; Konopka et al. 1998).

In response to GN MicroPlate ecological use, Biolog developed the EcoPlate™, containing, in triplicate, 31 organic carbon substrates and a control well with dye, but no substrate (Table 3.1). EcoPlates provide intraplate replication, allowing for greater statistical applications. EcoPlates contain 25 substrates common to GN plates, as well as 6 substrates unique to the EcoPlate. This suite of substrates was chosen to allow for substrate utilization by a variety of metabolically diverse and slower growing bacteria that are commonly found in environmental matrices (Preston-Mafham et al. 2002).

Table 3.1: List of all 31 carbon substrates in Biolog EcoPlates™, their corresponding classes, and plate code numbers.

Substrate	Plate Code	Substrate	Plate Code
Amino Acids (n = 6)		Carboxylic Acids (n = 9)	
L-Arginine	A4	γ-Hydroxybutyric Acid	E3
L-Asparagine	B4	α-Ketobutyric Acid	G3
Glycyl-L-Glutamic Acid	F4	D-Galacturonic Acid	B3
L-Phenylalanine	C4	D-Glucosaminic Acid	F2
L-Serine	D4	Itaconic Acid	F3
L-Threonine	E4	D-Malic Acid	H3
Carbohydrates (n = 10)		Pyruvic Acid Methyl Ester	B1
D-Cellobiose	G1	2-Hydroxybenzoic Acid	C3
i-Erythritol	C2	4-Hydroxybenzoic Acid	D3
D-Galactonic Acid γ-lactone	A3	Amines (n = 2)	
N-Acetyl-D-Glucosamine	E2	Phenylethylamine	G4
Glucose-1-Phosphate	G2	Putrescine	H4
β-Methyl-D-Glucoside	A2	Polymers (n = 4)	
D,L-α-Glycerol Phosphate	H2	α-Cyclodextrin	E1
α-D-Lactose	H1	Glycogen	F1
D-Mannitol	D2	Tween 40	C1
D-Xylose	B2	Tween 80	D1

While literature has addressed many Biolog MicroPlate limitations and applications, this investigation addresses these issues as they apply directly to freshwater

heterotrophic bacterial community studies. The value of this is threefold. (1) Unlike many environmental bacteria, aquatic bacteria differ in that their environmental matrix allows direct inoculation; therefore microbial ecologists need a reference to specifically address this significance. (2) Because heterotrophic bacteria dynamically impact organic matter cycling in freshwater ecosystems (Van Mooy et al. 2001; Chin-Leo 2002), CSUPs illustrate the potential complexity of organic matter cycling (Sinsabaugh and Foreman 2001; Tam et al. 2003). (3) CLPPs indicate bacterial community metabolic potential, which provides insight into the overall functional diversity of heterotrophic bacterial assemblages present in the ecosystem.

For general methodological and statistical issues involving a variety of Biolog MicroPlates, the reader should refer to Konopka et al. (1998), Mills and Garland (2002), and Preston-Mafham et al. (2002). Because of their design for ecological studies and ease of statistical analysis, only EcoPlate usage is discussed. However, the information presented is pertinent to other types of Biolog MicroPlates due to the similarity of their design and applicability to aquatic bacterial communities.

Specifically the following topics are addressed: (1) The effect of aquatic bacterial inoculum density on carbon substrate utilization rate, (2) The effects of incubation temperature on carbon substrate utilization rate and pattern, (3) Well color development due to non-bacterial entities in the inocula, (4) The extent of substrate selectivity, and (5) Adaptation of EcoPlates for use in anaerobic aquatic bacterial community studies.

Materials and Methods

Study Site

Bacterioplankton samples were collected from Belton Reservoir, Bell County, Texas, USA (31° 06' N, 97° 28' W), a subtropical, eutrophic monomictic reservoir (Christian et al. 2002). Collection depths ranged from 13.5 m to 25.2 m. Water temperature and dissolved oxygen concentration ranged from 10.9°C to 26.9°C and 0 mg l^{-1} to 10.6 mg l^{-1}, respectively, as measured with a YSI 600QS Sonde (YSI, Inc. Yellow Springs, Ohio, USA).

General Methodology

Samples were collected at various times before, during, and after autumnal overturn to allow for differences in lake temperature and dissolved oxygen, as necessary for analyses. Samples were taken from less than 0.1 m above the sediment-water interface using a 3.2 l horizontal Alpha Water Sampler (Wildlife Supply Company, Buffalo, New York, USA). Three hundred ml black BOD bottles were overflowed three times with the sample and immediately capped to maintain in situ oxygen concentration. The sampler was rinsed with bacteria-free (0.2 μm-filtered) water between sample hauls to flush out remaining bacteria. The bottles were immersed in coolers containing water collected at sampling depth so that in situ temperature was maintained. All samples were returned to the laboratory within 4 to 6 hours of sample collection for immediate processing.

EcoPlates were purchased directly from the manufacturer (Biolog Inc., Hayward, California, USA) and stored at 4°C until use. All plates were inoculated with 150 μl of sample water into each well using an 8-channel repeating pipettor fitted with 1 ml sterile

disposable pipettor tips. Plates were incubated (temperature depended on experiment) and read at OD_{590} on a daily basis with a Biolog MicroStation 3 plate reader and software. The data was transferred via the software to Microsoft Office Excel 2003. JMP™ 5.0 (SAS Institute, Inc., Cary, North Carolina, USA), Microsoft Office Excel 2003, and SigmaPlot® 2000 (SPSS, Inc., Chicago, Illinois, USA) were used for statistical and graphical analyses.

Mean OD_{590} for each substrate (n = 3) at each reading time (e.g. every 24 hours) was corrected by subtracting the mean optical density of the control wells (n = 3) and plotted against time. Because typical bacterial logistic growth was observed, a 3-parameter sigmoidal curve was fitted to the data:

$$y = \frac{a}{1 + e^{-(X-X_0/b)}} \tag{3.1}$$

where x and y denote time of incubation and mean OD_{590} of the substrate at time x respectively, a = the horizontal asymptote of maximum growth rate, b = the inverse of the exponential growth rate, and X_0 = the time at the midpoint of exponential growth (Lindstrom et al. 1998). Likewise, the 3-parameter sigmoidal curve was fitted to the average well color development (AWCD) at each reading time (y-axis) plotted against time (x-axis):

$$AWCD_{(t)} = \sum (C_{(t)} - R_{(t)})/n \tag{3.2}$$

where t = time of incubation, $C_{(t)}$ = mean color production (OD_{590}) of a substrate at time t, $R_{(t)}$ = mean color production in the control well at time t, and n = number of substrates (i.e. 31 for EcoPlates) (Garland and Mills 1991; Choi and Dobbs 1999).

Inoculum Density Effects

One ml aliquots from various samples collected before, during, and after autumnal overturn were enumerated for total bacterial abundance. Samples were preserved with an equal volume of 4% formalin and stored at 4°C until enumerated. Each sample was stained with DAPI fluorochrome (1 μg ml^{-1}, final concentration), filtered onto a 0.2 μm polycarbonate black membrane filter, and viewed under UV excitation (330 nm – 380 nm) with a Nikon Eclipse 6600 microscope at 1500 x total magnification (Porter and Feig 1980). All bacteria in 20 fields, or 300 bacteria, whichever came first, were counted. Initial bacterial abundance (inoculum density) was regressed against the AWCD at each reading time for all samples (n = 50) to observe correlation of inoculum density and extent of substrate utilization.

Incubation Temperature Experiment

Samples were collected from all five sampling sites after autumnal mixis. Sample collection temperatures ranged from 15.9°C to 16.5°C, and dissolved oxygen concentration ranged from 4.5 mg l^{-1} to 8.4 mg l^{-1}. Each sample was inoculated into two EcoPlates, one incubated aerobically at near in situ temperature (15°C), the other aerobically at room temperature (22°C). Substrate utilization rates and substrate utilization pattern similarity were compared between the plates.

A simple matching coefficient was used to compare similarities for each substrate response at each reading time between plates incubated at 15°C and 22°C. The use and disuse of a carbon substrate was scored as a 1 or 0, respectively, where use was any positive OD_{590} value when corrected for color development in the control well:

$$\text{Substrate Similarity} = S_{[15°C,\ 22°C]} / (N_{15°C} + N_{22°C} - S_{[15°C,\ 22°C]}) \quad (3.3)$$

where $S_{[15°C, 22°C]}$ = total number of similar responses in corresponding wells at 15°C and 22°C (either 1 and 1 or 0 and 0), $N_{15°C}$ = number of substrates measured at 15°C (i.e. 31) and $N_{22°C}$ = number of substrates measured at 22°C (i.e. 31).

Non-Bacterial Color Development Effects

Samples were collected from all five sampling sites after autumnal mixis, but before cooling. Sample collection temperatures ranged from 24.5°C to 24.7°C, and dissolved oxygen concentration from 6.5 mg l^{-1} to 7.4 mg l^{-1}. Each sample was partitioned, one half inoculated directly into an EcoPlate and the other half autoclaved for 15 min at 121°C and 103 kPa, cooled to room temperature, and inoculated into an EcoPlate. Plates were incubated aerobically at room temperature (22°C). Substrate utilization rates and AWCD at each reading point of raw samples were compared to substrate utilization rates and AWCD at each reading point of autoclaved samples.

Substrate Selectivity Effects

Equal volumes of the non-autoclaved portions of the water samples (n = 5) used for the non-bacterial color development experiment were pooled and plated onto two EcoPlates and incubated aerobically at room temperature (22°C). One ml of initial inoculum was enumerated as above for the inoculum density experiment. In addition counts were separated into total cocci and total bacilli. A 100 µl aliquot was removed from all 32 different wells every 24 hours for four days and enumerated, with counts separated into total cocci and total bacilli. Because each well was inoculated with 150 µl of sample, new wells were sampled daily.

Anaerobic Community-Level Physiological Profiling

Numerous samples were retrieved from all sites during a prolonged period of anoxia (summer stratification). Temperatures ranged from 14.9°C to 25°C. To process an individual sample in the laboratory, the BOD bottle containing collection water was uncapped and immediately purged with a stream of nitrogen gas via a 15 mm-diameter aquarium tube fitted into the bottle. Sample was drawn gravimetrically from the bottle, through 15 mm-diameter aquarium tubing, into a 25 ml sampling trough and overflowed three times volume. The sample in the trough was continuously purged with nitrogen gas while it was drawn into the 8-channel pipettor, in which the disposable pipet tips were purged with nitrogen before and after fitting them to the pipettor. Samples were immediately inoculated onto the plate under a blow down of nitrogen. The plates were immediately covered with a non-slit silicone plate seal, with small volumes of water displaced by the seal, preventing a headspace of air. The lid was placed on the plate and double-sealed with tape.

Results

Inoculum Density

There was a significant positive correlation between bacterial inoculum density and AWCD after 24 hours of incubation ($r^2 = 0.73$, $p < 0.0001$, $n = 50$) (Figure 3.1). This correlation decreased with increasing incubation time (Table 3.2). Because low initial cell inocula are associated with low rates of substrate utilization for short term incubations, the initial density of cells plated impacts the duration of lag before exponential growth is observed.

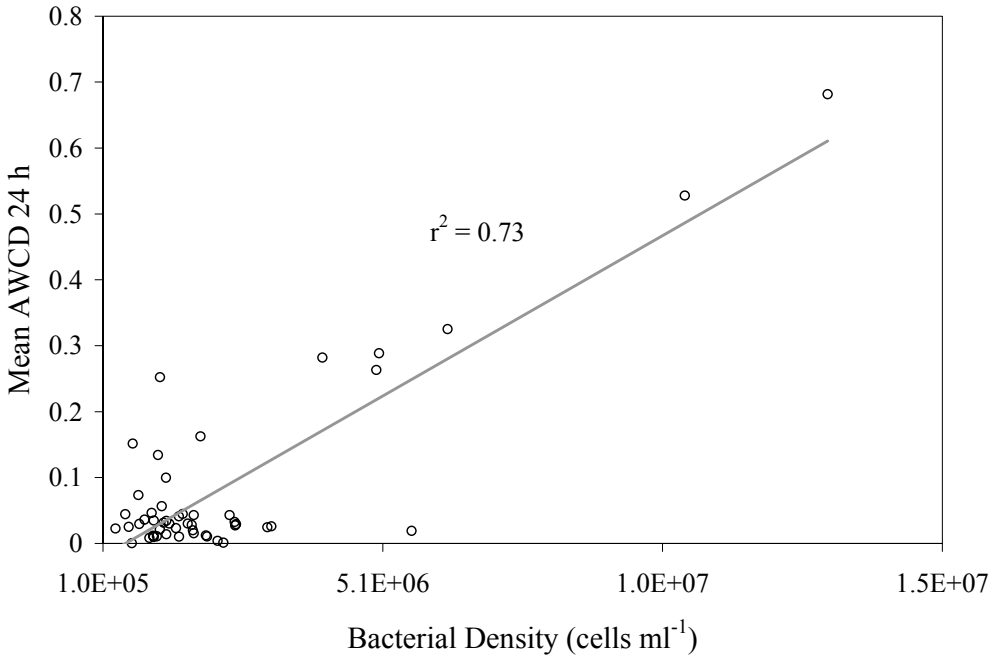

Figure 3.1: Correlation of bacterial inoculum density vs. average well color development for the 31 carbon substrates collectively after 24 hours of incubation. Based on a total of 50 samples.

Table 3.2: Coefficients of determination (r^2) for bacterial inoculum density vs. average well color development for all 31 carbon substrates collectively for various time-course incubations, based on 50 samples.

Incubation (h)	r^2	p-value
24	0.73	< 0.0001
48	0.38	< 0.0001
72	0.07	0.35
96	0.07	0.63

Incubation Temperature

In their study of four temperate lakes, Grover and Chrzanowski (2000) incubated water samples in Biolog GN Plates at various temperatures under the assumption that incubation temperature affects the rate of substrate oxidation, but not pattern. This assumption was tested by incubating water samples in EcoPlates at near in situ temperature and at room temperature. Room temperature of 22°C was chosen, as this

was the temperature used by Grover and Chrzanowski for most of their incubations (2000).

AWCD at each reading point and substrate utilization rates for the 15°C incubations were less than for incubations at 22°C (Figure 3.2). 15°C plates were further read at OD_{590} daily for a total of two weeks, each of which reached a μ_{max} of approximately 0.28 OD_{590} units (data not shown). It was therefore proposed that differences in substrate utilization rate and AWCD were due to the elevated incubation temperature causing a net increase in bacterial community metabolism. However, it was uncertain if the increased temperature selected for bacterial species that do not metabolize substrates at 15°C. Thus, substrate response similarity at multiple reading times between both sets of plates was compared. Results of the matching coefficient (Table 3.3) indicate that the highest similarity among plates incubated at 15°C and 22°C was approximately 60% after 96 incubation hours. These results indicate that incubation temperature has an effect CSUP, not just rate.

Table 3.3: Mean number of similar responses and mean similarity coefficient ± standard deviation for a series of paired plates (5 pair, one of the pair incubated at 15°C and the other at 22°C, each pair from a different water sample) at various times of incubation. Total possible number of similar responses (both wells $OD_{590} > 0$, or both wells $OD_{590} \leq 0$) per pair of plates is equal to the number of wells on a single plate (96).

Time of Incubation (h)	Mean Similar Responses	Mean Similarity Coefficient ± S.D.
24	67.5	0.552 ± 0.143
48	47.8	0.340 ± 0.117
72	63.2	0.502 ± 0.151
96	70.8	0.597 ± 0.165

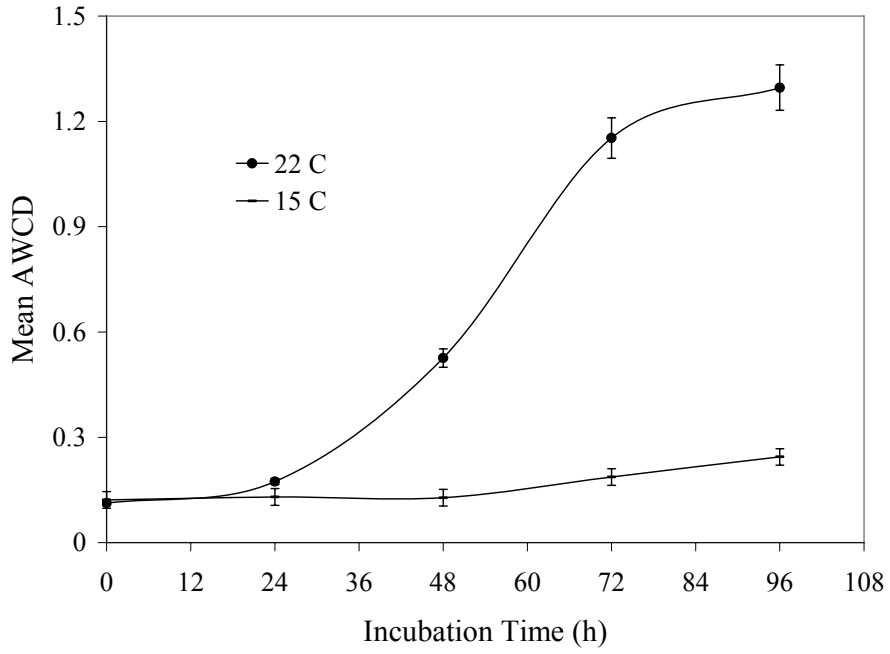

Figure 3.2: Mean average well color development through a time course incubation for plates (n = 5) incubated at near in situ temperature (15°C) or room temperature (22°C). Error bars indicate standard error of the mean.

Non-bacterial Color Development

Results from this experiment showed that mean AWCD of the autoclaved water sample at each reading point was minimal (Figure 3.3). However, the large standard error associated with the mean AWCD of the autoclaved samples was unexpected. All plates were incubated with the same homogenous autoclaved sample, and hence, a similar response was expected in all plates. A possible explanation is that the substrate formulations among plates were not equal in either substrate concentration and/or tetrazolium dye. Plates used for this experiment were purchased at different times, each from different production batches, potentially causing this source of variation.

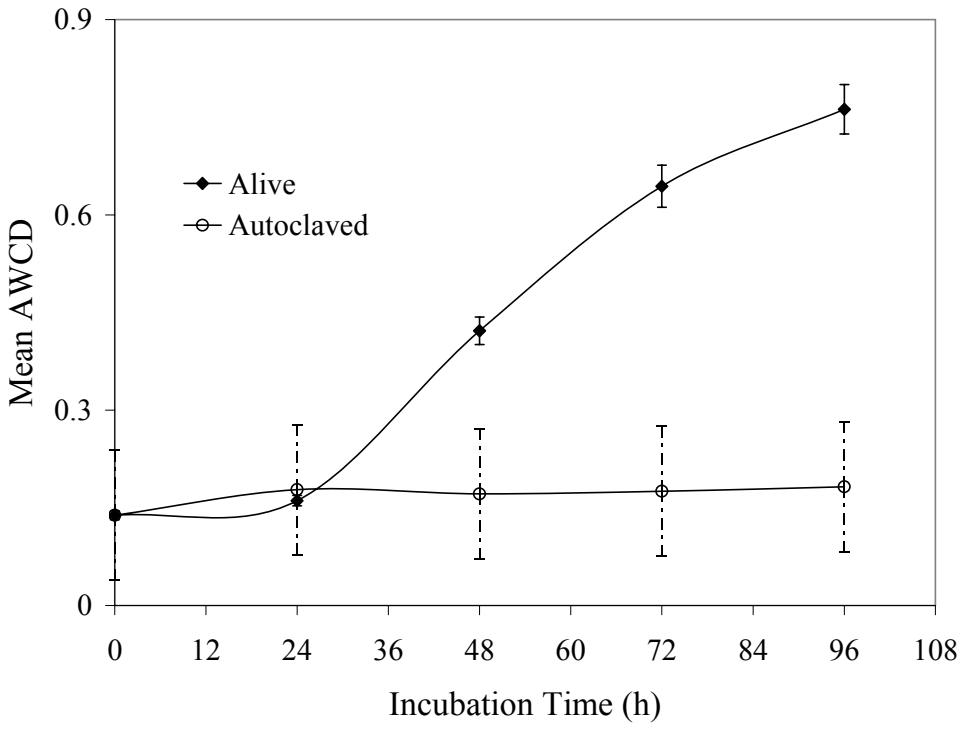

Figure 3.3: Mean average well color development through a time course incubation for plates incubated with a single water sample that was unamended (n = 5 plates) or autoclaved (n = 5 plates). Error bars indicate standard error of the mean.

Substrate Selectivity

All substrates exhibited changes in morphological ratios (Figure 3.4). After 96 hours all but two substrates showed greater than 25% change, while ten substrates exhibited more than 100% change in morphological ratio after just 24 hours of incubation. There was no pattern of percent change vs. substrate class. Two substrates selected for what appeared to be a pure culture, as only one size of cocci (L-phenylalanine) or bacilli (Tween 80) were observed after 96 hours of incubation. Some ratios fluctuated throughout incubation time (e.g. itaconic acid), while only one substrate (2-hydroxybenzoic acid) showed little net change in ratios (Figure 3.5).

