MICROBIAL PROCESSES AND CARBON UTILIZATION IN HIGH SULFATE WATERS AND SEDIMENTS

by

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ABSTRACT

The application of microorganisms for treating high sulfate effluents is proving to be an effective approach although the processes involved are not well understood. One example is the use of anaerobic passive systems such as mine pit lakes and subsurface flow wetlands. This work addresses the missing information on microbial processes in two high sulfate environments: a permanently stratified fjord and a subsurface flow wetland treating mine waste.

In Nitinat Lake fjord, although sulfide was present, no significant sulfate reduction occurred and quantitative polymerase chain reaction (qPCR) of the dissimilatory sulfite reductase gene (*dsr*) detected very few sulfate-reducing bacteria (SRB). Instead, the small subunit rRNA phylogenetic analysis revealed almost complete domination by novel *Arcobacter*-related species in deep anoxic water. In contrast, substantial sulfate reduction was measured in the fjord sediments. A rate of 250 ± 60 nmol cm^{-3} d^{-1} was determined, and 8.7 ± 0.7 x 10^6 copies of *dsr* mL^{-1} were found using quantitative PCR (qPCR). When the sediments were amended with carbon sources (acetate, lactate, or a mixture of compost, silage and molasses), acetate stimulated the highest rate of sulfate reduction.

An operating passive treatment system remediating metal-containing seepage near the Teck smelter in Trail, B.C. was used for a study of five carbon materials (silage, pulp mill biosolids, compost, molasses with hay, and cattails) as potential substrates for passive systems. Phylogenetic analyses of SSU rRNA and *dsr* genes were performed, as well as qPCR and chemical analyses of carbon parameters including easily degradable material (EDM), dissolved and particulate organic carbon (DOC and TOC), particulate nitrogen (PN), and carbon to nitrogen ratio C/N. Silage showed highest sulfate-reducing potential. The results showed that the initial C/N ratio of organic materials correlated positively with the SRB activity. However, phylogenetic analysis determined that the majority of bacterial species belonged to *Bacteroidetes* and *Firmicutes* phyla likely involved in complex carbon degradation. The lack of SRB in the actual system suggests that processes other than sulfate reduction are responsible for metal removal.

This study contributed to the understanding of microbial processes and therefore aids in improving design and monitoring of passive treatment systems.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

ABR	Anaerobic bioreactor
AMD	Anaerobic oxidation of methane
AOM	Anaerobic oxidation of methane
ARD	Acid rock drainage
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
dsr	Dissimilatory sulfite reductase
EDM	Easily degradable material
FISH	Fluorescence in situ hybridization
K_M	Saturation constant
MME	Metal mining effluents
MPN	Most probable number
OMZ	Oxygen minimum zone
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PN	Particulate nitrogen
POC	Particulate organic carbon
PRB	Permeable reactive barrier
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SRB	Sulfate-reducing bacteria
SRR	Sulfate reduction rate
SSU rRNA	Small subunit ribosomal RNA
VFA	Volatile fatty acids

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CO-AUTHORSHIP STATEMENT

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Environments with high concentrations of sulfur compounds, such as sulfate and sulfide, are found naturally such as in the ocean, near shore fjords and basins, thermal hot springs, saline surface waters and groundwater aquifers. Although these are naturally occurring, they can create problems for commercially important aquatic species. For example, the high sulfide concentrations that occur close to the surface in the fjord Nitinat Lake on Vancouver Island can cause massive mortalities of fish fry in a salmon hatchery located at the terrestrial end of the fjord (Pawlowicz et al., 2006). Other natural high sulfur environments, so-called oxygen minimum zones, are growing in extent and creating "dead zones" devoid of aquatic plants and vertebrates (Helly and Levin, 2004). Also, an increasing problem is the creation of high sulfur environments by human activities such as in the case of metal mining effluents (MME), many of which are characterized by high sulfate concentrations, elevated levels of metals, and low pH that are toxic to aquatic life. The Canadian mining industry generates 650 million tonnes per year of waste rock, tailings and other mining sources that have the potential to cause MME[1] (Canada, 1991). British Columbia is prominent among the provinces causing MME with 25 mines currently generating acidic MME and another 17 sites identified as potentially acid-generating. In 1994, there was an estimated 312 million tonnes of acid-generated waste rock and mine tailings in B.C., with this number growing by circa 25 million tonnes each year (BC, 2004). Effective treatment processes must be in place as allowed concentrations of deleterious metals in mining effluents released to the environment in Canada are controlled by the Metal Mining Effluent Regulations (Table 1.1).