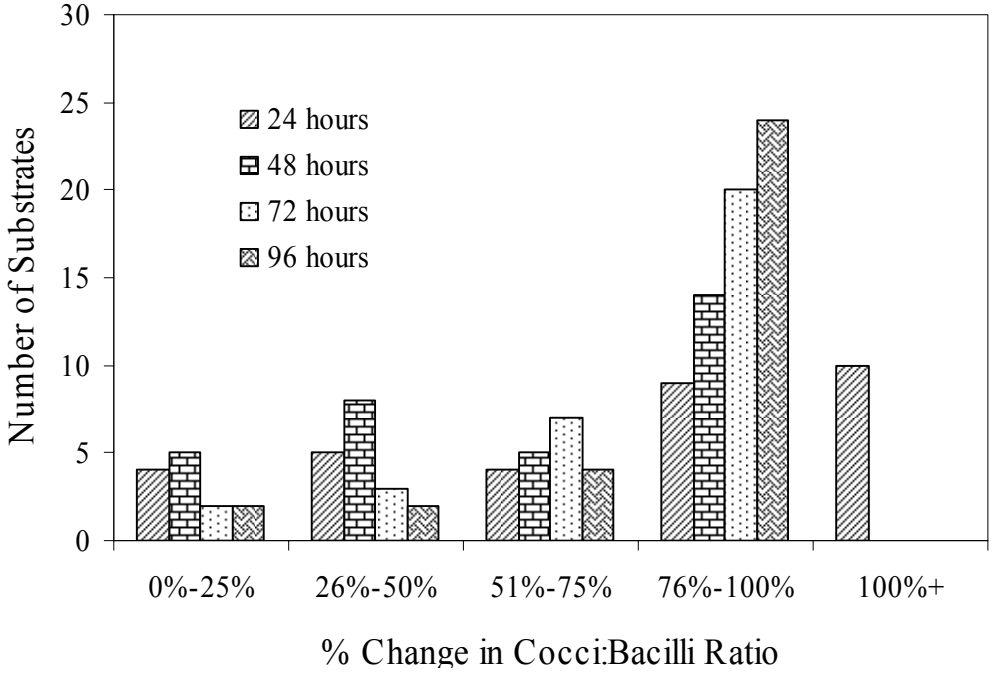

Figure 3.4: Number of substrates with their percent change in morphological (cocci to bacilli) ratios for each reading time. Incubation conducted at 22°C.

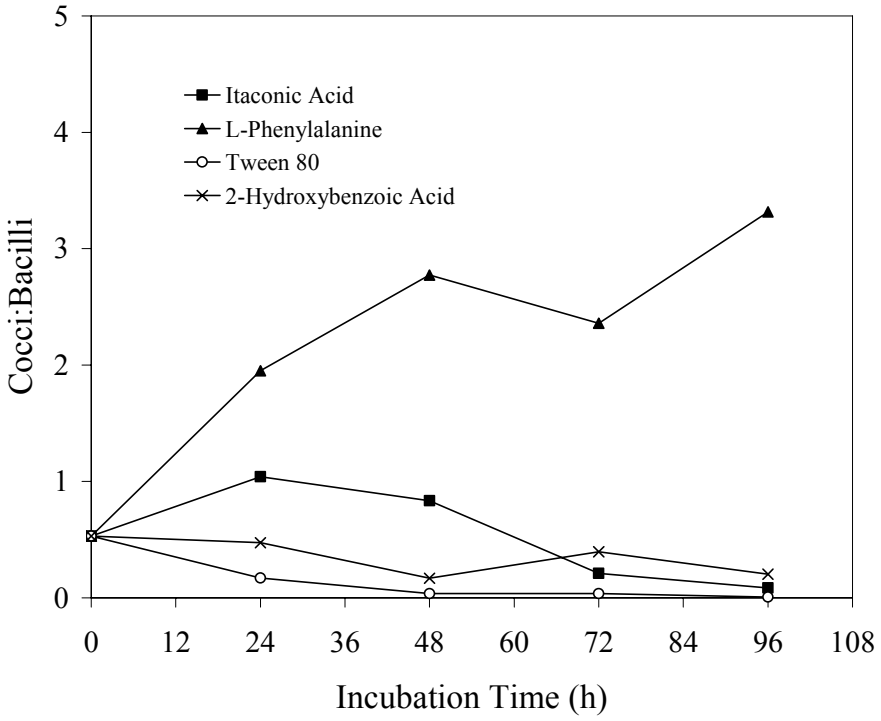

Figure 3.5: Change in morphological ratios (cocci to bacilli) through time for a bacterial community for various substrates.

Anaerobic Bacterial Community Analysis

The effectiveness of our anaerobic method was tested by simultaneously incubating anoxic water samples in a pair of EcoPlates, one prepared using our anaerobic method, the other prepared aerobically (both incubated at 22°C, n = 3). Plates were read at OD_{590} over the course of seven days. Plates grown aerobically showed a significantly diminished response in color development (mean μ_{max} anaerobic = 0.623 ± 0.138 vs. mean μ_{max} aerobic = 0.212 ± 0.087, student's $t = 4.358$, $p < 0.01$). Also the mean number of utilized substrates in the aerobically grown plates (26 substrates) was less than the mean number utilized in the anaerobically grown plates (31 substrates). Assuming all strict anaerobes were killed upon exposure to oxygen during aerobic sample processing, growth in the aerobically grown plates was due to facultative bacteria.

Discussion

Although numerous methods are used to analyze aquatic bacterial assemblages (e.g. epifluoresence microscopy, radiolabelled tracers, FAME analysis, RFLPs, DGGE analysis, etc.), each are used to describe different characteristics. Biolog MicroPlates are often described as a method of determining the functional diversity or functional potential of bacterial communities (Konopka et al. 1998; Preston-Mafham et al. 2002). However, it is unlikely that in situ function can be determined from the plates, because most organic substrates in aquatic systems are more complex than those found in Biolog EcoPlates and the carbon substrates may not be ecologically relevant (Smalla et al. 1998). Further, the plates are selective and may not represent activity of all community members, possibly confounding ecological interpretation (Haack et al. 1995). Yet, CLPPing has been used effectively to establish spatial and temporal changes in bacterial communities (Garland

1997) as well as providing insight into functional ability of bacterial community members (Preston-Mafham et al. 2002).

Unlike soil or sediment CLPPing, aquatic systems offer the advantage of being able to inoculate the environmental matrix (water) directly into the wells without dilution or major disruption of the community. Yet bacterial abundance in aquatic ecosystems is variable on both spatial and temporal scales (Christian and Capone 2002), therefore variable bacterial abundances inoculated directly from the environment without first standardizing the inoculum density will affect rate of substrate utilization. This investigation shows that for short term incubations, lower inocula densities results in a longer lag phase before substrate is utilized. It could be argued that enumerating the bacteria via DAPI or acridine orange staining followed by dilution to a known concentration and using the diluted consortia for inoculation is a valid method, however this may eliminate bacteria that show strong response to substrate utilization, but are present in low numbers. It is also possible that dilution may alter ratios of individual populations that strongly contribute to overall response, which will affect the rate.

This finding is also important from an analytical standpoint. For example, the OD_{590} of a substrate at a single time point divided by the AWCD at that time point is often used as the metric for determining CSUPs (Glimm et al. 1997; Insam and Goberna 2004). If the time point is unknowingly chosen before exponential growth has began for all substrates, the resulting metric will underestimate the actual carbon substrate utilization pattern. To alleviate this problem, the use of a kinetic instead of a single time-point approach is suggested, in which the rate of exponential growth (equation 1) is used as the metric to develop the CSUP. While using a kinetic approach is not novel, it allows

the investigator to preserve the relative abundance of the entire bacterial community, providing a better estimate of community functional potential.

Because CLPPs are a measure of 'functional potential', then incubation temperature should be reported with all results. This investigation has shown that altering incubation temperature results not only in variation of color response in all wells, but also response pattern. If various incubation temperatures are used for a spatial or time course study, CSUP differences will be confounding, making it impossible to assess spatial and temporal CSUP differences from differences due to the selectiveness of varying incubation temperature. Therefore incubation temperature should be standardized across all incubations if spatial and/or temporal community studies are to be undertaken. This is not to imply that any certain incubation temperature is 'optimum', because the temperature may not reflect in situ temperature. However if this temperature is applied consistently, one may eliminate possibility of CSUP differences due to varying temperatures.

When interpreting CSUPs, it has been assumed that all color development from tetrazolium dye reduction is due to bacterial carbon substrate oxidation, however previous to this investigation this remained untested. By plating an autoclaved water sample, all living organisms were killed (e.g. bacteria, viruses, microeukaryotes), leaving only complex organic and inorganic nutrients, waste products, metals, and toxins in the water sample to react with the carbon substrates and produce insoluble formazan. By plating autoclaved water samples and comparing the response to unamended water samples, it was deduced from the high standard error associated with utilization rates of the autoclaved samples that all plates for a single investigation should be from the same

production batch to minimize any differences in substrate and tetrazolium dye concentration. Each well is said to contain 0.3 mg of carbon substrate (Mills and Garland 2002), but even small deviations from this amount may drastically affect substrate utilization rate. Plates from the same batch are likely to contain more similar and consistent formulations of substrates than plates produced in different batches.

If high rates of non-bacterial tetrazolium dye reduction are suspected, killed controls should be run under the same conditions as sample plates and a correction factor applied to the color development of each well. Furthermore, while the carbon substrates are formulated in a concentration for bacterial use, it is uncertain if a portion of the total substrate oxidation is due to heterotrophic eukaryotic microorganisms.

Many researchers assume that individual wells of Biolog MicroPlates are analogous to selective culturing media, however the extent of individual substrate selectivity has not been tested on aquatic microbial assemblages. If the carbon substrates are not strongly selective, at any given time point, bacterial morphological ratios (i.e. cocci to bacilli) would be similar to their ratios in the initial inoculum. Significant changes in morphological ratios would indicate selectivity for certain populations. This investigation empirically shows that Biolog is a selective culturing technique. The results indicate that selectivity varies among substrates, but all substrates exhibit some degree of selectivity. This selectivity may be due to several factors: (1) only organisms that can grow under the constrained conditions imposed will contribute to color response in the individual wells, (2) inoculum density, and/or (3) activity/dormancy of specific populations. Further, the results support the suggestion that CLPPs are a measure of functional potential, not in situ ecological potential, therefore any attempts to imply

ecological function should be approached with caution. Yet, previous studies suggested that incubations of less than 24 h may closely represent in situ function (Mills and Garland 2002). However, use of a preferred class of substrate by a bacterial community (e.g. carbohydrates, amino acids) may indicate nutritional needs of the bacteria and therefore provide insight into community metabolic status.

Many aquatic systems contain anaerobic habitats (e.g. anoxic hypolimnia of lakes, anaerobic wetlands), but obtaining anaerobic bacterial CLPPs from these habitats is difficult and thus lacking in literature. Traditional anaerobic incubations require specialized, expensive anaerobic chambers and disruption of the anaerobic conditions to optically read plates. Therefore a simpler method was devised for anaerobic incubations that does not require anaerobic chambers.

Some indicate that formazan is not produced in Biolog plates incubated under anaerobic conditions (Preston-Mafham et al., 2002). However other reports contradict this (Mills and Garland, 2002). Preliminary experiments for this investigation showed that formazan is indeed produced under anaerobic conditions, but may be sensitive to incubation temperature (data not shown). Recently Biolog has developed an AN Plate, with reformulated redox indicators specific for anaerobic bacteria, however few microbial ecological studies have used these plates.

This investigation shows that the protocol given in the above methods for anaerobic incubations is effective. The only items required that are not used for aerobic inoculations are a source of nitrogen gas (preferably of a high purity), simple aquarium tubing, and silicone plate seals. While various types of plate seals are available, seals that

contain indentations that will minimize any headspace of air are suggested. Gases other than nitrogen (e.g. helium, argon) may be substituted if necessary.

Conclusions

While the techniques for Biolog MicroPlate usage in aquatic microbial ecology are simple, analyzing CLPPs has often proved confounding. However, based on our recommendations, sources of confusion and error can be minimized. The low cost and ease of use of Biolog plates provides valuable insight into bacterial substrate utilization patterns and metabolic functional potential in aquatic ecosystems. Furthermore, because our suggestions also address Biolog use with anaerobic systems, CLPPing is now readily applicable to a wider variety of aquatic habitats. We believe this will serve as a valuable reference for aquatic microbial ecologists choosing to use Biolog.

Acknowledgments

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CHAPTER FOUR

Multiple Carbon Substrate Utilization by Bacteria at the Sediment-Water Interface: Seasonal Patterns in a Stratified Eutrophic Reservoir

Introduction

Secondary production via heterotrophic bacterial uptake and metabolism of dissolved organic carbon (DOC) substantially contributes to organic matter cycling in aquatic ecosystems (Sinsabaugh et al. 1997; Chin-Leo 2002). Sediment-water interfaces (SWIs) of lakes provide ample habitats for these heterotrophic bacteria (Liikanen and Martikainen 2003; Bloesch 2004). High and low molecular weight DOC (HM-DOC and LM-DOC) as well as particulate organic carbon (POC) and other dissolved and particulate organic matter (DOM and POM) not utilized by pelagic microbes collects and concentrates at the SWI (Dean 1999; Vreča 2003; Heinen and McManus 2004). The DOC fraction contributes a substantial portion of total organic matter in these freshwater sediments (O'Loughlin and Chin 2004). Bacteria that blanket the SWI incorporate DOC into their biomass and oxidize DOC into inorganic carbon using a variety of electron acceptors in respiratory pathways, releasing chemically-reduced compounds into the water column (Vreča 2003; Bloesch 2004). Further, in situ studies and theoretical mixing models have shown that SWI bacteria exhibit high activities (Butorin 1989; Gantzer and Stefan 2003). However seasonal stratification and mixing events and their effects upon changes in SWI bacterial populations and DOC utilization remain unknown.

Physically, the SWI is the transition layer from a fluid (water) to solid (sediment) matrix (Danovaro et al. 1998). This includes the water layer near the sediment surface

that contains a steep gradient of physical and chemical dynamics (Boudreau and Jørgensen 2001). As SWI bacteria incorporate and oxidize organic carbon, electron acceptors are utilized in order of decreasing free energy yield. Dissolved oxygen, being the energetically favored electron acceptor, is quickly depleted resulting in the active SWI bacterial consortia shifting to assemblages that can utilize less energetically-favored acceptors (e.g. nitrate, sulfate). This process lowers redox potential and increases concentrations of chemically-reduced nutrients (e.g. ammonium, sulfides) (Liikanen and Martikainen 2003). The chemically-reduced SWI rapidly disintegrates during episodes of hypolimnetic overturn (Gantzer and Stefan 2003). The reintroduction of dissolved oxygen restores oxidizing redox potentials and shifts active SWI bacterial assemblages towards species that favor aerobic respiration, halting anaerobic respiration and production of reduced nutrients (Stumm 2004). These cycles of thermal stratification and overturn also alter the quantity, and possibly the type, of DOC substrates present at the SWI (Dean 1999).

Various methods have been used to study bacterial DOC utilization in aquatic ecosystems including culture-dependent (e.g. incubation cultures, selective plating) and culture-independent (e.g. respiration rates, biomass production) methods (Jahnke and Craven 1995; Rosenstock et al. 2005). For this investigation Biolog EcoPlates, a phenotypic assay, were utilized to observe SWI seasonal bacterial utilization of LM-DOC (< 1 kDa) substrates at the SWI of a monomictic, eutrophic reservoir. Biolog EcoPlates contain 96 wells, each containing a distinct DOC substrate and redox-sensitive tetrazolium dye. Thirty-one different carbon substrates in triplicate and three carbon-free control wells provide intraplate replication. These substrates include various

carbohydrates, amino acids, carboxylic acids, amines, and small polymers (Table 3.1). Carbon substrate utilization rates (CSURs) are generated by inoculating unamended bacterial samples into the plate wells, incubating the plates, and spectrophotometrically measuring the optical density (OD_{590}) of purple formazan dye formation from tetrazolium reduction, which is proportional to carbon substrate oxidation rate (Mills and Garland 2002).

While Biolog assays suffer from the same inherent biases as selective culturing, they are a valuable and inexpensive way to elucidate functional potential changes in various microbial communities (Choi and Dobbs 1999; Mills and Garland 2002; Chapter Three; Christian and Lind 2006). Also, recent studies have addressed and minimized problems that have historically plagued Biolog assays including inoculum size, incubation temperature effects, and incubation of anaerobic bacterial communities (Chapter Three; Christian and Lind 2006). While the carbon sources in Biolog assays may not represent the DOC compounds found in situ, oxidation of these substrates may serve as a proxy for understanding various classes and patterns of substrates that are preferred under various physicochemical conditions (Grover and Chrzanowski 2000).

Using Biolog EcoPlates, SWI bacterial community CSURs were measured from samples taken during autumnal overturn, winter mixing, early (onset of) summer stratification, and late (prolonged) summer stratification. Corresponding bacterial abundance and physicochemical variables (e.g. temperature, dissolved oxygen, redox potential) were also measured. The objectives were to: (1) determine substrate classes preferentially used by SWI bacterial consortia during each seasonal mixing and stratification event; (2) determine the amount of variation in seasonal substrate utilization

explained by corresponding physicochemical variables; (3) detect correlations among individual substrate CSURs and individual physicochemical variables; and (4) analyze similarities and differences in seasonal community-level physiological profiles (CLPPs) derived from the CSURs.

Materials and Methods

Field Sampling

Belton Reservoir, Bell County, Texas, a monomictic, eutrophic reservoir, served as the sampling location. It thermally stratifies in late spring, maintains an anaerobic hypolimnion throughout summer, and overturns in late autumn (Christian et al. 2002). Five sampling stations representing the depth gradient below the photic zone were chosen (Table 4.1). Depth (m), water temperature (°C), dissolved oxygen (mg l^{-1}), and redox potential (mV) of the SWI were measured using a YSI 600QS sonde by lowering the sonde to the sediment surface and waiting for it stabilize. Two dates were sampled per mixing event: autumnal overturn, winter mixing, early (onset of) stratification, and late (prolonged) stratification (Table 4.2). The late stratification and autumnal overturn samples were collected over two years due to the short time span of stratification and overturn events.

Samples were retrieved from the SWI via a 3.2 l horizontal PVC Alpha water sampler (Wildlife Supply Company, Buffalo, New York, USA). The sampler was lowered to the sediment, raised approximately 0.5 m, moved 2 m horizontally and gently lowered to the sediment surface. This technique minimized disruption of the SWI. Two samples were taken from each sample site, with the second sample collected approximately 10 m from the first as measured via GPS. This minimized sediment

Table 4.1: Morphometric characteristics of Belton Reservoir and corresponding average depth of each sampling site located within the reservoir.

Location	31° 07' N, 97° 29' W
Surface Area (km^2)	49.8
Volume (x 10^8 m^3)	5.45
Secchi Depth (m)	1.2 - 2
Mean Depth (m)	
Whole Lake	10.9
Sampling Site 1	13.7
Sampling Site 2	16.4
Sampling Site 3	20.5
Sampling Site 4	22.8
Sampling Site 5	25.8

resuspension of the first sample affecting the second sample. This sampling scheme was used to sample the benthic boundary layer. This layer is a component of the sediment-water interface, often defined as an area of high discontinuity in wet bulk density and high rates of sinking particles, thus differing from the water column and sediment column (Austen et al. 2002; Hulbert et al. 2002). Thus the samples were turbid, yet liquid in consistency. Equal volumes of the duplicate samples from each station (water with sediment particles) were pooled in a 300-ml dark dissolved oxygen bottle and capped to maintain in situ dissolved oxygen concentration. The sampler was rinsed with 0.45-µm filtered and/or deionized water between samples. Samples were held at collected temperature until returned to the laboratory. Samples were processed within 5 h of collection.

Laboratory Analyses

Biolog EcoPlates (Biolog, Inc., Hayward, California, USA) were inoculated with 150 µl of pooled sample per well. A single EcoPlate was inoculated for each pooled sample, with one pooled sample per station. EcoPlates contain each of the 31 substrates

Table 4.2: Physicochemical data and bacterial abundance at the sediment-water interface for all sampling sites and dates. Dates are classified based on mixing characteristics of the lake.

Classification	Date	Site	Temperature (°C)	Dissolved Oxygen (mg l^{-1})	Redox Potential (mV)	Bacteria x 10^6 ml^{-1}
Late Stratification	16-Oct-03	1	23.7	3.1	225	1.2
		2	17.1	0.6	276	1.0
		3	17.0	0.0	93	5.0
		4	15.5	0.0	38	1.9
		5	14.9	0.0	4	0.6
Fall Overturn	6-Nov-03	1	21.1	3.0	296	1.5
		2	21.0	4.8	364	1.3
		3	21.1	5.5	219	3.0
		4	21.1	5.1	342	1.2
		5	18.6	1.2	103	2.5
Winter Mixing	31-Jan-04	1	11.4	9.7	348	10.5
		2	11.3	10.0	347	5.0
		3	11.4	10.1	367	13.1
		4	11.4	10.6	392	4.0
		5	11.5	10.6	383	6.3
Winter Mixing	12-Mar-04	1	11.8	7.6	384	1.4
		2	11.4	7.1	380	1.3
		3	11.2	7.7	394	1.0
		4	10.9	9.9	391	1.3
		5	10.9	8.6	383	2.2
Early Stratification	6-May-04	1	19.4	8.0	217	1.1
		2	18.8	6.0	231	1.1
		3	17.9	6.9	409	0.7
		4	16.4	2.5	293	0.5
		5	15.1	1.5	255	0.6
Early Stratification	1-Jul-04	1	25.8	4.4	381	1.2
		2	22.9	1.1	258	1.5
		3	21.0	0.4	186	1.0
		4	19.6	0.2	155	1.6
		5	19.4	0.0	120	2.5
Late Stratification	9-Sep-04	1	27.0	2.9	230	1.7
		2	26.8	1.3	157	2.4
		3	23.7	0.8	108	1.9
		4	20.6	0.0	37	1.7
		5	20.1	0.0	15	1.2
Fall Overturn	14-Oct-04	1	24.6	7.4	431	1.1
		2	24.6	7.2	405	1.5
		3	24.6	5.3	411	2.3
		4	24.6	6.8	415	2.2
		5	24.5	6.6	341	3.1

in triplicate, thus allowing intraplate replication for each pooled sample. For samples with dissolved oxygen concentrations less than 0.2 mg l^{-1}, anaerobic inoculation, processing, and incubation techniques were used as outlined in Chapter Three.

Plates were incubated at 22°C and read at OD_{590} (i.e. absorption spectrum of the formazan precipitate) once daily for five days using a Biolog MicroStation 2 plate reader. 22°C was chosen for the incubation temperature, not as an optimum, but to maintain consistency throughout the experiment. Varying incubation temperatures instead of a pre-selected incubation temperature may confound spatial and temporal CSUR variation with differences due to incubation temperature variation (Chapter Three; Christian and Lind 2006).

For each plate, mean OD_{590} for each carbon substrate at each reading time was corrected by subtracting the mean OD_{590} of the control (no substrate) wells at the same reading time. Subtraction of control wells eliminated color and turbidity effects due to varying amounts of sediment (clay) particles in the samples. The corrected OD_{590} for each substrate was plotted against time. Logistic bacterial growth rates were observed; therefore a three-parameter sigmoidal curve was fitted to each substrate for each sample using Sigma Plot 2000. The rate of exponential growth determined by the curve was the CSUR metric (Lindstrom et al. 1998; Christian and Lind 2006).

In addition to Biolog assays, aliquots of each pooled sample were preserved in formalin (2% final concentration) for total bacterial enumeration. The bacteria were stained with DAPI fluorochrome (1 µg ml^{-1} final concentration), filtered onto 0.2-µm blackened polycarbonate filters, and viewed under UV excitation at 1500 x magnification. Total bacteria ml^{-1} were estimated by counting 20 fields or 300 bacteria

per filter (Porter and Feig 1980). For bacteria attached to clay particles, a correction factor of 2 x was applied (Lind and Dávalos-Lind 1991).

Statistical Analyses

Summary and univariate statistics (i.e. correlation/regression, One-way ANOVA, Student's t) were performed using JMP 5.0 and Microsoft Office Excel 2003. A significance level of $\alpha = 0.05$ was used. CANOCO 4.5 was used for all multivariate analyses. CanoDraw for Windows, a graphical analysis package included with CANOCO 4.5, was used for graphical analyses of the multivariate data.

The complete multivariate data set consisted of four subsets, each subset including all sites and dates corresponding to a specific season (mixing event). Each subset consisted of two parts: (1) response (dependent) variables (CSURs) for the 31 substrates (listed in columns) for each corresponding date and site (listed in rows); and (2) supplemental environmental (independent) variables (i.e. temperature, dissolved oxygen, redox potential, bacterial abundance) (columns) for each corresponding date and site (rows). This grouping by subset allowed individual analyses for each mixing event, and partitioned out the covariable effects of sample site. All CSUR data were centered to a mean of zero and standardized to unit variance before conducting the multivariate analyses.

Preliminary data analysis using detrended correspondence analysis (DCA) on the response (CSUR) data for each subset, detrended by segments, established first gradient lengths of 1.4 – 2.9 standard deviation units. Gradients less than 2.0 indicate relatively low beta diversity; hence these data should be subjected to linear ordination methods (e.g. principal components analysis) rather than unimodal ordination methods (e.g.

correspondence analysis). However, gradients ranging from 2.0 – 4.0 work well with either ordination method (Lepš and Šmilauer 2003). For consistency and comparison among subsets, principal components analysis (PCA) was used. This analysis extracted orthogonal linear combinations of variables (principal components) that corresponded to the maximum amount of variation in the subset. PCA produces as many principal components as there are variables. However, only significant components are retained for analyses (ter Braak and Šmilauer 2002).

The environmental variables were projected onto the PCA axes a posteriori to assess the amount of variation in the PCA data that could be attributed to the environmental variables. While this approach was indirect, it did not require stringent assumptions as does direct gradient analyses (e.g. Redundancy Analysis, Canonical Correspondence Analysis) and therefore precludes the use of significance tests (Grover and Chrzanowski 2000).

Results

Seasonal Carbon Substrate Utilization Patterns

Figure 4.1 (a – d) shows loadings of SWI bacterial CSURs onto the first two PCA axes for each seasonal mixing event. Only utilizations that had greater than 50 percent fit (i.e. significant loadings) onto the two axes collectively were included for interpretation. Additionally, only the first two PCA axes were retained for interpretation as the other axes did not account for a significant percentage of the total variance as determined by application of the broken-stick model (Lepš & Šmilauer, 2003; Peres-Neto et al., 2003).

During early summer stratification, the first two PCA axes accounted for 68.2 percent of the total substrate utilization variance. Significant loadings were observed for 15 of the 31 substrates, all strongly positively associated with the first axis and weakly, but significantly associated with the second axis (Figure 4.1a). These loadings included 9 carbohydrates, 3 carboxylic acids, 2 polymers and 1 amino acid. Hence, carbohydrates contributed the highest relative utilization by SWI bacteria during early stratification.

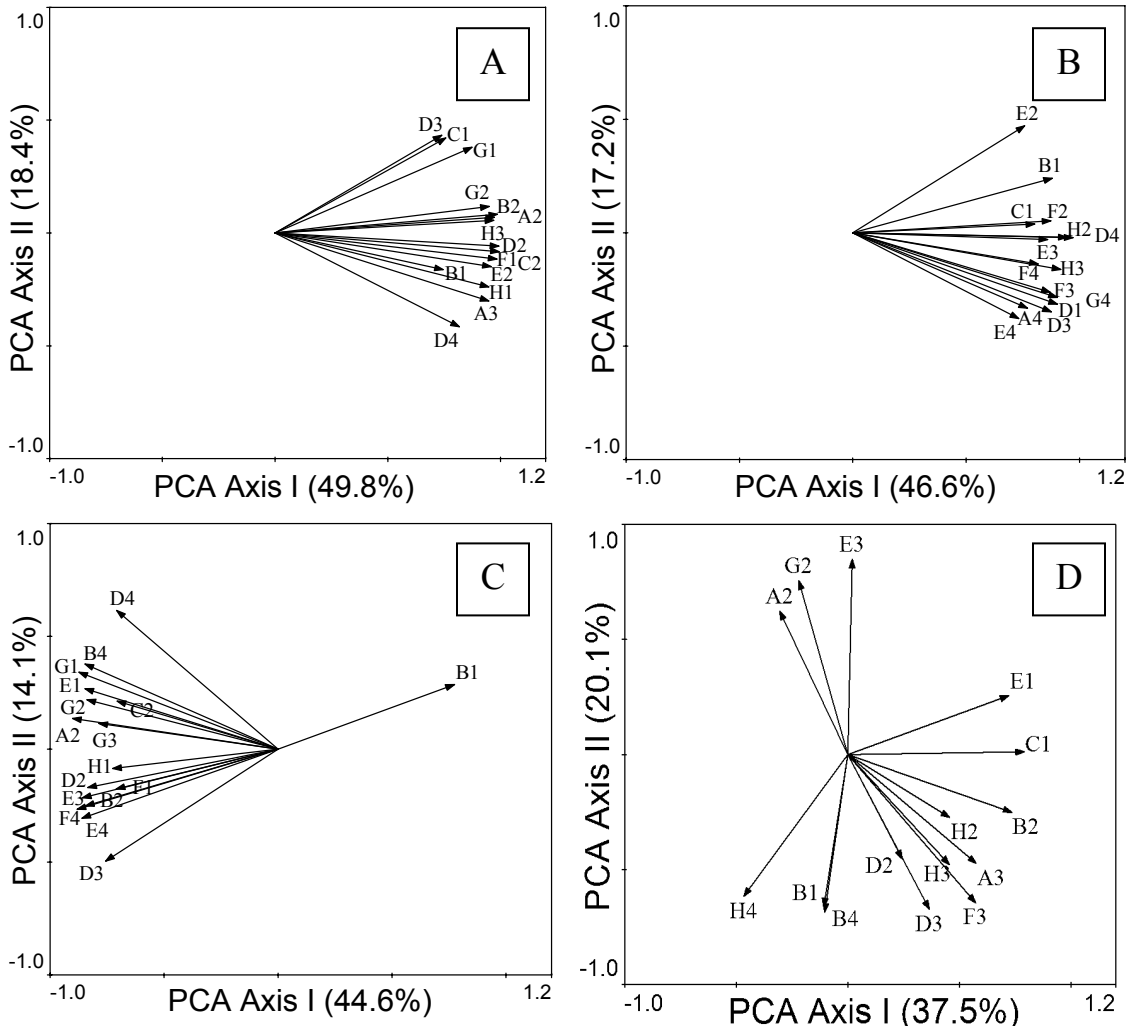

Figure 4.1(a –d). Principal components analyses showing loadings on principal components axes I and II for substrates that exhibited greater than 50% fit to the axes for early stratification (a), late stratification (b), autumnal overturn (c), and winter mixing (d), respectively. Percent variance explained by each axis is given. Arrows connect at origin. Code numbers correspond to substrates given in Table 1.

During late season (prolonged) stratification, measured during two consecutive years, the first two PCA axes accounted for 63.8 percent of the total substrate utilization variance. Significant loadings of 15 substrates were observed, all strongly positively loading onto the first axis and weakly onto the second axis (Figure 4.1b). These loadings included six carboxylic acids, four amino acids, two polymers, two carbohydrates, and one amine. Opposite of early stratification, late season stratification was characterized by the high SWI bacterial utilization of amino acids and carboxylic acids and low utilization of carbohydrates.

At the onset of autumnal overturn, as measured during two consecutive years, significant loadings for 17 substrates were observed, all except pyruvic acid methyl ester explained by high negative loadings on PCA axis I and moderate loadings on axis II (Figure 4.1c). The first two PCA axes accounted for 58.7 percent of the total variance. Substrates exhibiting significant loadings included seven carbohydrates, four amino acids, four carboxylic acids, and two polymers. Because of the negative loadings on the first axis (which explained 44.6 percent of the substrate utilization variance), these substrates contributed little to SWI bacterial carbon substrate utilization during overturn, specifically most carbohydrates and amino acids.

During winter mixing, significant loadings were observed for 15 substrates. The first two PCA axes accounted for 57.6 percent of the total variance (Figure 4.1d). These substrates exhibited diverse loadings on both PCA axes. Three carbohydrates and two polymers exhibited positive loadings on PCA axis I, while two carbohydrates and one carboxylic acid exhibited positive loadings on PCA axis II. Four carboxylic acids, and a single carbohydrate, amino acid, and amine display negative loadings on PCA axis II.

These data indicate that during cold, mixing conditions carbohydrates and polymers contributed the highest utilization responses, yet trends were not as defined as during other seasons.

Carbon Substrate Utilization Variation Attributed to Environmental Variables

While only the first two PCA axes were significant for each seasonal data set, there are a total of as many PCA axes as there are substrates (n = 31), in total accounting for 100 percent of the substrate utilization variance. Of this total variance, linear combinations of measured environmental variables (i.e. bacterial abundance, SWI temperature, dissolved oxygen, redox potential) were fitted onto the PCA axes a posteriori to determine the percentage of total variance attributable to the environmental variables (Table 4.3). For each seasonal mixing event, environmental variables accounted for no less than 50 percent of the total variance, with the largest percentage accounted for during autumnal overturn (62.3 percent). Temperature was the largest attributable variable during early stratification (19.2 percent) and autumnal overturn (40 percent). The largest amount of variation accounted for by dissolved oxygen and redox potential individually was during autumnal overturn (32.4 and 30.8 percent, respectively) and the least during late stratification (7.3 and 11 percent, respectively). The relationship for dissolved oxygen and redox potential individually also held for the combined effect of dissolved oxygen and redox potential. Their combined effect is more ecologically interpretable than either variable individually due to their high correlation and codependence. The sum of the variances of the individual variables was greater than the total variance explained by the variables collectively due to significant correlations among several of the variables (Lepš & Šmilauer, 2003).

Table 4.3: Percent variance in seasonal CSUR data explained by individual environmental variables and all variables collectively. Sum of percentages of individual variables are greater than percentage of all variables collectively due to correlation of the individual variables.

Variable	Winter Mixing	Early Stratification	Fall Overturn	Late Stratification
All Variables	49.8	54.0	62.3	62.1
Bacteria ml^{-1}	24.0	14.4	5.7	20.5
Temperature (C)	6.4	19.2	40.0	16.4
Dissolved Oxygen (mg l^{-1})	15.1	14.3	32.4	7.3
Redox Potential (mV)	16.1	14.6	30.8	11.0
Oxygen * Redox	13.6	19.9	38.6	6.7

During late season stratification and winter mixing, bacterial abundance accounted for the largest proportion of the CSUR variance (20.5 and 24 percent, respectively), while during autumnal overturn it accounted for little variance (5.7 percent). A significant difference was observed among seasonal bacterial abundance (One-way ANOVA, $F_{3,36} = 4.36$, $p < 0.01$). However, the only pairwise difference was greater abundance during winter mixing than during early stratification (Tukey's HSD, $q = 2.69$, $\alpha = 0.05$). While higher bacterial abundance during winter mixing explained a high percentage of variance, this trend did not hold for all seasons. There was no significant difference between bacterial abundance during late stratification and autumnal overturn (Student's $t_{.05, 9} = 0.06$, $p = 0.81$), yet variance explained by bacterial abundance was 20.5 and 5.7 percent, respectively.

Carbon Substrate Utilization and Environmental Variable Correlations

Because a large percentage of variance in SWI bacterial substrate utilization was explained by the measured environmental variables, we explored relationships among those environmental variables and individual substrates (Table 4.4). These correlations

Table 4.4: Pearson product-moment correlation coefficients between environmental variables and substrates among a total of 40 samples. Only significant correlations are shown. Data are inclusive of all seasons. Single, double, triple, and quadruple asterisks indicate p-values less than 0.05, 0.01, 0.001, and 0.0001, respectively.

Temperature (°C)		Bacteria ml^{-1}	
Substrate	Correlation Coefficient (r)	Substrate	Correlation Coefficient (r)
L-Asparagine	-0.46**	L-Asparagine	+0.52***
γ-Hydroxybutyric Acid	-0.33*	N-Acetyl-D-Glucosamine	+0.33*
D-Glucosaminic Acid	-0.46**	Itaconic Acid	+0.43**
Itaconic Acid	-0.58****	Pyruvic Acid Methyl Ester	+0.33*
Phenylethylamine	-0.48***	Phenyethylamine	+0.48**
Putrescine	-0.41**	Putrescine	+0.84****
Dissolved Oxygen (mg l^{-1})		Dissolved Oxygen x Redox (mV)	
Substrate	Correlation Coefficient (r)	Substrate	Correlation Coefficient (r)
L-Asparagine	+0.41**	L-Asparagine	+0.39**
Putrescine	+0.46**	Pyruvic Acid Methyl Ester	+0.30*
		Putrescine	+0.42***

were derived from data of all seasons inclusive. Six significant correlations occurred for temperature, all of which were negative. These included three carboxylic acids, one amino acid, and both amines. For these substrates, all exhibited higher utilization as SWI temperature decreased. Six significant correlations also occurred for bacterial abundance, however these correlations were positive. These included one amino acid, one carbohydrate, two carboxylic acids, and both amines. For dissolved oxygen, two positive significant correlations occurred, one amino acid and one amine. No significant correlations occurred for redox potential alone, but three significant positive correlations occurred for the combined effect of dissolved oxygen and redox potential. These included one amino acid, one carboxylic acid, and one amine. Only L-asparagine (amino

acid) and putrescine (amine) utilization showed significant correlation with every environmental variable, indicating strong utilization of these substrates by all seasonal SWI bacterial communities.

Community-level Physiological Profiles

Figure 4.2 shows sample loadings for each sampling date and site (as classified by season) used in the study (Table 4.2) projected onto PCA axes I and II. Each sample point was derived from linear combinations of all substrate utilization scores for a specific site and date. The graph is a composite overlay of all seasons; therefore percent

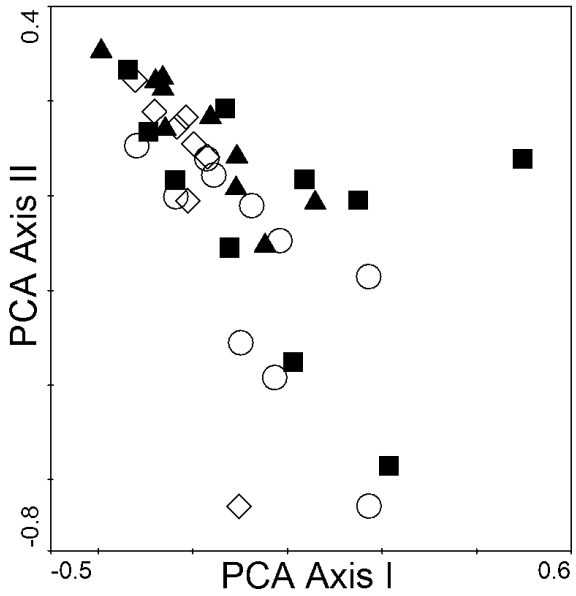

Figure 4.2: Sample loadings (CLPPs) on PCA axes I and II for all seasons. Sample loadings are derived from principal components scores of all substrates. Symbols close together imply sample similarity. Graph is a composite overlay of four separate PCA graphs, therefore percent variance is not listed. Two samples from early stratification were removed from the graph, but not analyses, due to their extreme outlying values. ◊ = early stratification, ■ = late stratification, ▲ = autumnal overturn, ○ = winter mixing.

variance is not listed for the axes. The distance between points is proportional to similarity among individual samples (i.e. further apart is less similar). Highest similarity among samples occurred during early stratification, with all but one sample clustered

together with low eigenvalues along PCA axis I and high eigenvalues along PCA axis II. The two furthest outliers from early stratification were excluded from the graph, but not from analyses, due to their extreme distance away from other samples. High similarity also occurred during autumnal overturn, even though these samples were collected during two different years. Lowest similarity was observed during late stratification, with a wide range of eigenvalues spread along PCA axes I and II. Low similarity was also observed during winter mixing, explained primarily by PCA axis II.

Discussion

Organic carbon utilization by heterotrophic bacteria strongly influences internal nutrient cycling in freshwater ecosystems and impacts eutrophication processes (Sinsabaugh et al. 1997; Qu et al. 2005). Specifically, bacterially-mediated sediment nutrient releases often input nutrients into the water column at greater quantities than allochthonous sources (Heinen and McManus 2004; Song et al. 2004). Organic carbon utilized by heterotrophic bacteria is commonly classified into three groups: particulate organic carbon (POC), high molecular dissolved organic carbon (HM-DOC), and low molecular dissolved organic carbon (LM-DOC) (Wirtz 2003). This investigation specifically explored SWI bacterial utilization of the LM-DOC via the use of Biolog EcoPlates. CSUR data generated from the EcoPlates represents bacterial community functional potential and has been used to assess functional ability and changes in various environmental bacterial communities (Garland 1997; Preston-Mafham et al. 2002). By considering the limitations of EcoPlates (i.e. inoculum density, incubation temperature, oxygen contamination) and modifying our protocol to avoid these limitations (i.e. using

growth rate metrics, incubation temperature standardization, anaerobic techniques), results were robustly interpreted (see Chapter Three).

Seasonal Carbon Substrate Use

Few explanations for preferential seasonal use of DOC substrates by freshwater (specifically, SWI) bacteria have been proposed (Pettine et al. 1999; Brugger et al. 2001) therefore this investigation served an exploratory, not confirmatory purpose. Future investigations (i.e. in situ chemical measurements; stable isotope analysis) into the presence or absence of these substrates at the SWI will aid the results obtained in this study. Most substrates in the Biolog EcoPlates were used at various extents during all seasons; however the first two PCA axes for each seasonal data subset shows only those substrates that contributed to significant variation in increased and/or decreased substrate utilization. Therefore different seasonal patterns of SWI bacterial substrate use were elucidated from the PCA graphs (Figure 4.1a-d).

During the onset of spring stratification, characterized by warming temperatures and depletion of dissolved oxygen and decreasing redox potential, SWI bacterial assemblages preferentially used carbohydrates over carboxylic acids, amino acids, and amines. As summer stratification progressed (i.e. late season stratification), indicated by warm temperatures and depleted dissolved oxygen and reduced redox potential, substrate preference shifted to amino acids, carboxylic acids, and amines with little use of carbohydrates. At the onset of autumnal overturn marked by decreasing temperatures, replenishment of dissolved oxygen, increasing (oxidized) redox potentials and presence of turbulent mixing, SWI bacteria decreased use of amino acids and continued little use

of carbohydrates. Throughout winter mixing, SWI bacteria had high preference for carbohydrates and polymers.