A common engineered treatment method for MME is metal precipitation with a neutralizing agent (Younger et al., 2002). This approach requires building and operating a

[1] Also known as acid mine drainage (AMD) or acid rock drainage (ARD) if acidic.

treatment plant with continuous reagent supply, process control and waste disposal. The end products of lime treatment are metal (oxy)hydroxides, which are high in volume and unstable under changing environmental conditions so that they generally require further treatment. In cases where low cost and minimal maintenance are required, such as in widespread remote areas and discontinued mine operations, an alternative remedial approach using passive bioremediation is often considered.

Passive bioremediation involves the use of natural microbial processes to degrade or immobilize environmental pollutants. The biggest advantages over chemical treatment are simplicity, lower costs and minimal site disturbance. On the downside, design and control of passive treatment systems is challenging, contributing to their lower efficiency, and their performance is less predictable than chemical treatment systems (Johnson and Hallberg, 2005a; Neculita et al., 2007). This is because passive treatment systems are complex environments composed of a wide variety of organic and inorganic compounds and include diverse microbial communities. Thus, the key approach to improving these systems is understanding the dynamic interactions within these systems.

Passive systems for treating metal mine drainage, such as subsurface-flow wetlands, permeable reactive barriers (PRB) and sometimes mine pit lakes, rely on consortia of organisms for sustained removal of metals. The intention, in most of these passive treatment systems, is to create an organic-rich, circum-neutral-pH, anaerobic environment that is favorable for sulfate-reducing bacteria (SRB). These obligate anaerobes reduce sulfate to sulfide, which combines with metal ions to form, depending on the pH, precipitates with a low solubility product. SRB have also been used successfully in active (reactor-based) treatment of sulfate-rich industrial wastewater (Hulshoff Pol et al., 2001). Anaerobic packed bed reactors (Maree and Strydom, 1987), gas-lift reactors (van Houten et al., 1994; Esposito et al., 2003), and upflow sludge blanket reactors (La et al., 2003) are among the most popular industrial reactor designs used.

In contrast to active chemical processes, the application of passive SRB bioreactors has met with mixed results (Doshi, 2006). Their effectiveness in meeting MME water quality requirements is not always consistent and therefore they are viewed as unreliable by some regulatory agencies. This is in part due to the many different designs and configurations. For example, a wide variety of organic amendments is used and although experience dictates which are more successful at supporting SRB than others, there are few science-based studies of how these organics influence the presence of different microbial groups. In general, although some work has been done correlating SRB presence and sulfate reduction rate (SRR) with

environmental parameters in natural high sulfur environments, there is a paucity of data on microbial characterization in man-made passive treatment systems. Therefore, the overall objective of this thesis is to adapt and use molecular-based tools, such as SSU rRNA clone libraries and quantitative polymerase chain reaction, to study the presence of SRB and other microbes in passive treatment systems. These tools can be incorporated ultimately into monitoring programs for diagnosing operating problems with passive treatment systems. Also, the presence and abundance of certain microbial species can be correlated with the system characteristics and performance to inform more effective design. I used the water column of an anaerobic fjord, Nitinat Lake, a typical natural high sulfur environment, to adapt and test molecular methods for characterizing bacterial diversity, particularly SRB and their activities. The fjord and its water column are easily accessed and sampled. As well, collection of microbes from water and extraction of their DNA is uncomplicated when compared to more heterogeneous terrestrial environments. The main hypothesis that was tested in Nitinat Lake was that high sulfate anaerobic environments support SRB as one of the more functionally important microbial groups. Another objective was to determine the potential sulfate reduction rates after the Lake sediments were amended with organic material. Different organic amendments were compared with respect to the extent to which they stimulate SRB in experiments with the fjord sediment and in an operating passive treatment system in Trail, BC.