Highest carbohydrate use during early stratification, along with high carbohydrate use during winter mixing may suggest that SWI bacterial communities prefer carbohydrate substrates under oxic conditions and pre-anoxia, under a wide variety of temperatures. Indeed, the preferred catabolic pathway of many aerobic and facultative heterotrophs involves oxidation of a simple or complex carbohydrate and oxygen as a terminal electron acceptor (Madigan et al. 1997; Rosenstock and Simon 2003). In addition, fixed carbon in the form of carbohydrates synthesized via phytoplankton photosynthesis and carbohydrate-rich allochthonous organic matter is flushed in and sinks to sediments during lake mixing (Vreča 2003; Heinen and McManus 2004); hence conditions present during winter mixing may select for SWI bacterial populations that readily utilize carbohydrates. Lowest carbohydrate utilization during late season stratification and onset of autumnal overturn are possibly attributed to SWI carbohydrate depletion from lack of mixing combined with high hypolimnetic bacterial activity that oxidizes sinking carbohydrates before they can reach the sediments (Cole and Pace 1995; Seiter et al. 2005).

Amino acids were preferentially used during late stratification. Amino acids were used to a much lesser extent during autumnal overturn, winter mixing, and onset of stratification. Unlike carbohydrates and carboxylic acids, amino acids are nitrogen-rich (Madigan et al. 1997). Thus SWI bacteria may utilize amino acids as a nitrogen, in addition to a carbon, source by assimilating the ammonium side-chain (Pettine et al. 1999). In turn, the ammonium is incorporated into organic molecules such as other

amino acids and proteins (Hollibaugh and Azam 1983). High use of amino acids suggests that bacteria may be nitrogen deprived during late stratification, even though during this time ammonium is produced from bacterial nitrate reduction. Thus nitrogen deprivation at the SWI during late stratification may result from decreased sinking of nitrogen-rich organic matter due to lack of reservoir mixing (Hodell and Schelske 1998). Because of the opposing catabolic and anabolic processes of amino acid utilization, the breakdown of these extracellular amino acids and incorporation into cellular amino acids may be performed by separate bacterial taxa in a synergistic interaction rather than both processes conducted simultaneously by the same taxon (Atlas & Bartha 1998). In addition, previous studies on Lake Belton have indicated that larger bacteria are present during late summer stratification and their size is a function of anoxia (Christian et al. 2002); hence the larger bacteria may require a greater number of enzymes and proteins for metabolic functions, which can be synthesized via uptake of ammonium from amino acid breakdown in the environment.

Carboxylic acid utilization was greatest during late season stratification, but also had marked utilization during other seasons. Of the EcoPlate substrates, the carboxylic acids are the most diverse substrates in terms of molecular weight and chemical configuration. Little is known about free carboxylic (organic) acids in aquatic and sediment systems. Naturally occurring organic acids such as carboxylic acids are often a product of bacterial fatty acid catabolism, photochemical degradation of HM-DOC, or as an end product of fermentative metabolism which occurs independent of dissolved oxygen and redox potential (Bertilsson and Tranvik 2000; Ding and Sun 2005). Studies at SWIs in marine systems have shown that organic acids degrade more quickly under

aerobic than anaerobic conditions and their concentrations are unrelated to bacterial abundance (Ding and Sun 2005). However, the results show highest utilization of carboxylic acids during anaerobic, reducing conditions of late stratification. Two possible reasons for this phenomenon are suggested: (1) increased photoperiod during late stratification increases photodegradation of HM-DOC and POC in the photic zone, increasing the amount of organic acid-rich DOC that sinks to the SWI, possibly selecting for organic acid-utilizing bacteria (Tranvik et al. 1999); (2) fermentative bacteria may be more prevalent during anoxic conditions, which require the use of an organic compound, such as a carboxylic acid as an electron acceptor in a fermentative pathway (Madigan et al. 1997).

Selective Pressures on SWI Bacterial Assemblages

Several SWI environmental (physicochemical and biological) variables exhibited seasonal change, including: temperature, dissolved oxygen, redox potential and bacterial abundance. Thus Beijerinck's adage was invoked, 'everything is everywhere, the environment selects' when assessing these variables affects on CSUR variance, hence the environmental selective pressures upon SWI bacterial assemblages. While the EcoPlates were themselves selective, the measured environmental variables collectively were associated with at least 50 percent of the total CSUR variance each season, thus were influential environmental selectors for the SWI bacterial assemblages (Table 4.3).

During seasons marked by high variance explained by bacterial abundance, not just abundance, but also, bacterial per-cell activity (i.e. ability to utilize substrate) may attribute to this variance. A previous study on Belton Reservoir SWI bacterial

communities showed that specific per-cell activity varies on a seasonal basis, supporting this evidence (see Chapter Two).

The SWI exhibited wide seasonal temperature variation. The highest amount of CSUR variance explained by temperature was during autumnal overturn, while the lowest was during winter mixing. CSUR variance due to temperature differences was proportional to the temperature range exhibited during that season; with the large CSUR variance during autumnal overturn corresponding to a wide temperature range (18.6°C – 24.6°C) and the small variance during winter mixing corresponding to a small temperature range (10.9°C – 11.8°C). All plates were incubated at 22°C to maintain consistency throughout duration of the experiment. However this incubation temperature may have affected SWI bacterial growth rates, but presumably not pattern (Grover and Chrzanowski 2000). Therefore CSUR variance due to temperature was a function of collected temperature as well as the normalized temperature of incubation (22°C).

At the SWI, dissolved oxygen depletion was coupled with lowered redox potential; therefore their effects upon CSUR variance are best understood when considered in tandem. The combined effect accounted for the largest percentage of CSUR variance during autumnal overturn. It also accounted for a large percentage of CSUR variance during early stratification. These seasons had the greatest ranges of dissolved oxygen and redox potential. Large redox potential changes are defined by bacteria that utilize various electron acceptors, implying tremendous changes in SWI bacterial community composition (Sweerts et al. 1991). Autumnal overturn and early stratification had the combined effect of dissolved oxygen and redox potential working in

reverse for their respective seasons. Therefore it was not surprising that CSUR variance also showed an opposite relationship (Figure 4.1a and 4.1c).

Individual Substrate Utilization and Environmental Variable Correlations

Individual substrates whose CSUR was significantly positively or negatively correlated with the individual physicochemical variables were assessed, all seasons inclusive. Carbohydrates showed strong utilization during two seasons marked by a wide variation in physicochemical variables (early stratification and winter mixing); therefore no individual carbohydrate CSUR was significantly correlated with any environmental variable. Most significant correlations occurred with carboxylic acids (Table 4.4), possibly due to their highest utilization during a single season marked by similar physicochemical conditions (late stratification). Interestingly, two substrates were correlated with all measured environmental variables. These substrates were L-asparagine (amino acid) and putrescine (amine). Positive correlations of these substrates CSURs occurred with bacterial abundance, dissolved oxygen, and the combined effect of dissolved oxygen and redox potential. Negative correlations occurred with temperature. These two substrates are characterized by low C/N ratios. However, a significant trend was not observed with every low C/N ratio substrate. Yet a recent study utilizing EcoPlates demonstrated a preferential utilization of high nitrogen-containing substrates by bacteria in marine aquatic environments (Sala et al. 2006).

Community-level Physiological Profiles

In addition to individual CSURs, Biolog plates have been used to profile total bacterial community similarities (Mills and Garland 2002). Applying this to our data

(Figure 4.2), highest SWI bacterial community similarities occurred during early stratification and autumnal overturn, while lowest similarity occurred during winter mixing and late stratification. High similarity in the autumnal overturn samples was unexpected because sampling events were conducted during two separate years. This evidence suggests possible similar SWI bacterial community succession occurs during overturn. However, late stratification samples were also collected during consecutive sampling years and exhibited high dissimilarity. Yet, some late stratification samples from the first year were more similar to samples collected during the second year than samples collected during the same year. Such seasonal succession in aquatic bacteria has been observed among differing temperate lakes (Grover and Chrzanowski 2000).

Conclusions

This investigation explored differences among SWI bacterial CSURs, CLPPs, and several related environmental variables throughout seasonal mixing, stratification, and anoxia in a eutrophic lake. Using Biolog EcoPlates, distinct seasonal shifts in CSURs were observed. Seasonal differences in SWI bacterial abundance, temperature, dissolved oxygen and redox potential accounted for a large percentage of CSUR variance. Preferential use of amino acids during late summer stratification suggests seasonal SWI nitrogen limitation. Seasonal similiarity of CLPPs from two separate years suggest a possible predictable succession of SWI bacterial communities, a phenomenon still poorly understood among aquatic bacterial communities. While many steadfast conclusions cannot be obtained from Biolog assays, these data contribute to the ever expanding knowledge of carbon cycling in aquatic ecosystems and relays the importance of SWIs and their associated biota to the understanding of lake ecosystem nutrient dynamics and

processes. Future investigation into reservoir SWI bacterial dynamics can integrate these findings into developing specific carbon cycling models and establishing in situ carbon measurements utilizing a variety of other tools.

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CHAPTER FIVE

Organic Matter at the Sediment-Water Interface of a Stratified, Eutrophic Reservoir: Sources, Fates, and Stoichiometry

Introduction

Organic matter (OM) in reservoirs is produced and supplied by various autochthonous and allochthonous sources (Dean and Gorham 1998). Much of this OM is assimilated or oxidized by microorganisms in the water column. The remaining OM sinks to the sediment-water interface (SWI) where further microbial assimilation and oxidation of OM occurs, often at significantly higher rates than the overlying water column (Heinen and McManus 2004). These SWI microbial (i.e. bacterial) processes are often accompanied by additional nutrient transformations that release nitrogen, phosphorus, and sulfur-containing compounds into the water column (Nealson 1997). These nutrients (e.g. ammonia, hydrogen sulfide) contribute to reservoir eutrophication and aesthetic problems (Nealson and Stahl 1997). Therefore SWI OM sources and fates are important in the overall carbon budgets and nutrient dynamics of reservoirs. However, SWI contributions to reservoir OM cycling are less understood than open-water OM dynamics (Syväranta et al. 2006).

Stratified reservoir mixing events may profoundly influence OM sinking rates to the SWI, affecting the OM that deposits in sediments (Beutel 2003). In addition, SWI bacterial metabolism is dependent upon reservoir mixing and stratification (see Chapter Two). Hence seasonal oxic and anoxic cycles may affect rates of SWI bacterial OM degradation, as well as the types and fraction (i.e. carbon, nitrogen) of OM degraded.

During winter mixing, the total organic carbon (TOC) fraction of SWI OM is transformed and/or oxidized by aerobic and/or facultative anaerobic heterotrophic bacteria (Rosenstock et al. 2005). These bacteria utilize oxygen, provided to the SWI via mixing, as a terminal electron acceptor in respiratory pathways (Ding and Sun 2005). During warm and tranquil weather conditions, thermal stratification develops between shallow and deeper waters thus bacteria consume the remaining dissolved oxygen in the hypolimnion, resulting in an anoxic hypolimnion that blankets the SWI (Bloesch 2004). As a result, active SWI bacterial assemblages utilize electron acceptors other than oxygen (e.g. facultative anaerobes, strict anaerobes), or undergo fermentative metabolism. This may also alter the type and quantity of OM at the SWI.

Overall, sources and fates of carbon and nitrogen are important indicators of reservoir productivity and trophic dynamics. However, measures of C and N inputs and quantities at SWIs of reservoirs remains poorly understood. In this investigation total bulk OM, total inorganic and organic carbon (C_{in} and C_{org}) and total nitrogen (N_{tot}) were measured at the SWI of a monomictic, eutrophic reservoir on a seasonal and spatial basis. Relationships among these nutrient concentrations were examined in addition to C/N ratios and C_{org} and N_{tot} stable isotopic signatures ($\delta^{13}C_{org}$ and $\delta^{15}N_{tot}$). These data provided seasonal and spatial SWI stoichiometric profiles and isotope signatures of SWI OM that were defined by OM inputs and SWI bacterial metabolism. Spatially differing $\delta^{13}C$ values of organic carbon ($\delta^{13}C_{org}$) were used to suggest possible sources of SWI OM inputs, while temporally differing $\delta^{15}N$ values of nitrogen were used to suggest seasonal changes in SWI OM sources as well as bacterial nitrate utilizations (Lehmann et al. 2002).

Materials and Methods

Study Site and Physicochemical Variables

Belton Reservoir, a subtropical monomictic and eutrophic reservoir located in central Texas, was the study location (Figure 1.1). The lacustrine region thermally stratifies during late spring and develops an anoxic hypolimnion that decreases in redox potential throughout summer. Thermal mixing begins in mid-autumn and persists throughout winter (Chapters 2, 3, 4). Reservoir depth varies approximately ± 2 m depending on the amount of rainfall, inflow, discharge, and water consumption. The reservoir basin is defined by steep limestone cliffs and little emergent vegetation. Sediments are clay-rich.

SWI samples were collected from five sites along a longitudinal linear transect representing the depth gradient of the lacustrine region (Figure 1.2, Table 4.3). Hence, each site was not equally spaced apart; however each site was separated by a horizontal distance of at least 200 m. The depth of the hypolimnion blanketing these sites during summer stratification varied on a spatial and temporal basis. Therefore, this sampling design provided variability in SWI physicochemical variables (e.g. dissolved oxygen, pH, redox potential) under a variety of stratification conditions, ranging from weakly stratified (Site A) to stratified and anoxic (Site E) during summer. Likewise, all sites underwent overturn in sequential order from Site A to Site E. Samples were collected from October 2003 through February 2005 during periods of stratification, autumnal overturn, and winter mixing.

Table 5.1: Physicochemical Variables of Belton Reservoir for all sites and dates in this study.

Classification	Date	Site	Temperature (°C)	Dissolved Oxygen (mg l-1)	pH	Redox Potential (mV)
Stratification	16-Oct-03	A	23.7	3.1	6.5	225
		B	17.1	0.6	6.9	276
		C	17.0	0.0	6.7	93
		D	15.5	0.0	6.4	38
		E	14.9	0.0	6.6	4
Fall Overturn	13-Nov-03	A	19.7	4.6	7.1	298
		B	19.7	4.3	7.2	305
		C	19.7	4.0	7.0	258
		D	19.8	4.2	6.9	259
		E	19.9	7.1	7.1	388
Winter Mixing	4-Dec-03	A	16.5	8.5	7.4	396
		B	16.4	5.9	7.3	387
		C	16.3	5.6	7.2	363
		D	16.2	4.8	7.2	345
		E	16.0	4.5	7.1	314
Winter Mixing	12-Mar-04	A	11.8	7.6	7.6	384
		B	11.4	7.1	7.4	380
		C	11.2	7.7	7.4	394
		D	10.9	9.9	7.6	391
		E	10.9	8.6	7.7	383
Stratification	6-May-04	A	19.4	8.0	7.2	217
		B	18.8	6.0	7.2	231
		C	17.9	6.9	7.3	409
		D	16.4	2.5	7.0	293
		E	15.1	1.5	6.9	255
Stratification	1-Jul-04	A	25.8	4.4	6.8	381
		B	22.9	1.1	6.1	258
		C	21.0	0.4	6.0	186
		D	19.6	0.2	6.1	155
		E	19.4	0.0	6.1	120
Stratification	3-Aug-04	A	26.3	0.6	6.7	269
		B	25.7	0.3	6.5	250
		C	23.8	0.2	6.3	230
		D	22.6	0.1	6.4	218
		E	22.1	0.1	6.5	198
Stratification	9-Sep-04	A	27.0	2.9	7.0	230
		B	26.8	1.3	6.9	157
		C	23.7	0.8	7.1	108
		D	20.6	0.0	6.6	37
		E	20.1	0.0	6.3	15
Fall Overturn	14-Oct-04	A	24.6	7.4	7.1	431
		B	24.6	7.2	6.9	405
		C	24.6	5.3	6.9	411
		D	24.6	6.8	6.9	415
		E	24.5	6.6	6.5	341
Winter Mixing	3-Feb-05	A	10.9	9.5	7.2	398
		B	10.9	9.4	7.1	405
		C	10.9	9.7	7.2	393
		D	11.2	9.1	7.1	454
		E	11.1	9.4	7.4	459

SWI temperature (°C), dissolved oxygen (mg l^{-1}), pH, and redox potential (mV), were measured at the SWI using a YSI QS 600 Data Sonde (Table 5.1). Measurements were taken by gently lowering the sonde to the sediment surface and allowing measurements to stabilize. Though SWI physicochemical gradients differed on milli and micrometer scale, the sonde measurements were representative of the overall physicochemical conditions that blanketed the sediment surface.

Microcosm Incubations for Determination of SWI Layer

Bulk sediment and water were retrieved from the five sample sites during winter mixing via a Peterson grab sampler and a 3.2 l PVC Alpha water sampler, respectively. Sediments from all sites were combined in a single deionized water-rinsed mixing tub, returned to the laboratory, and thoroughly homogenized. Water was stored in deionized water-rinsed 4 l plastic bottles, returned to the laboratory, combined and mixed in a 5 gallon carboy. One hundred 250 ml glass jars were filled with 150 ml of homogenized sediment and topped off with mixed sample water. The jars were sealed with lids and holes were drilled in 50 of the jar lids to allow aerobic incubation. The remaining 50 jars were wrapped with parafilm to allow anaerobic incubation. All jars were incubated for 1 month in the dark at 20ºC (approximately annual median hypolimnetic temperature) to allow a stable SWI bacterial community to establish.

After incubation, the 50 aerobic samples were randomly divided into groups of 10. Ten samples served as controls (mean ± s.d. dissolved SWI oxygen concentration 8.6±1.2 mg l^{-1}). The other groups of samples were purged with nitrogen gas to establish a SWI dissolved oxygen concentration of 4, 2, 1, or 0 mg l^{-1}, respectively as measured by an Orion model 97-08 dissolved oxygen electrode. Samples were resealed and re-

incubated for 24 hours. For the 50 anaerobic samples (all samples dissolved oxygen ≤ 0.1 mg l^{-1}), ten samples served as controls while other groups of 10 samples were purged with oxygen to obtain a dissolved oxygen concentration of 1, 2, 4, and 8 mg l^{-1}, respectively. These samples were resealed and re-incubated for 24 hours.

After the 24 hour incubation, a Unisense© RD-10 redox electrode was used to measure the sediment depth (mm) in each sample at which the lowest redox potential (mV) occurred. The average depth of the lowest redox potential (< 0 mV) in the aerobic samples (i.e. samples purged with dissolved oxygen) was approximately 11 mm, while this depth for anaerobic samples (i.e. samples purged with nitrogen) was approximately 9 mm. Henceforth these depths were used to define the SWI, with 10 mm being the depth that all future collected samples were subsampled for all analyses.

Sediment-Water Interface Sampling and Storage

SWI samples were retrieved via a 15 cm x 15 cm Ekman dredge, modified to minimize SWI disruption. Three samples were collected per site on each date listed n Table 5.1. Immediately upon retrieval, excess water was gently siphoned away from the sediment surface, leaving approximately 1 cm of water overlay. Liquid nitrogen was slowly poured into a corner of the dredge to freeze the sediment into a solid block being careful to maintain SWI integrity and composition. The frozen sediment block was gently removed from the dredge, wrapped in aluminum foil, bagged in plastic, and placed into a cooler of dry ice. The frozen blocks were returned to the laboratory and held at -80°C until further processing.

Sediment Processing

The frozen sediment blocks were subsampled with a scalpel to a depth of approximately 10 mm. This 10 mm sediment layer was thawed and homogenized in a 50 ml plastic conical centrifuge tube. The samples were held at -30°C until further processing.

Total Organic Matter Quantification

Approximately 10-20 g of the thawed and homogenized sediment was added to a pre-weighed crucible, dried at 100°C for 24 h, and pulverized to a fine powder. Percent water was calculated as the difference between wet mass (gram wet weight (gww)) and dry mass (gram dry weight (gdw)) divided by gww. Approximately 2 g of the powdered sediment was preserved for carbon and nitrogen analysis and stable isotope analysis. The remaining powdered sediment was ignited in a crucible at 550°C for 1 hour. Loss on ignition (LOI) was recorded as the difference between the mass of the dried sample and the ignited (ashed) sample. Percent TOM was calculated as the LOI divided by gram dry weight (Dean 1974).

Carbon and Nitrogen Content

Approximately 30 mg of dried, but not ashed, sediment samples were measured into tin capsules and analyzed for total carbon (C_{tot}) and total nitrogen (N_{tot}) on a ThermoQuest Flash EA™ 1112 Elemental Analyzer. 30 mg aliquots of ashed sediments were analyzed via the same process for total inorganic carbon (C_{in}). Total organic carbon (C_{org}) was calculated as the difference between C_{tot} and C_{in}. No inorganic nitrogen was present in the samples; therefore N_{tot} was equal to total organic nitrogen. C/N ratios were

calculated as the ratio of C_{org} to N_{tot}. The ratio was then converted from mass ratio to atomic ratio by multiplying the mass ratio by 1.167, which is equal to the ratio of atomic weights of carbon to nitrogen (Meyers and Teranes 2001).

Stable Isotope Analysis

Triplicate dried, but not ashed, samples from each site were pooled in equal amounts, and approximately 20 mg these pooled samples were placed into silver capsules, moistened with 50 µl – 100 µl of deionized water and fumigated with 12 N HCl for 24 hours in a dessicator to remove all C_{in} (Harris et al. 2001). Fumigated samples were analyzed for stable isotopes of C_{org} and N_{tot} ($\delta^{13}C$ and $\delta^{15}N$) using a Thermo Finnigan Delta^{Plus} Mass Spectrometer connected to a CE 2500 Elemental Analyzer via a Finnigan Conflo II. Acetanilide a, bovine liver, low organic soil, and NIST Peach 1547 were used as internal calibration standards. $\delta^{13}C$ and $\delta^{15}N$ were expressed per mil (‰) relative to Vienna Peedee Belemnite for $\delta^{13}C$, and atmospheric N_2 for $\delta^{15}N$, respectively.

Statistical Analyses

All statistical and graphical analyses were performed with Microsoft Office Excel XP and/or JMP version 5.0 statistical packages. A significance level of $\alpha = 0.05$ was used for all pairwise and non-pairwise comparisons (e.g. Two-way ANOVA, Student's t, etc.). For most statistical applications, spatial and temporal analyses were conducted to observe differences among sites (all dates inclusive) or among dates (all sites inclusive), respectively.

Results

Physicochemical Conditions of the Sediment-Water Interface

Table 5.1 summarizes SWI physicochemical variables throughout the study. The sites range from shallowest (Site A, mean depth 13.7 m) to deepest (Site E, mean depth 25.8 m), thus differing in their physicochemical characteristics on a spatial and temporal basis. The study commenced on 16 Oct 2003, during late season stratification. Samples were subsequently collected during autumnal overturn, winter mixing, summer stratification, and ending during winter mixing on 3 Feb 2005.

Table 5.2. F-ratio table showing results of two-way ANOVA and corresponding significance of carbon and nitrogen variables for site and date. Degrees of freedom by site: MSBG = 4, MSWG = 45; by date: MSBG = 9, MSWG = 40.

Dependent Variable	Independent Variable	F-ratio	p-value
% C_{tot}	Site	7.34	< 0.0001
	Date	0.84	0.59
% C_{org}	Site	1.38	0.26
	Date	0.71	0.69
% C_{in}	Site	17.60	< 0.0001
	Date	0.28	0.98
% N_{tot}	Site	5.04	< 0.01
	Date	1.27	0.28
% OM	Site	0.78	0.54
	Date	0.80	0.62
C/N ratio	Site	1.32	0.28
	Date	0.89	0.54
$\delta^{13}C$	Site	11.90	< 0.0001
	Date	0.49	0.87
$\delta^{15}N$	Site	2.39	< 0.05
	Date	0.84	0.59

Total Organic Matter

Percent total organic matter (% OM) did not differ significantly by site or date (Table 5.2). However, a wider range of values were observed at Site A (6.6%-14.5%) and B (7.6%-13.1%), than at Sites C (6.4%-10.9%), D (8.6%-11.2%) and E (7%-11.1%). Highest % OM concentrations were at Site B on 13 Nov 2003 and Site A on 3 Aug 2004 (Figure 5.1). A slight negative correlation exists between % OM and dissolved oxygen concentration (Table 5.3).

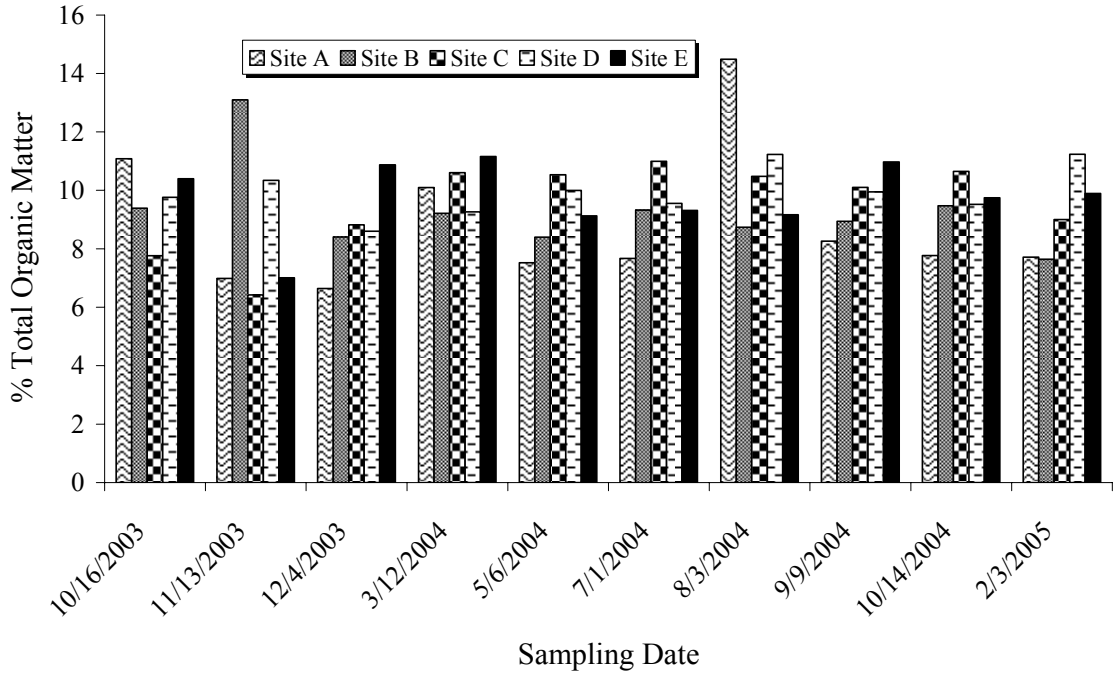

Figure 5.1: Percent total organic matter among sites and dates.

Carbon Dynamics

% C_{tot} significantly differed among sites, but not dates. These differences were explained by significant differences in % C_{in}, with values significantly lower at Sites A and B. There were no significant differences in % C_{org} (Table 5.2). C_{in} accounted for a larger percentage of C_{tot} than did C_{org}. Site A exhibited little range in % C_{in} (7.1%-8%),

as did Site B (6.3%-7.3%) and Site C (6.4%-7.5%). Site D (4.7%-6.9%) and Site E (4.6%-6.4%) exhibited wider ranges of % C_{in} (Figure 5.2). % C_{tot} was weakly, but positively correlated with SWI temperature, while % C_{org} and % C_{in} were not significantly correlated with any measured physicochemical variables (Table 5.3). However, % C_{org} and % C_{in} were negatively correlated with each other (Table 5.4).

Table 5.3: Pearson product-moment correlations and corresponding p-values for carbon and nitrogen correlations with various physicochemical variables. Significant values occur at $p < 0.05$.

Variable	Temperature (°C)		Dissolved O_2 ($mg\ l^{-1}$)		p H		Redox Potential (mV)	
	r	p-value	r	p-value	r	p-value	r	p-value
% C_{tot}	0.26	0.05	0.10	0.35	0.14	0.38	0.03	0.81
% C_{org}	0.07	0.62	0.17	0.24	0.20	0.16	0.02	0.86
% C_{in}	0.22	0.11	0.00	0.98	0.03	0.83	0.02	0.91
% N_{tot}	0.08	0.55	-0.37	0.01	-0.36	0.01	0.20	0.15
% OM	0.06	0.64	-0.25	0.05	0.20	0.17	0.14	0.35
C/N ratio	0.01	0.97	0.04	0.76	0.01	0.91	0.20	0.14
$\delta^{13}C$	0.10	0.44	0.10	0.41	0.06	0.65	0.10	0.48
$\delta^{15}N$	0.02	0.89	-0.24	0.09	0.20	0.19	0.20	0.15

Table 5.4: Significant Pearson product-moment correlations and corresponding p-values between carbon and nitrogen variables.

Correlation	r	p-value
% C_{tot} & % N_{tot}	0.41	< 0.01
% C_{tot} & C/N ratio	0.26	< 0.05
% C_{tot} & $\delta^{13}C$	0.30	< 0.05
% C_{org} & % OM	0.32	< 0.05
% C_{org} & % C_{in}	-0.32	< 0.05
% C_{org} & % N_{tot}	0.36	< 0.01
% C_{in} & C/N ratio	-0.42	< 0.01
% OM & % N_{tot}	0.28	< 0.05
$\delta^{13}C$ & % N_{tot}	-0.39	< 0.01

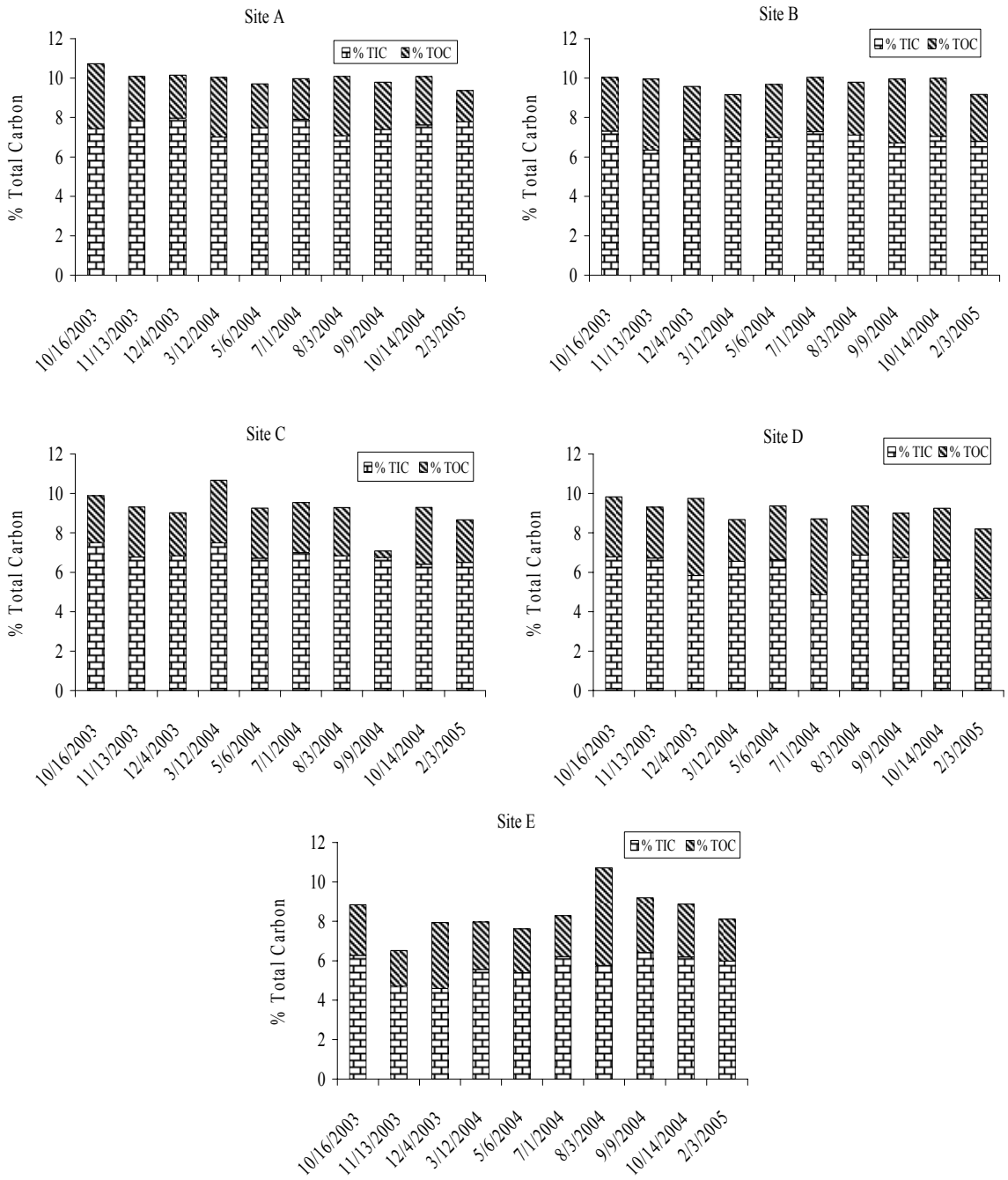

Figure 5.2: Percent total carbon (% C_{tot}) for each sampling site and date.

Nitrogen Dynamics

Because no inorganic nitrogen was present in the SWI samples, % N_{tot} is equivalent to % N_{org}. % N_{tot} significantly differed by date, but not among site (Table

5.2). When all SWI sites were categorized as 'stratified' or 'mixing', a significant difference existed between % N_{tot} for these groupings (Student's $t = -2.07$, $p < 0.05$). Large fluctuations were observed among many % N_{tot} concentrations, some dates exhibiting similar or identical values at several sites (e.g. 4 Dec 2003; 8 Aug 2004), others exhibiting a wide range of values (e.g. 13 Nov 2003; 1 Jul 2004) (Figure 5.3). % N_{tot} was negatively correlated to both dissolved oxygen and pH (Table 5.3). Significant positive correlations occurred between % N_{tot} and % C_{tot}; % N_{tot} and % C_{org}; and % N_{tot} and % OM (Table 5.4).

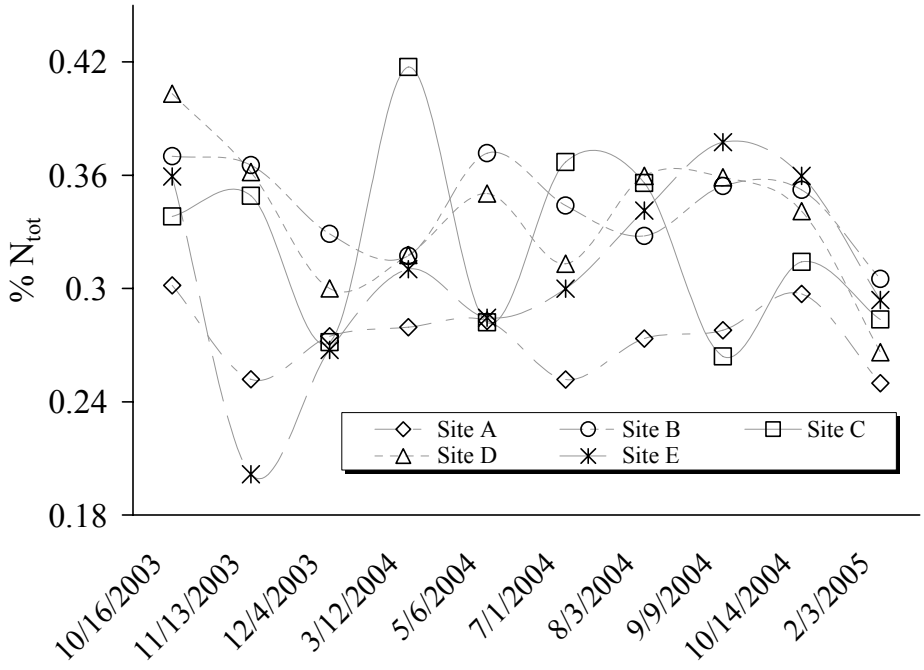

Figure 5.3: Percent total nitrogen (%N_{tot}) at each site throughout the course of the study.

Carbon to Nitrogen Ratios

SWI C/N ratios (% C_{org} atomic / % N_{tot} atomic) did not significantly differ by site or date (Table 5.2), nor were the ratios significantly correlated to any measured physicochemical variable (Table 5.3). On several dates, C/N ratios were similar among

several sites (e.g. 16 Oct 2003; 5 May 2004). One extremely low C/N ratio was observed at Site C (9 Sep 2004) (Figure 5.4). A significant positive correlation exists between % C_{tot} and C/N ratio, while a significant negative correlation exists between % C_{in} and C/N ratio (Table 5.4). When % C_{org} was plotted against % N_{tot}, and a linear regression forced through the points, the model indicates % N_{tot} is approximately 11.5% of % C_{org}. This gives an omnibus C/N ratio of approximately 8.7 (Figure 5.5).

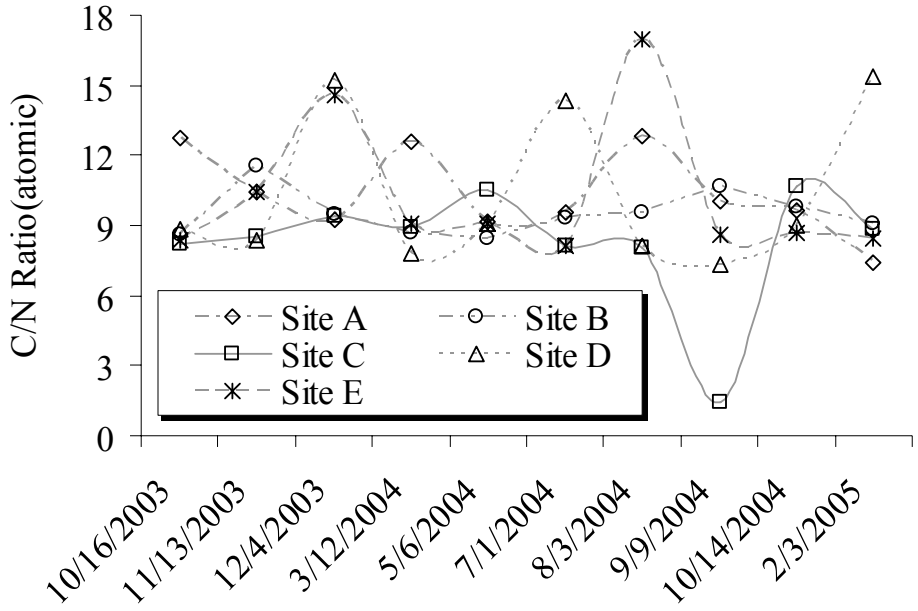

Figure 5.4: Atomic C/N ratios at each site throughout the course of the study.

Stable Isotope Analyses

Stable isotope values of SWI C_{org} ($\delta^{13}C$) and N_{tot} ($\delta^{15}N$) significantly differed by site, but not date (Table 5.2). No correlations existed among $\delta^{13}C$ or $\delta^{15}N$ and the measured physicochemical variables (Table 5.3). Largest ranges of $\delta^{13}C$ occurred at Site A (-11.4‰ to -26.2‰), while the other sites showed very little range (-23.5‰ to -27.6‰). $\delta^{13}C$ was significantly positively correlated with % C_{tot} and significantly negatively correlated with % N_{tot} (Table 5.4). Largest ranges of $\delta^{15}N$ occurs at Site A

(6.5‰ to 10.1‰), while the other sites do not have as wide of range, including Site B (6.9‰ to 8.9‰), Site C (6.9‰ to 9.4‰), Site D (6.9‰ to 9.8‰), and Site E (7.3‰ to 10‰) (Figure 5.6).

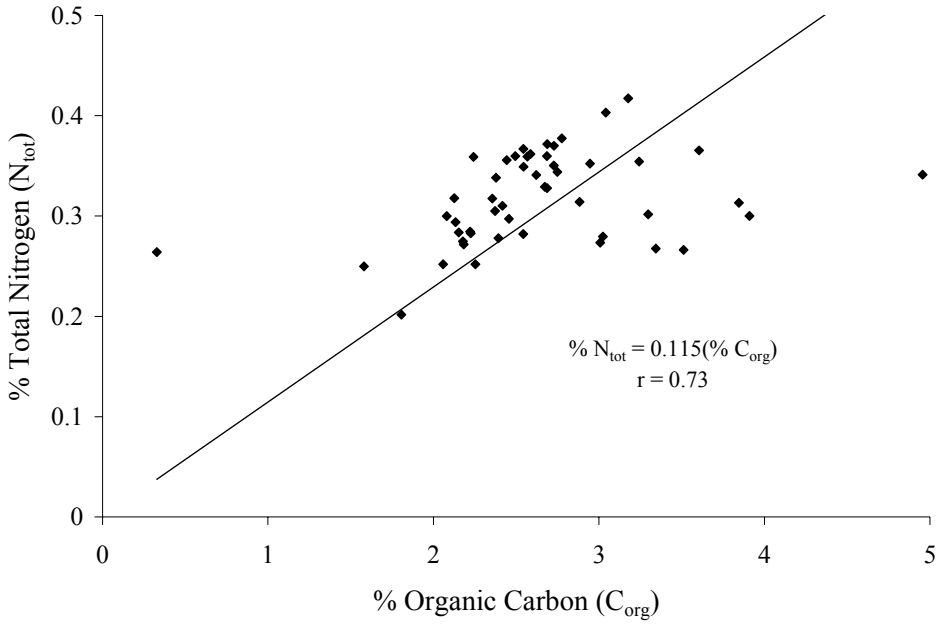

Figure 5.5: Linear regression equation for % C_{org} and % N_{tot} correlation. Line is forced through origin.