1.2 LITERATURE REVIEW

1.2.1 MME Treatment Systems

Metal mine effluents containing sulfate originate from mine wastes comprising sulfide minerals, such as pyrite (FeS_2), when they decompose in the presence of air and water:

$$4FeS_{2(s)} + 14O_{2(g)} + 4H_2O_{(l)} \rightarrow 4Fe^{2+}{}_{(aq)} + 8SO_4{}^{2-}{}_{(aq)} + 8H^{+}{}_{(aq)} \quad (1)$$

This oxidation reaction creates acidity although in some cases the MME has a circum-neutral pH due to the presence of sufficient dissolved alkaline compounds. Although sulfide mineral oxidation occurs abiotically, certain autotrophic prokaryotes (e.g., *Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans, Acidimicrobium ferrooxidans, Sulfolobus metallicus*) can increase the rate of reaction by many orders of magnitude (Baker and Banfield, 2003). In case of

pyrite, these microorganisms accelerate its dissolution by re-generating ferric ion, which acts as an oxidant in mineral dissolution. The ferric ions then hydrolyze in water to form ferric hydroxide ($Fe(OH)_3$), thus releasing more hydrogen ions (Equation 2).

$$4Fe^{3+}_{(aq)} + 12H_2O_{(l)} \rightarrow 4Fe(OH)_{3(s)} + 12H^{+}_{(aq)} \quad (2)$$

Among the heavy metals commonly found in MME in elevated concentrations are copper, zinc, arsenic, and manganese. MME composition from several British Columbia mining sites is presented in Table 1.2. If left untreated, MME can acidify the soil, kill aquatic organisms, and contaminate the groundwater with high metal concentration and acidity. In addition, it causes corrosion of engineered structures such as bridges and pipes.

Treatment options of MME can be divided into active and passive processes. Further, these can be split into chemical and biological systems (Table 1.3). The most commonly used approach is active precipitation with alkaline compounds such as CaO, $Ca(OH)_2$, $Mg(OH)_2$, NaOH, or Na_2CO_3. The addition of alkalinity increases the pH, which causes metals to precipitate as hydroxides and carbonates. The second step in the process separates the metal-rich sludge from the clean aqueous effluent. This so-called high density sludge treatment process is effective for high MME flows that are very acidic and contain high metal concentrations that would be too toxic for a microbial process. However, the operational costs are substantial and the large volume of waste sludge produced is a major liability since further storage and treatment are required. Also, because of the fact that metals have a wide range of isoelectric points (ranging from pH 6 to 11), this technique is not ideal when MME contains a variety of metals. More cost-efficient chemical treatment systems utilizing the same principles are limestone channels and anoxic limestone drains. Due to the low limestone solubility and surface coating with $Fe(OH)_3$, this method is often not sufficient to meet water quality requirements. Biological processes, specifically sulfate reduction, are used to generate sulfide, precipitate metals as metal sulfides and increase alkalinity. Active processes, such as the Paques (Balk, NL) SULFATEQ™ and BioteQ (Vancouver, BC, Canada) BioSulphide® systems include sulfidogenic bioreactors that are fed by simple organic molecules such as acetate, lactate, or ethanol. They prove to be effective for metal and sulfate removal, and the metal sulfides produced in these bioreactors are more stable and less bulky than precipitates formed after alkaline addition. The disadvantage of such approach is high construction and operation costs due to constant supply of pure organic substrates and bioreactor maintenance (i.e., retaining anaerobic conditions, excess biomass and