Discussion

Aquatic ecosystems that receive large OM inputs undergo various chemical changes at the SWI that indirectly affect eutrophication (Tankéré et al. 2002). Belton Reservoir is such an ecosystem. Due to seasonal stratification and anoxia, organic C and N components of SWI OM inputs (C_{org} and N_{tot}) are utilized by anaerobic bacteria which also produce reduced compounds via anaerobic respiration, such as ammonia and hydrogen sulfide. A previous study showed that these SWI anaerobic bacterial communities are marked by high activity and biomass production during summer

stratification (see Chapter 2). The study contradicted conventional wisdom that advocates greater bacterial activities and biomass production (requiring C_{org} and N_{tot})

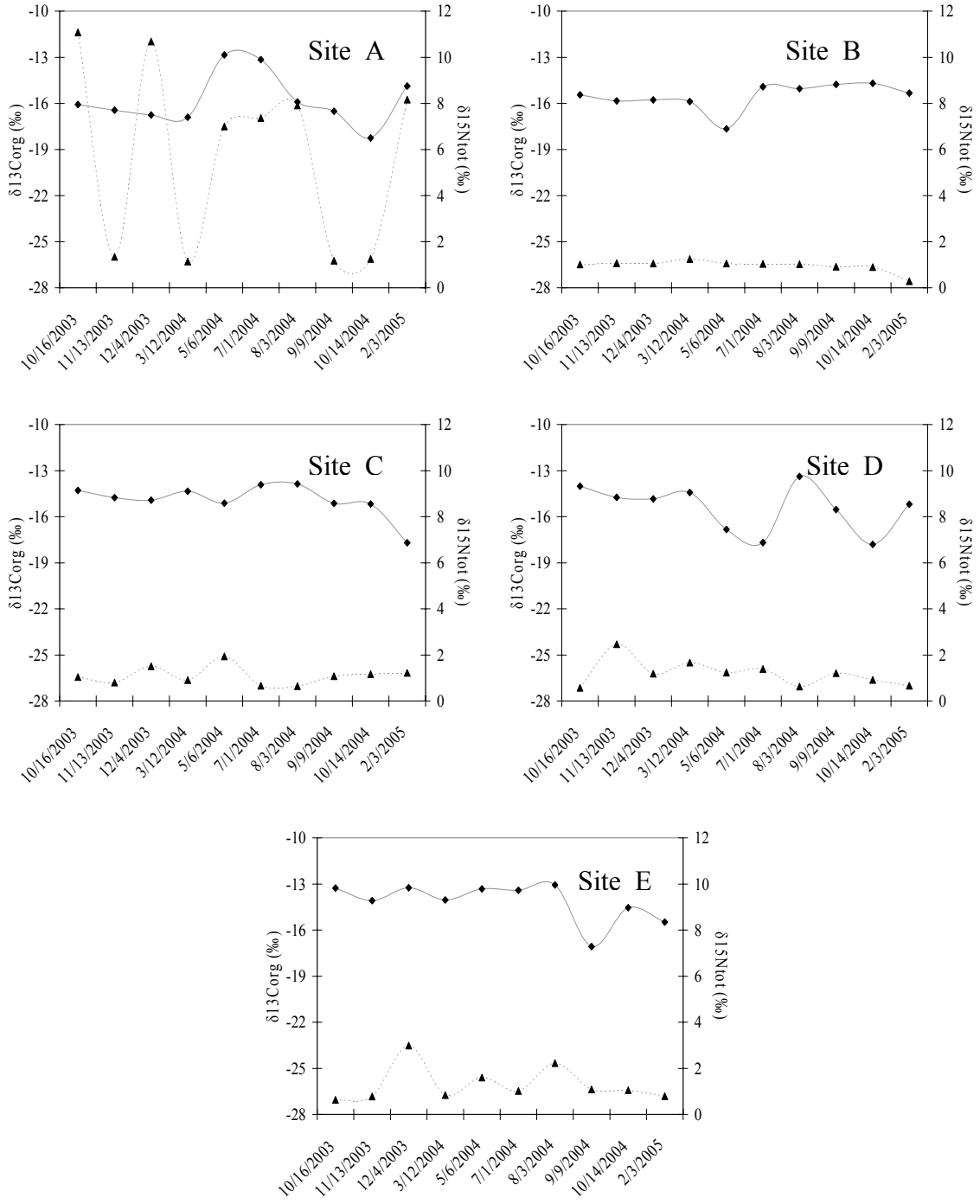

Figure 5.6: Stable isotope values for $\delta^{13}C$ (▲) and $\delta^{15}N$ (♦) at each site over the course of the study.

when oxygen, with its large free energy potential, is the preferred electron acceptor (Wirtz 2003). The goal of this study was to propose various ways that these SWI bacterial communities quantitatively and qualitatively affect SWI OM due to seasonal stratification and mixing events on a spatial and temporal basis.

Bulk Organic Matter Sources and Sinks

The significant negative correlation between OM concentration and dissolved oxygen suggests higher OM degradation under higher dissolved oxygen concentration. This seemingly contradicts the study that concluded higher SWI bacterial activity, thus degradation of OM, occurs under anoxic conditions (see Chapter 2). However, a substantial portion of SWI bacterial activity under anoxia also includes chemolithotrophic metabolism and fermentative metabolism of small dissolved organic compounds that may go undetected in bulk OM measurements.

Another possibility accounting for the inverse relationship between dissolved oxygen and OM concentration is increased SWI OM deposition during summer stratification due to binding of OM to dissolved calcite ($CaCO_3$) under anoxic conditions, which increases the OM sinking to the SWI (Hodell and Schelske 1998). $CaCO_3$ commonly precipitates from epilimnetic waters in the summer, and exists in high concentrations in Belton Reservoir (Dr. Steve Dworkin, Baylor University, personal communication).

However, SWI OM concentrations were not significantly different among seasons. This in and of itself does not imply that SWI OM inputs were quantitatively similar among seasons, but that net burial of OM to the sediments is relatively equal, regardless of oxic or anoxic conditions. For example, rates of OM degradation may be

dependent not only on rates of aerobic or anaerobic bacterial mineralization, but also the quality of organic matter present. Inputs of simple carbohydrates, lipids, and proteins are readily utilized by both aerobic and anaerobic bacteria (Lehmann et al. 2002). Thus it is possible that bacteria completely utilize all labile OM under oxic and anoxic conditions, but all recalcitrant OM, which comprises the majority of OM, exhibits little difference in quantity among seasons and is thus permanently deposited (Jonsson et al. 2001).

Carbon Dynamics

Differences in SWI C_{tot} occurred on a spatial, but not seasonal scale. C_{org} did not significantly differ among site, which was not surprising considering that OM, of which C_{org} is a component, did not differ spatially. Instead, the spatial C_{tot} differences were attributed to high concentrations of inorganic carbon (C_{in}) at Sites A, B, and C. Limestone cliffs (composed of $CaCO_3$) surrounding Belton Reservoir possibly serves as an allochthonous source of C_{in} due to weathering and runoff during rain events. Sites D and E had low C_{in}, and were located further away from the cliffs than Sites A, B, and C, possibly accounting for the C_{in} differences.

However, autochthonous processes may also account for higher C_{in} at Sites A, B, and C. Algal photosynthesis in the epilimnion during the summer consumes CO_2, which increases pH and produces $CaCO_3$ (Horne and Goldman 1994). If high pH is maintained at the SWI (such as Site A due to only weak stratification), $CaCO_3$ directly precipitates to the sediment surface. Under prolonged anoxic conditions (Sites D and E), pH and redox potential are low, thus $CaCO_3$ dissolves and is only deposited if bound to organic matter from the epilimnion (Hodell and Schelske 1998). At intermediate pH and redox potentials, such as Sites B and C during stratification, redox potential is within a range

that results in ferric iron reduction. Iron reduction, coupled with pH that is low enough to dissolve $CaCO_3$, produces siderite ($FeCO_3$) that precipitates to the SWI. However this process does not completely remove all dissolved $CaCO_3$, thus the amount of C_{in} at the SWI will not be as large as those that have direct deposition of $CaCO_3$ (Dean 1999). Sites A through E follow this gradient, as observed in Figure 5.2.

Nitrogen Dynamics

Significant differences in % N_{tot} among dates suggest that SWI OM sources and/or use of nitrogen-containing OM by SWI bacteria vary by season. Referring to Figure 5.3, lowest % N_{tot} occurred during winter mixing (4 Dec 2003; 3 Feb 2005; and with the exception of Site 3, 12 Mar 2004). This is supported by the negative correlation between % N_{tot} with dissolved oxygen and pH. This contradicts studies that suggest wind and rain events during winter mixing often increases inputs of allochthonous OM in reservoirs, which contain nitrogen-rich compounds (Thornton and McManus 1994). Further, differences in % N_{tot} among sites are due to lower values at Sites A and B, possibly due to higher cellulose-rich allochthonous inputs (i.e. lower C/N ratio) at these sites due to closer proximity to the shoreline.

During winter mixing in Belton Reservoir, SWI bacteria preferred utilization of carbohydrates which lack nitrogen over amino acids which contain nitrogen. Amino acids were preferred by SWI bacteria during stratification (see Chapter 4). When interpreted in context of this study, non-nitrogen containing compounds are preferred by SWI bacteria when SWI N_{tot} is low. Thus it is possible that during winter mixing SWI bacteria are selected for taxa that obtain a sufficient amount of nitrogen under low N_{tot}. Higher N_{tot} present during stratification may select for bacteria that require higher

concentrations of nitrogen, thus those that readily use amino acids. An alternative explanation is that high amino acid utilization during stratification is due to bacterial nitrogen limitation, suggesting that the nitrogen present during stratification, while abundant, may be unusable by the anaerobic bacteria (Hollibaugh and Azam 1983).

C/N Ratios

C/N ratios are used as a proxy to determine OM that originates from autochthonous as opposed to allochthonous sources. These ratios are often used in conjunction with stable isotope measurements of C and N. C_{org}/N_{tot} ratios between 4 and 10 generally indicate OM present from sinking phytoplankton, while ratios greater than 20 indicate OM from cellulose-rich allochthonous sources (i.e. C_3 land plants) (Meyers and Teranes 2001).

With the exception of one anomalous sample, all C/N ratios range from 7.3 to 16.9, median 9.1. Thus these SWI OM samples are close to the predicted range of OM from sinking autochthonous production. This median ratio falls between the sediment C/N ratios for Lake Ontario (median = 8) and Lake Baikal (median = 11) (Hodell and Schelske 1998; Qiu et al. 1993).

The positive correlations between % N_{tot} with % C_{tot} and % C_{org} explain the lack of significant difference of C/N ratios (% C_{org} / % N_{tot}) among site or date. Had differences in C/N ratios existed, the positive correlation between % Ctot and % Ntot would not be observed. A linear model of best-fit, forced through the origin predicts an omnibus C/N ratio of 8.7, only 4.3% less than the actual median value.

Conceivably, seasonal differences in bacterial activity coupled with selective preferences in C-rich or N-rich compounds, could affect the measured C/N ratio. Thus

the ratio would not only be a function of allochthonous or autochthonous inputs, but also of bacterial utilization of OM. However, bacterial utilization of C and N is usually not significant enough to affect interpretation of C/N ratios being from algae or land plants (Meyers and Teranes 2001).

Stable Isotope Dynamics

Ratios of ^{13}C to ^{12}C ($\delta^{13}C_{org}$) and ^{15}N to ^{14}N ($\delta^{15}N_{tot}$) in lake sediment OM are used to assess OM sources, C and N utilization by various organisms, and reconstruction of past productivity. This is based on evidence that algae preferentially uptake ^{12}C over ^{13}C, thus $\delta^{13}C$ values of sediment from autochthonous production are lower (i.e. isotopically lighter) than $\delta^{13}C$ values of sediment from terrestrial OM sources, which has a preference for ^{13}C uptake. Algae also preferentially uptake ^{15}N over ^{14}N because of high ^{15}N values in nitrate, the preferred algal nitrogen source. Terrestrial plants are higher in ^{14}N because their preferential nitrogen uptake is atmospheric which does not contain ^{15}N. Thus δ15N values are higher from sediment whose sources are autochthonous (Meyers and Teranes 2001; Hoefs 2004).

Spatial differences in stable isotope values for $\delta^{13}C$ and $\delta^{15}N$ were attributed to significantly different isotope signatures at Site A as opposed to the other sites. The median $\delta^{13}C$ signature at Site A was -17.2 ‰. This $\delta^{13}C$ signature at Site A corresponds to the signature commonly seen in C_4 vascular plants, unlike the $\delta^{13}C$ signatures at the other sites that correspond to algae. Unlike C/N ratios, this stable isotope signature clearly shows the difference in Site A OM sources. Thus, Site A SWI OM is influenced by allochthonous inputs unlike the other sites that are clearly dominated by autochthonous production.

While the $\delta^{15}N$ signature was significantly lighter at Site A than other sites, its median value was 7.83 ‰, still in the range commonly seen in lake sediments, which normally ranges from 0 ‰ to 10 ‰ (Hoefs 2004). The lighter signature was possibly due to input of allochthonous OM which has a typical $\delta^{15}N$ signature of -1 ‰ to 3 ‰, which dilutes the overall $\delta^{15}N$ signature. Because sites B through E exhibit strong seasonal anoxic patterns, the range of $\delta^{15}N$ signatures observed at the SWI of these sites is possibly a function of both OM source and fate. The source corresponds to that of sinking algae and phytodetritus, while fate (i.e. transformation) corresponds to denitrification from bacteria under anoxic conditions (Meyers and Teranes 2001).

Conclusions

SWI bacterial metabolism is often much greater than bacterial metabolism in the overlying water column, therefore SWI bacterial degradation and mineralization of OM substantially affects reservoir carbon and nitrogen cycling. In a stratified, eutrophic reservoir with highly variable SWI bacterial activities, OM quantity was not only a function of the bacteria, but also of the depth and surrounding landscape of the reservoir. Allochthonous processes play an important role in OM burial at shallow sites, more so than at deeper sites in which OM burial is completely of autochthonous origin. Shallow sites that are near shore also only weakly undergo stratification, thus pH, redox, and mixing activities affected C_{in}, C/N ratios, $\delta^{13}C$, and $\delta^{15}N$. This demonstrates that quantitatively important SWI OM dynamics are a function of physical and chemical, as well as biological, processes.

Acknowledgments

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CHAPTER SIX

Presence and Diversity of Total and Sulfate-Reducing Bacteria at the Sediment-Water Interface of a Stratified, Eutrophic Reservoir

Introduction

Sediment-water interfaces (SWIs) in thermally stratified eutrophic reservoirs are seasonally blanketed by anoxic hypolimnia due to water column temperature gradients and bacterial consumption of hypolimnetic dissolved oxygen. Oxygen depletion selects for hypolimnetic bacterial assemblages that anaerobically oxidize various organic carbon substrates (Tammert et al. 2005). In anaerobic respiration, electron acceptors (e.g. nitrate, ferric iron, sulfate) are reduced in order of their decreasing free energy and often form a vertical redox potential gradient (Nealson and Stahl 1997). These redox processes also occur among the bacterial assemblages at the SWI on a much smaller, often micrometer, scale. The depth of the SWI redox gradient partially depends on the mixing conditions overlying water. Further, SWIs contain bacteria at abundances higher than the water column, and these bacteria are highly active (see Chapter Two). Various studies have addressed many SWI bacterial processes, however an adequate measure of SWI bacterial community similarity and diversity, specifically on a seasonal basis in stratified reservoirs is lacking.

Historically, bacterial taxonomic and diversity studies have lacked an applied aspect, relegating such studies to lesser importance in understanding reservoir ecosystem dynamics. Much of this relies on the fact that bacterial diversity and function measurements historically have suffered from methodological limitations. Yet,

understanding bacterial diversity in freshwater ecosystems has become increasingly important in understanding aspects of eutrophication and in development of theoretical models that predict reservoir trophic dynamics (Torsvik et al. 2002).

Traditional measures of bacterial diversity in environmental matrices involved selective culturing methods. However, culturing drastically underestimates true bacterial diversity because the vast majority of naturally occurring bacteria are unculturable (Kirk et al. 1998). Molecular biology techniques have reduced these biases and limitations by preserving the original sample bacterial diversity. Diversity includes richness which is the number of different operational taxonomic units (OTUs) (also known as taxon or species), and evenness which is the amount of each OTU relative to the entire community (Hewson and Fuhrman 2004). Molecular fingerprinting methods that have been utilized to measure bacterial diversity included DNA reassociation kinetics, restriction and terminal-restriction fragment length polymorphisms (RFLPs and t-RFLPs), denaturing gradient gel electrophoresis (DGGE), and automated intergenic ribosomal spacer analysis (ARISA). Each of these methods provide various degrees of resolution, therefore multiple methods are often used to profile bacterial communities (MacGregor 1999). We utilized both DGGE and ARISA to measure seasonal diversity and similarity of SWI bacterial communities in a seasonally stratified reservoir. ARISA was used to profile the entire bacterial community, while DGGE was used to profile the richness of sulfate reducing bacteria (SRB).

The microbial loop concept redefined traditional understanding of aquatic food chain dynamics (Pomeroy 1974; Sherr and Sherr 1988). Novel to the microbial loop was the role of heterotrophic bacteria in organic matter cycling. SWIs are quantitatively

important as organic matter sinks and sources in lakes, mediated by SWI bacterial communities. However, studies of SWI bacterial dynamics lags behind those of open water. This investigation measured total SWI bacterial community diversity and similarity on a seasonal basis and related these measurements to seasonal reservoir mixing dynamics. Because of the importance of sulfur cycling in freshwater ecosystems, SRB diversity was also measured. The SWI of the study site, Belton Reservoir, has a highly active bacterial community with various populations utilizing a wide variety of organic carbon sources (see Chapters 2 and 4). However, until now, the relative diversity of this SWI bacterial community has remained unknown.

Materials and Methods

Study Location

The investigation was conducted on the SWI of Belton Reservoir, a warm, monomictic reservoir in central Texas. Belton Reservoir is eutrophic and undergoes lacustrine zone thermal stratification during late spring and summer, developing an anoxic hypolimnion (Christian et al. 2002; Figure 1.1). The lacustrine zone exhibits a steep depth gradient along its longitudinal axis. The anoxic hypolimnion blanketing this gradient varies spatially and temporally in depth and duration. Five SWI sites along a linear transect of this gradient were chosen for sampling, with each site of increasing depth (Figure 1.2, Table 4.3).

Physicochemical characteristics of the SWI [e.g. temperature (ºC), dissolved oxygen (mg l^{-1}), redox potential (mV), pH] were measured with a YSI 600QS Sonde, lowered to the sediment surface and allowed to stabilize (Table 5.1).

Sample Collection and Processing

SWI samples were collected from the five sampling sites over multiple seasons, corresponding to the various seasonal mixing and stratification conditions of the reservoir (Table 5.1). Sediment samples were collected from the SWI using a 15 cm x 15 cm Ekman dredge. Three samples were collected at each site. Upon retrieval, excess water was carefully siphoned from the sample surface and liquid nitrogen was poured slowly into the corner of the dredge so that disruption of the SWI matrix was minimized. The frozen sediment block was removed from the dredge, wrapped in aluminum foil, sealed in plastic, and held on dry ice. Blocks were returned to the laboratory and held at -80ºC until processing.

For each sediment block, the upper 10 mm of sediment was shaved from the top, placed into a 50 ml plastic centrifuge tube and homogenized. This layer was defined as the SWI, and was determined based on the average depth of the sediment that achieved the lowest redox potential as measured on aerobically and anaerobically incubated microcosm samples using a Unisense© RD-10 redox microelectrode (see Chapter Five).

From each sample, approximately 1 g of the homogenized sediment was preserved in 9 ml of formalin and refrigerated at 4°C for later measurement of bacterial abundance. Another 1 g of sediment was added to a bead solution tube (MoBio) for DNA extraction. The remaining sediment was added to a pre-weighed crucible for organic matter analysis (see Chapter Five).

Bacterial Abundance Measurements

To separate bacteria from sediments, a combination of chemical and physical dispersion was used. Formalin-preserved sediment samples were vortexed briefly,

followed by addition of 50 µl of Tween 80. The samples were shaken on a rotary shaker at 500 rpm for 20 minutes, followed by sonication at 50 W for 30 s. One-hundred µl of the slurry was added to 900 µl of bacteria-free water (0.2-µm filtered) in a 2 ml microcentrifuge tube and centrifuged at 15,000 x g for 2 minutes. The supernatant was transferred to a clean 2 ml microcentrifuge tube. The precipitate was resuspended in 1 ml of bacteria-free water and centrifuged again at 15,000 x g for 2 minutes. The supernatant was transferred to the centrifuge tube containing the previous supernatant. Random samples were chosen for a third resuspension/centrifugation step, but the supernatant from this step did not contain a significant number of bacteria ($< 10^6$ bacteria ml^{-1}).

One-hundred µl of supernatant was added to 900 µl of bacteria-free water and stained with DAPI fluorochrome (5 µg ml^{-1} final concentration) for 3 minutes. The stained sample was filtered onto a 0.2-µm Nuclepore blackened polycarbonate filter and viewed under UV light at 1500 x magnification (Porter and Feig 1980). For any remaining particle-attached bacteria, a 2 x correction factor was applied (Lind and Dávalos-Lind 1991). Bacterial concentration was converted to total cells per gram dry weight of sediment (bacteria gdw^{-1}) (Ellery and Schleyer 1984; Ramsay 1984; dos Santos Furtado and Casper 2000).

DNA Extraction

A MoBio UltraClean™ Soil DNA kit was used to extract and purify total genomic DNA from sediment samples. Approximately 1 g of sediment was used per extraction, with the exact amount weighed into a bead solution tube. Two separate extractions were performed on separate subsamples of a sample.

Resulting DNA was quantified and checked for purity using a GeneQuant™ Pro DNA calculator. If both subsamples were of sufficient purity (A_{260}/A_{280} = 1.8 - 2.0), then they were combined and quantified. If a subsample was not of sufficient purity, then it was discarded. DNA concentration of all samples were recorded as ng μl^{-1} and normalized per gram dry weight of sediment (see Chapter Five).

ARISA Analysis

The primer set ITSF and ITSReub (Integrated DNA, Coralville, Iowa) complementary to portions of the 16S and 23S rRNA genes (16s rDNA) of eubacteria was used in a polymerase chain reaction (PCR) to amplify bacterial DNA corresponding to the intergenic spacer region between 16S and 23S rDNA (Cardinale et al. 2004) (Table 6.1). The 5' end of the ITSF primer was labeled with WellRED D4 phosphoramidite dye to allow fluorometric analysis of the PCR amplified fragments using an automated fragment analyzer.

Table 6.1: Primer sequences used in this study, their common name, and gene targeted.

Target Gene	Primer Name	Sequence (5' - 3')	Reference
16S rRNA	ITSF	GTCGTAACAAGGTAGCCGTA	Cardinale et al. 1998
23S rRNA	ITSFReub	GCCAAGGCATCCACC	Cardinale et al. 1998
dsrB	DSRp2060F[a]	CAACATCGTYCAYACCCAGGG	Geets et al. 2006
dsrB	DSR4R	GTGTAGCAGTTACCGCA	Wagner et al. 1998
	GC Clamp[b]	CGCCCGCCGCGCCCCGCGCC-CGTCCCGCCGCCCCCGCCCG	Schäfer and Muyzer 2001

[a] where Y = C/T

[b] GC clamp is attached to 5' end of DSRp2060F primer

Two µl of sediment-extracted DNA was added to a reaction mixture containing (final concentration) 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 400 ng μl^{-1}

bovine serum albumin, 1.25 U of *Taq* polymerase, and 15 pmol of each primer in a final volume of 50 µl. PCR conditions were a 94ºC initial denaturation for 3 min, followed by 35 cycles of a 1 min denaturation at 94ºC, 1 min annealing at 53ºC, and 2 min elongation at 72ºC, followed by a final extension at 72ºC for 5 min. All PCR products were electrophoresed on a 1% horizontal agarose gel at 120 V for 90 min to insure that proper amplified products (50 bp – 1200 bp) were obtained.

A mixture containing 3 µl of the PCR product and 1 µl of a Bioventures size standard (50-1000 bp in 20 and/or 50 bp increments) was added to 36 µl of deionized formamide and injected into a Beckman Coulter™ CEQ 8000 Genetic Analysis System using a modified FRAG-4 separation method with a 50ºC capillary temperature and a 5 kV separation voltage for 150 min.

Resulting separation profiles (electropherograms) were analyzed with the Beckman Coulter Fragment Analysis Package v.8.0. A quartic analysis model was used with a slope threshold parameter of 5 and PA version 1 dye mobility calibration.

Electropherograms consisted of a series of peaks. Because each bacterial taxon has a specific size and composition of their intergenic space, each peak represented an individual operational taxonomic unit (OTU). The area underneath each peak was divided by the total area under the entire electropherogram to calculate relative abundance of each OTU (Figure 6.1). Relative abundance and richness (i.e. number of peaks in an electropherogram) were used to calculate the Shannon-Weiner diversity index (H) and Shannon-Weiner evenness (E_H). Higher H indicates more diverse communities (i.e. higher number of species and comparative relative abundance), while E_H values indicate the proportion of individuals among the species, indicating if there are dominant

populations (i.e. E_H values closer to 1 indicate complete evenness) (Begon et al. 1996; Atlas and Bartha 1998).

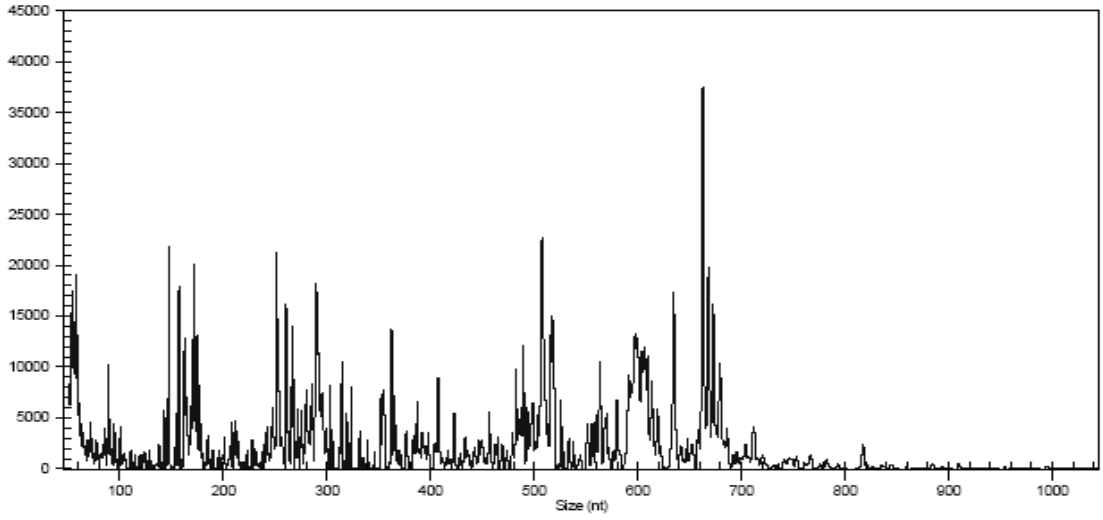

Figure 6.1: Example of an electropherogram. From Site D, 13-Nov-03.

DGGE Analysis of Sulfate Reducing Bacteria

The primer set DSRp2060F and DSR4R (Integrated DNA, Coralville, Iowa) that amplifies a 350 bp segment of the *dsrB* gene in sulfate reducing bacteria (SRB) was used (Geets et al. 2006) (Table 6.1). A 40 bp GC-clamp was added to the 5' end of the forward primer to allow products to run on denaturing gradient gels (Muyzer et al. 1993).

The PCR amplification consisted of a reaction mixture of (final concentration) 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 400 ng μl^{-1} bovine serum albumin, 2.5 U of *Taq* polymerase 50 pmol of each primer, and 2 µl of sediment-extracted DNA in a total volume of 100 µl. PCR conditions were an initial denaturation for 4 min at 94ºC, followed by 35 cycles of a 1 min denaturation at 94ºC, 1 min annealing at 55ºC, and 1 min elongation at 72ºC, followed by a final 10 min extension at 72ºC (Janse et al. 2004). The PCR products were electrophoresed on a 1% horizontal agarose gel at 120 V for 90

min to insure that proper amplified products were obtained. All amplified products were the same length (350 bp), but of various sequences.

10 µl of amplified PCR product was loaded onto a 1 mm-thick 8 % (w/v) polyacrylamide gel with a 40% - 60% gradient of urea/formamide denaturant. The vertical gels were electrophoresed at 75 V for 16 h at 60ºC in 1 x TAE using a dual-cassette DGGE System (Model 2401, CBS Scientific, California). After electrophoresis, gels were stained with SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes, Oregon) diluted 1:10,000 in 1 x TAE buffer for 45 min followed by a 15 min rinse in deionized water. Gels were photographed under UV trans-illumination (540 nm) with an Omega Ultra Lum™ gel imaging system. Further gel imaging and processing was conducted with Omega UltraQuant™ imaging and analysis software v 6.0 and Adobe® Photoshop® v 9.0.

Statistical Analyses

Descriptive and univariate statistics (e.g. linear regression, one-way ANOVA) were performed with JMP v 5.0 (SAS Institute, California) and Microsoft Excel XP. A significance level of $\alpha = 0.05$ was assumed. Microsoft Excel was also used for graphical analyses.

For ARISA analyses, a spreadsheet was generated containing individual samples (columns) and base pair sizes from 50 to 1200 (rows). The presence or absence of a specific base pair size for each sample was recorded as a 1 or 0, respectively. From this binary matrix, a Jaccard similarity index was generated, using PopTools v 2.7.5 (CSIRO, Australia). Using PHYLIP v 3.65 phylogeny inference package, the Neighbor-Joining method of clustering analysis was performed on the Jaccard matrix to generate a

dendrogram that allowed comparison of sample similarities. Dendrograms were optimized using Phylodendron v 0.8 phylogenetic tree software.

Results

Bacterial Abundance

No significant differences existed among SWI bacterial abundance (cells gdw^{-1}) among sites (one-way ANOVA, $F_{4,45} = 0.56$, $p = 0.69$) or among dates ($F_{9,40} = 0.88$, $p = 0.56$). However notable patterns occurred on various dates (Figure 6.2). In March 2004, Sites B, D, and E had near-identical bacterial abundance, possibly a function of reservoir mixing resulting in a homogenous SWI matrix. Also, clusters of similar bacterial abundance among various sites occurred in May, August, September, and October 2004. The May, August, and September dates corresponded to summer stratification, while October corresponded to fall overturn. Yet, other dates corresponding to summer stratification (July 2004) had widely variable bacterial abundances among sites.

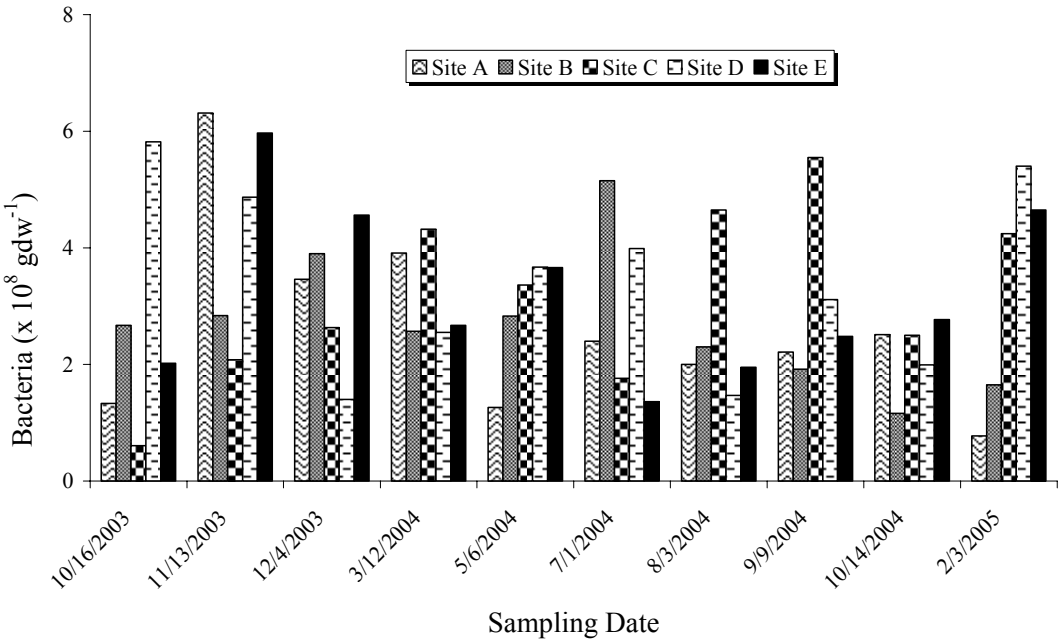

Figure 6.2: Bacterial abundance across all sites and dates of the sampling study.

DNA Concentration

DNA concentration (μg gdw^{-1}) did not differ significantly among sites ($F_{4,45}$ = 1.02, p = 0.41), however it did significantly differ among dates ($F_{9,40}$ = 3.67, p < 0.01). This was made evident by a negative correlation between DNA concentration and dissolved oxygen (r = -0.32, p < 0.05). Lowest overall DNA concentrations occurred at the onset of fall overturn (November 2003) and onset of summer stratification (May 2004). On four separate dates Site D had the highest DNA concentration, all occurring at the onset or during summer stratification. Highest absolute DNA concentrations occurred during October 2003 (Site B) August 2004 (Site D) and September 2004 (Sites C and D), all of these dates corresponding to late season stratification (Figure 6.3).

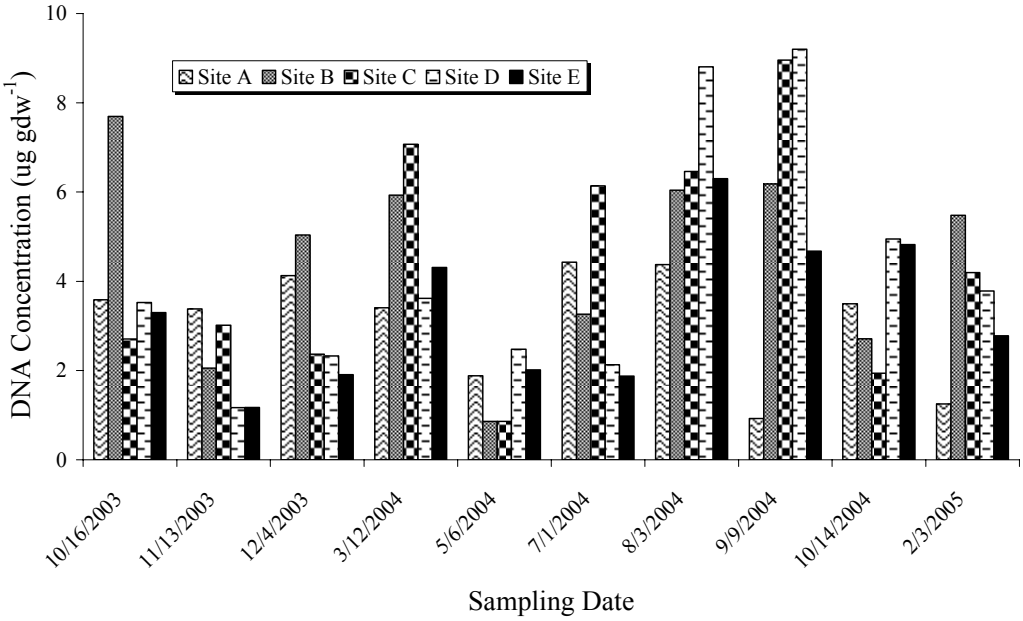

Figure 6.3: Mean DNA concentration across all sites and dates of the sampling study.

ARISA Analyses

Significant differences occurred in species richness (i.e. number of OTUs) among dates ($F_{9,40}$ = 2.81, p < 0.01), but not among sites ($F_{4,45}$ = 0.98, p = 0.43) (Table 6.2).

Highest richness was not correlated with any specific season, as significantly higher richness was present in May 2004 (onset of stratification), July 2004 (summer stratification), October 2004 (autumnal overturn), and February 2005 samples (winter mixing). Lower richness was present in March 2004 (winter mixing), as well as August and September 2004 samples which exhibited total anoxia and lowest redox potential.

No significant difference existed for Shannon-Weiner Diversity (H) by date ($F_{9,40} = 1.41$, $p = 0.22$) or by site ($F_{4,45} = 0.78$ $p = 0.54$) (Table 6.2). In addition, no significant difference existed for Shannon-Weiner Evenness (E_H) by date ($F_{9,40} = 0.77$, $p = 0.64$) or by site ($F_{4,45} = 0.35$, $p = 0.85$) (Table 6.2).

Figure 6.4 relates the similarity among the SWI bacterial communities. Samples during stratification and anoxia were highly similar. For example, in July 2004 Sites C, D, and E were highly similar; during August 2004 all sites were highly similar; and in September 2004 all sites were highly similar with the exception of Site A. In October 2003, Sites B and E were similar as were Sites C and D. The high similarities correspond directly to anoxia or hypoxia (< 1 mg l^{-1}). The dissimilar sites all have > 1 mg l^{-1} dissolved oxygen.

Similarities among sites also occurred during winter mixing, but were not as pronounced and sequential as during stratification. In December 2003, Sites A, B, and D were similar; in March 2004 Sites A, B, and C were similar; in February 2005 sites had high similarity with the exception of Site D.

During overturn during October 2004, high similarity occurred; however during overturn in November 2003, with the exception of Sites A and E, samples were highly

dissimilar. During onset of stratification during May 2004 Sites A, B, and C were similar while Sites D and E were dissimilar from the other sites and from each other.

Table 6.2: Richness, Shannon-Weiner Diversity Index (H), and Shannon-Weiner Evenness (E_H) for the SWI bacterial communities at each site and date.

Date	Site	Species Richness	Diversity (H)	Evenness (E_H)	Date	Site	Species Richness	Diversity (H)	Evenness (E_H)
16-Oct-03	A	224	4.75	0.88	1-Jul-04	A	307	4.85	0.85
16-Oct-03	B	239	4.28	0.78	1-Jul-04	B	311	4.75	0.83
16-Oct-03	C	267	4.91	0.88	1-Jul-04	C	304	4.88	0.85
16-Oct-03	D	289	4.92	0.87	1-Jul-04	D	282	4.77	0.85
16-Oct-03	E	232	4.33	0.80	1-Jul-04	E	293	4.78	0.84
13-Nov-03	A	277	4.69	0.83	3-Aug-04	A	227	4.61	0.85
13-Nov-03	B	297	4.89	0.86	3-Aug-04	B	201	4.50	0.85
13-Nov-03	C	84	3.94	0.89	3-Aug-04	C	160	4.39	0.86
13-Nov-03	D	313	5.11	0.89	3-Aug-04	D	75	2.84	0.66
13-Nov-03	E	300	5.04	0.88	3-Aug-04	E	214	4.68	0.87
4-Dec-03	A	306	4.88	0.85	9-Sep-04	A	26	2.60	0.80
4-Dec-03	B	214	4.75	0.88	9-Sep-04	B	231	4.63	0.85
4-Dec-03	C	92	4.01	0.89	9-Sep-04	C	221	4.24	0.79
4-Dec-03	D	275	5.01	0.89	9-Sep-04	D	264	4.74	0.85
4-Dec-03	E	271	4.73	0.84	9-Sep-04	E	238	4.28	0.78
12-Mar-04	A	258	4.91	0.88	14-Oct-04	A	284	4.76	0.84
12-Mar-04	B	94	3.91	0.86	14-Oct-04	B	287	4.84	0.86
12-Mar-04	C	105	3.23	0.70	14-Oct-04	C	312	4.85	0.84
12-Mar-04	D	253	4.85	0.88	14-Oct-04	D	283	4.71	0.83
12-Mar-04	E	295	4.73	0.83	14-Oct-04	E	321	4.85	0.84
6-May-04	A	334	4.96	0.85	3-Feb-05	A	276	4.79	0.85
6-May-04	B	316	5.00	0.87	3-Feb-05	B	296	4.76	0.84
6-May-04	C	274	3.93	0.70	3-Feb-05	C	313	4.92	0.86
6-May-04	D	311	4.88	0.85	3-Feb-05	D	284	4.81	0.85
6-May-04	E	264	4.89	0.88	3-Feb-05	E	316	4.75	0.82

DGGE Analyses of Sulfate Reducing Bacteria

Figure 6.5 shows banding patterns obtained in gels from DGGE analysis of sulfate-reducing bacteria (SRB). Each band represents an individual OTU, thus total number of bands in a lane is equal to species richness of a sample (Table 6.3). Care must

be taken when assuming one band equals one OTU, as some bands can harbor more than one OTU (Sekiguchi et al. 2001). Significant differences in richness exist by date ($F_{9,40}$ = 8.18, $p < 0.0001$), but not by site ($F_{4,45}$ = 0.23, $p = 0.92$). Significant differences are due to higher richness during December 2003 and March 2004, both during winter mixing; and lower richness in July 2004 during stratification.

Table 6.3: Richness of sulfate-reducing bacteria at the SWI throughout the course of the study.

Lane	Date	Site	SRB Richness	Lane	Date	Site	SRB Richness
1A	16-Oct-03	A	11	6A	1-Jul-04	A	8
1B	16-Oct-03	B	12	6B	1-Jul-04	B	9
1C	16-Oct-03	C	13	6C	1-Jul-04	C	11
1D	16-Oct-03	D	9	6D	1-Jul-04	D	8
1E	16-Oct-03	E	15	6E	1-Jul-04	E	8
2A	13-Nov-03	A	11	7A	3-Aug-04	A	12
2B	13-Nov-03	B	8	7B	3-Aug-04	B	13
2C	13-Nov-03	C	10	7C	3-Aug-04	C	11
2D	13-Nov-03	D	12	7D	3-Aug-04	D	5
2E	13-Nov-03	E	11	7E	3-Aug-04	E	8
3A	4-Dec-03	A	17	8A	9-Sep-04	A	10
3B	4-Dec-03	B	16	8B	9-Sep-04	B	15
3C	4-Dec-03	C	16	8C	9-Sep-04	C	13
3D	4-Dec-03	D	20	8D	9-Sep-04	D	13
3E	4-Dec-03	E	13	8E	9-Sep-04	E	11
4A	12-Mar-04	A	16	9A	14-Oct-04	A	12
4B	12-Mar-04	B	16	9B	14-Oct-04	B	11
4C	12-Mar-04	C	17	9C	14-Oct-04	C	13
4D	12-Mar-04	D	15	9D	14-Oct-04	D	14
4E	12-Mar-04	E	14	9E	14-Oct-04	E	13
5A	6-May-04	A	12	10A	3-Feb-05	A	13
5B	6-May-04	B	11	10B	3-Feb-05	B	13
5C	6-May-04	C	10	10C	3-Feb-05	C	12
5D	6-May-04	D	10	10D	3-Feb-05	D	12
5E	6-May-04	E	12	10E	3-Feb-05	E	10

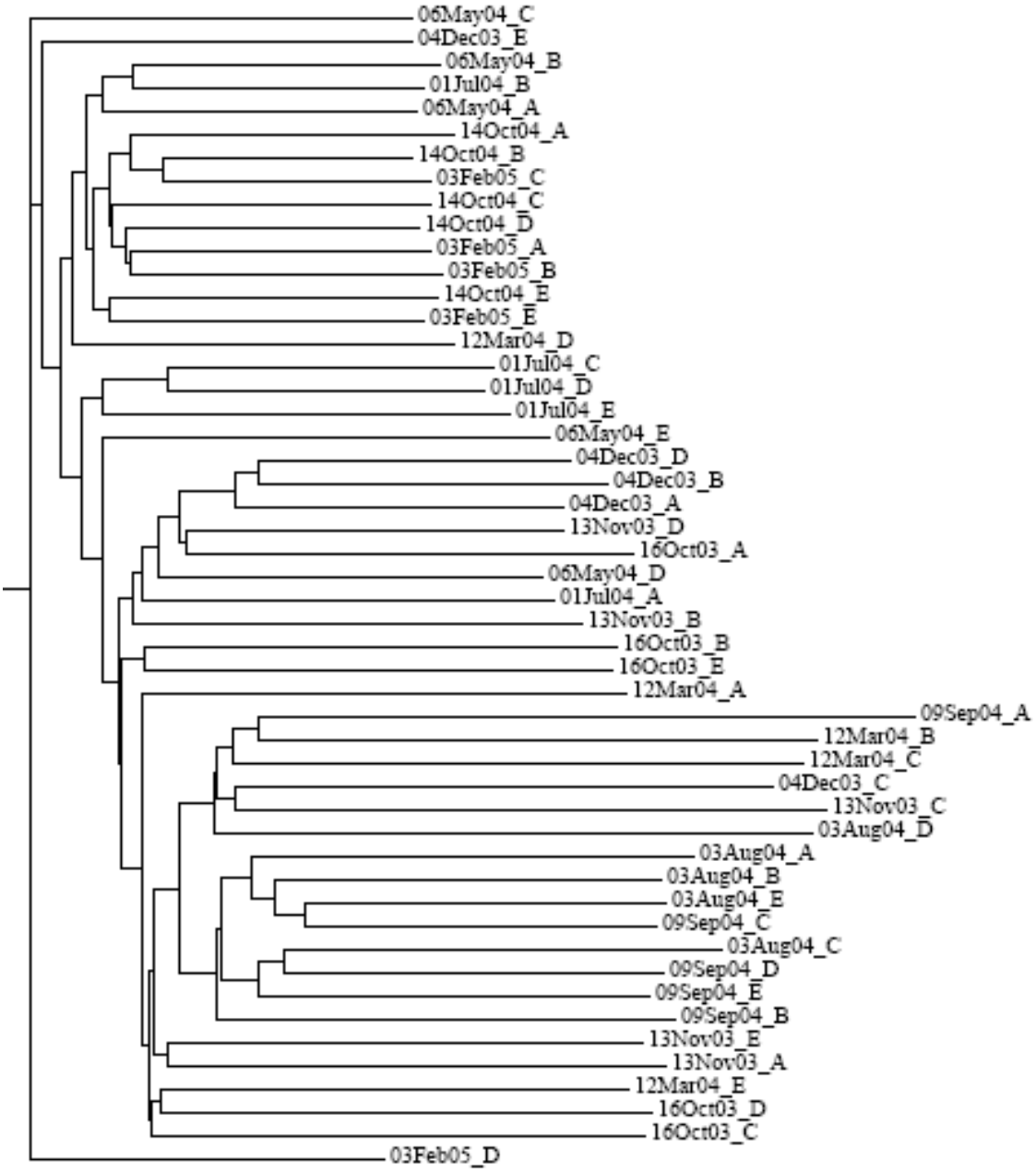

Figure 6.4: Neighbor-Joining cluster tree (dendrogram) derived from the Jaccard similarity index comparing SWI bacterial community similarity at each site and date. Similar samples are clustered close together. Samples further apart are more dissimilar.

Discussion

Though SWI bacterial abundance did not differ among seasons, the taxa that composed these abundances did show seasonal and spatial differences. In addition,

previous studies showed that seasonal differences exist among these SWI bacterial activities (see Chapter Two), thus bacterial abundance as a stand alone analysis is not a sensitive measurement of SWI bacterial differences.

DNA concentration was not a function of bacterial abundance ($r = 0.006$, $p = 0.96$). In some instances, high DNA concentrations were associated with low bacterial abundances (e.g. February 2005, Site B), others had high DNA concentrations and high bacterial abundances (e.g. September 2004, Site C). The extracted DNA is believed to be of bacterial origin for three reasons: 1) No benthic macroinvertebrates were present in the SWI samples; 2) Few protists were detected in several random sediment samples enumerated for autotrophic and heterotrophic nanoflagellates (Bloem et al. 1986); and 3) In some instances higher DNA concentrations occurred under anoxic conditions, when few, if any, other organisms other than bacteria inhabit the sediment.

The seasonal differences in DNA concentration suggest a possibility that some SWI bacterial taxa contain more per-cell DNA than others. However, DNA was extracted from bulk sediment, thus extracellular DNA was co-extracted with cellular DNA. Studies on marine sediments have shown that sediment extracellular DNA degradation is 7 to 100 times higher than in open water, but due to high quantities of DNA in sediments, its correlation to bacterial abundance remains uncertain (Dell'Anno and Corinaldesi 2004). Thus a second possibility is that under anoxic conditions, increased preservation of extracellular DNA occurs, as accounted for by the negative correlation between dissolved oxygen and DNA concentration.

Two molecular processes, DGGE and ARISA were used to profile SWI bacterial communities. With DGGE, a primer set specific for a 16s rRNA gene (16s rDNA)

hypervariable region was used in polymerase chain reaction (PCR) to produce DNA fragments of identical sizes but different in base pair sequences. The fragments were electrophoresed on a vertical denaturing gradient of urea and formamide that separated

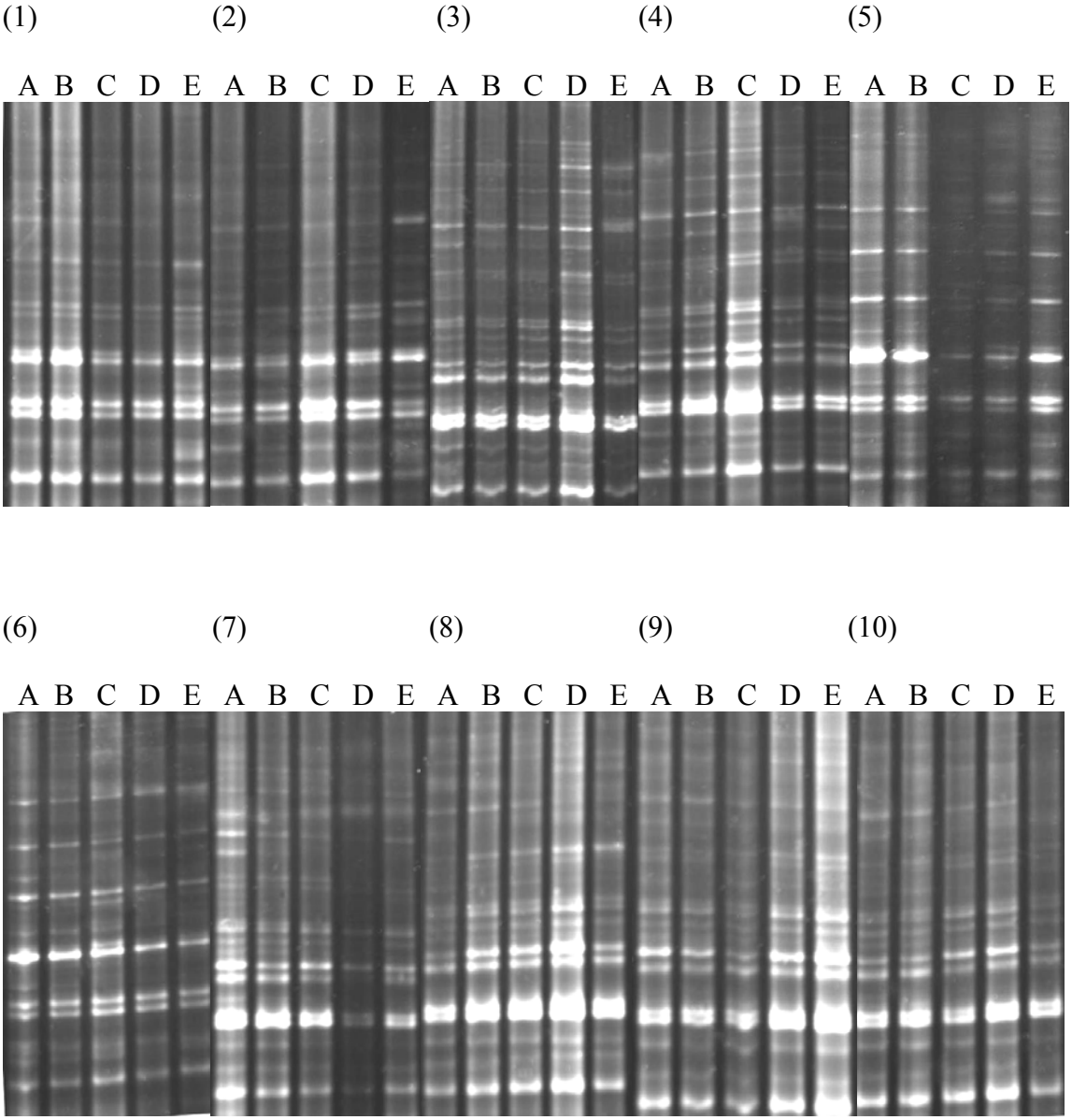

Figure 6.5: Photographs of DGGE gels for each site and date throughout the study. Numbers 1 through 10 indicate the consecutive order of dates sampled.

the fragments based on their mobilities due to differing sequences. Resulting fragments are distinct bacterial OTUs (Muyzer et al. 1993; Muyzer and Smalla 1998; Schäfer and Muyzer 2001). With ARISA, a primer set flanking the intergenic space between the 16s and 23s rRNA genes was used in PCR. Amplified PCR fragments included this intergenic space, which differs in both size and sequence for each OTU. Fragments were separated on an automated fragment analysis system, in which an electropherogram produced distinct peaks for each OTU (Fisher and Triplett 1999).

Molecular techniques, while powerful, have limitations that merit caution when interpreting results. With PCR, the concentration of template DNA, PCR cycle parameters (e.g. number of cycles, cycle temperatures), and multiple batches of PCR runs (i.e. differing ingredient concentrations) may bias the final PCR product. These biases may alter the proportions of genes present in the original gene pool (Casamayor et al. 2002). In DGGE, the primary limitation is that individual bands indicate OTUs composing greater than 0.3-0.4% of the total community. Thus populations of low abundance may go undetected in analyses (Reche et al. 2005). In addition, 'double banding' may occur, in which more than one OTU is present in a single band (Sekiguchi et al. 2001; Janse et al. 2004). Inter-gel variability resulting from variations in gradient concentration may also confound results when comparing multiple DGGE gels (Schäfer and Muyzer 2001). While few limitations have been reported with ARISA, there is a possibility that two or more OTUs may share the same intergenic space length, thus a single peak could represent more than one taxon, underestimating diversity measurements (Fisher and Triplett 1999).

Significant variation occurred in the number of OTUs (richness) at the SWI throughout the course of the study. However this difference did not correspond to a specific season. High richness occurred under various mixing and stratified conditions. The seasons that exhibited lowest richness occurred under two seasonal processes: 1) complete anoxia and lowest observed redox potential during late summer stratification (August and September 2004), and 2) winter mixing signified by coldest temperatures (March 2004). Lower richness would be expected during times of stress such as low redox potential or low temperature, however high richness occurred during February 2005 when temperatures were cold. No other studies have assessed bacterial richness at the SWI, however some studies have assessed bacterial richness and diversity in oxic and anoxic pelagic zones of various lakes. These studies either noted little change in seasonal richness (i.e. Mono Lake, California) or higher richness in oxic waters (Lake Sælenvannet, Norway) (Øvreås et al. 1997; Hollibaugh et al. 2001). An ARISA-based study on several Wisconsin lakes of various trophic states noted substantial decreases in pelagic bacterial richness during summer due to a clear-water phase, however these differences were less pronounced in increasingly oligotrophic lakes (Yannarell et al. 2003).

No significant differences in Shannon-Weiner Diversity or Evenness were observed based on relative peak areas produced by ARISA electorpherograms. High evenness suggests that no single SWI bacterial population dominates the community. Anoxia, low redox potential, and low temperatures are often considered stressful in terms of most biota, which would lower evenness; however many bacteria are highly specialized to occupy these habitats (Nealson 1997). In addition, some of the bacteria

detected in the ARISA analyses may have been present in samples, but dormant. This may artificially inflate evenness measurements. Thus evenness does not imply function of the community. It is possible that relatively few metabolically specific populations conduct the bulk of the total community metabolism at any given time (Stevenson 1977).

Little seasonal difference in diversity does not suggest there are not seasonal changes in the SWI bacterial composition. The Neighbor-Joining dendrogram (Figure 6.4) illustrates these differences. As a general observation, highest similarities appear among the various sites after prolonged periods of stratification or mixing. This suggests similar selective pressures at each site select for similar bacterial communities. During transition periods (i.e. onset of stratification or onset of overturn) similarity decreases, possibly as a function of heterogeneous selective pressures at the different sites. For example, during the onset of stratification in May 2004, Sites D and E that have undergone stratification and decreasing dissolved oxygen exhibit similar communities but are dissimilar from Sites A, B, and C which are similar to one another because these sites are still mixing.

Sulfate-reducing bacteria (SRB) are a diverse group of anaerobic bacteria that reduce sulfate (SO_4^{2-}) as a terminal electron acceptor in the presence of organic matter, producing hydrogen sulfide (H_2S). H_2S is toxic to many organisms, contains antibiotic properties, and causes aesthetic problems in reservoirs (Hines et al. 2002). Further, sulfides can combine with various metals to produce metal sulfides in the sediments, generating a characteristic black precipitate (Geets et al. 2006). Therefore elucidating the diversity and distribution of these causative organisms (i.e. SRB) is important in understanding rates and processes of sulfur cycling.

Common to all sulfate reducing bacteria is the dissimilatory sulfite reductase (DSR) enzyme that catalyzes reduction of sulfite to sulfide. Thus the gene that codes for the β-subunit of the DSR gene (*dsrB*) was used as a biomarker for diversity of SRB (Wagner et al. 1998). While the *dsrB* gene is highly conserved, the SRB in which it is contained are found in many diverse lineages including the δ subdivision of *Proteobacteria*, some gram-positive bacteria, the subdivision *Thermodesulfobacterium*, and the archaeal domain *Euryarchaeota* (Karr et al. 2005).

Although all SRB are anaerobic, presence of the DSR gene was found in all samples under oxic and anoxic conditions. In addition, SRB richness was highest during two winter mixing dates and lowest during a date corresponding to stratification and anoxia. Recent studies have shown that active SRB have been found in oxic zones of algal mats and biofilms (Dar et al. 2005). Other studies suggest that SRB violate the paradigm of electron acceptors being utilized in the order of decreasing free energy (Minz et al. 1999). Instead, SRB activity is possibly primarily controlled by the presence of SO_4^{2-}. Under aerobic conditions, dissolved oxygen and aerobic chemolithotrophic bacteria would be expected to oxidize H_2S to SO_4^{2-}, thus providing a large pool of potentially available SO_4^{2-} to the SRB (Nealson 1997). These SRB could subsequently reduce the SO_4^{2-} under oxic conditions. If the sulfur-oxidizing aerobic chemolithotrophs are more abundant and exhibit higher activities than SRB, equilibrium would shift to SO_4^{2-}. As oxygen becomes depleted due to stratification and aerobic bacterial metabolism, the obligately aerobic sulfur oxidizers would decrease while the SRB would continue to produce H_2S. Thus H_2S would accumulate under anaerobic conditions.

Conclusions

Seasonal physicochemical changes at the SWI in Belton Reservoir have various effects upon the SWI bacterial communities due to mixing and stratification. Total bacterial abundance and diversity do not change on a spatial or seasonal scale. Total concentration of SWI DNA shows significant decreases during onset of overturn and onset of stratification. In addition, there are significant changes in the richness and similarity of the bacterial communities among dates. While there are various exceptions, higher similarity is associated with prolonged stratification or prolonged mixing. Changes in richness did not follow a distinct seasonal pattern. Sulfate-reducing bacteria are highly diverse, yet show greater richness under oxic conditions, implying that they may be present but dormant. In contrast, SRB may be active under oxic conditions, implying that they do not necessarily follow the traditional order of redox-specific reductions.

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CHAPTER SEVEN

Conclusions

Sediment-water interfaces (SWIs) of monomictic, eutrophic reservoirs undergo physical and chemical changes associated with mixing and stratification. Few other ecosystems experience such an extensive habitat alteration on such a small spatial and temporal scale. In addition, SWIs in reservoirs accumulate organic carbon at higher rates than sediments of marine and other freshwater systems (Dean and Gorham 1998). Bacteria metabolize much of this organic carbon, thus reservoir SWIs are unique ecosystems for the study of bacterial community dynamics. The SWI of Belton Reservoir presents such an ecosystem, thus it was used as the study location.

Various methods were used to investigate SWI bacterial community seasonal dynamics, however the purpose of the study is summed into four basic ecological questions: 1) How diverse is the (bacterial) community? 2) How active are the community members? 3) Do community members preferentially feed on (i.e. uptake) certain substrates? 4) How do internal and external (i.e. autochthonous and allochthonous inputs) processes physically and chemically influence the habitat?

High seasonal SWI bacterial diversities were observed, and these seasonal diversities were highly similar. High community diversities suggest establishment of stable communities (Atlas and Bartha 1998). Unexpectedly this high diversity was maintained under abiotic SWI stresses during onset of stratification and onset of overturn. From the perspective of higher trophic levels, the SWI mixing and stratification

conditions are unfavorable to support stable communities. However specific bacterial populations are readily able to exploit these environments marked by anoxia and low redox potential. High, but similar, bacterial abundances were observed among all seasons, further supporting evidence of high community stability.

A related, but more specific ecological question asks 'how abundant are individual taxa?' This was explored with sulfate reducing bacteria (SRB). Higher richness observed during oxic conditions challenges two widely-accepted principles: 1) SRB are obligate anaerobes, only present during anoxic conditions, and 2) SRB are only present when redox conditions are favorable. However, undetected micro-habitats may exist that harbor SRB under their favored conditions. Yet, presence of these SRB populations in previously undescribed habitats may redefine their niche.

The SWI bacterial community exhibited higher activity, production, and generation time during anoxia and stratification. This evidence supports the diversity data, suggesting that conditions often deemed 'unfavorable' are not at all unfavorable to certain bacterial populations. The selective pressures brought upon by anoxia and low redox potential gives way to bacterial populations that are readily able to exploit the SWI. This concept is not new to microbial ecology, given Beijerinck's principle, 'everything is everywhere, the environment selects'. However, classical community theory states that there is a strong inverse relationship between diversity and productivity (Abrams 1995). Results of this study appear to contradict this.

Because a spatial habitat gradient was built into the study design, widely varying internal and external influences defined the chemical composition of the SWI. Shallowest sites were defined by greater allochthonous inputs. Thus organic matter

composition varied among the sites, which potentially influence the taxa present. While this investigation did not link presence of specific bacterial taxa to organic matter composition, a further investigation examined the types of organic carbon preferred by the bacteria under various mixing conditions. The marked differences in seasonal utilization of specific substrates indicate that bacteria shift preference for certain substrates based on their need of high carbon-containing or high nitrogen-containing compounds. Alternatively, the types of organic matter present may select for communities that optimally utilize the most easily oxidized substrate. The former suggests that SWI bacteria are decidedly stenotolerant, while the latter suggests rapid community succession occurs with changes in carbon inputs. Future investigations into this phenomenon will clarify this uncertainty.

APPENDIX

APPENDIX

Publications Related to This Research

Chapter Two

Christian BW, Lind OT. In Press. Increased sediment-water interface bacterial [3H]-L-serine uptake and biomass production in a eutrophic reservoir during summer stratification. Archiv für Hydrobiologie.

Chapter Three

Christian BW, Lind OT. 2006. Key issues concerning Biolog use for aerobic and anaerobic freshwater bacterial community-level physiological profiling. International Review of Hydrobiology 91(3):257-268.

Chapter Four

Christian BW, Lind OT. In Press. Multiple carbon substrate utilization by bacteria at the sediment-water interface: seasonal patterns in a stratified eutrophic reservoir. Hydrobiologia.

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