precipitate removal). Passive bioremediation systems for MME treatment such as natural and constructed wetlands, and permeable reactive barriers (PRB) are specifically targeted for low flow, less toxic MME. They are often used for MMEs that affect large, remote areas where other treatment options are not feasible. Constructed wetlands are built typically as either aerobic (surface-flow) or anaerobic (subsurface-flow) systems, or a combination thereof. Aerobic wetlands are used to treat MME that is net alkaline and rely on iron oxidation to precipitate iron (oxy)hydroxides, onto which metals can adsorb. MME in anaerobic (subsurface-flow) wetlands flows through a layer of organic substrate, which is typically a low-cost agricultural by-product or waste material, such as compost, hay, leaf mulch, and composted manures (Vile and Wieder, 1993; Waybrant et al., 1998; Edenborn and Morrow, 2002; Johnson and Hallberg, 2005b). The surface is usually capped with an impermeable layer and covered with plants such as *Typha latifolia* and *Sphagnum* moss or other native grasses. The plants serve several purposes such as stimulation of microbial processes, adsorption of metal precipitates, creation of wildlife habitat, and blend in visually with the environment. Several attempts were made to enhance SRB activity in mine pit lakes affected by MME by addition of carbon source and nutrients (Miller et al., 2006). Increased biological productivity induces stratification and bottom anoxic layer removes target analytes by either transforming them to another form (e.g., acidity, sulfate) or inducing them to precipitate as insoluble minerals that settle out of solution (e.g., heavy metal sulfides). These passive biological systems are good alternatives to conventional treatment, although their unpredictable and variable performance and long-term stability are currently the biggest engineering concerns that need to be addressed.

1.2.2 Design and Performance of Anaerobic Treatment Systems

Initially, sorption onto organic materials contributes to 50 to 80 % of metal removal and compensates for the limited bacterial activity during the wetland establishment (Skousen et al., 2000). After this period, even though other chemical and physical metal removal mechanisms still take place in anaerobic systems, long-term metal removal relies on bacterial sulfate reduction by SRB and subsequent metal precipitation (Equations 3- 5),

$$SO_4^{2-} + 2CH_2O + 2H^+ \rightarrow H_2S + 2H_2CO_3 \quad (3)$$

$$H_2S \leftrightarrow HS^- + H^+ \qquad \log K \cong 7 \quad (4)$$

$$HS^{-}(aq) + M^{2+}(aq) \leftrightarrow MS(s) + H^{+}(aq) \quad (5)$$

where CH_2O represents a simple organic molecule (such as acetate) that serves as an electron donor for SRB, K is equilibrium constant for ionization of H_2S, and M^{2+} denotes a divalent metal such as Fe, Cd, Ni, Cu, Co, or Zn. The rate of metal sulfide formation (Equation 5) is ca. 10^2-10^6 times higher than that of biological sulfate reduction; therefore, the latter is rate-limiting step in metal removal (Rickard, 2005).

Design parameters that are currently considered for passive bioremediation systems are the size of the system, retention time, flow hydraulics and the choice and amount of organic substrate (Wieder, 1989; Hellier et al., 1994). Wildeman et al. (1993) lists following criteria to be considered when designing the size of the wetland:

- Area/flux – Darcy's Law relates the flow (Q) to the surface area (A), hydraulic gradient (i) and the permeability of the substrate (K): $Q = K \cdot i \cdot A$ [$m^3\ s^{-1}$]
- Acidity mass loading – the acidity loading should not exceed the reaction rate of SRB (~100-300 nmol $SO_4^{2-}.cm^{-3}.day^{-1}$)
- Precipitated metals volumetric loading – availability of void space in the substrate for formed metal precipitates
- Biomass accumulation – accounting for accumulation of dead vegetation
- SRB stoichiometry – amount of carbon required for metal precipitation
- Evaporation losses

The performances of full-scale constructed wetlands reported in the literature are highly variable. Average iron removal efficiency in the 3000 m^2 Simco wetland in Coshocton Ohio treating coal mining effluent was 62 % (Stark et al., 1990). Total manganese concentration, on the other hand, increased by 5 %, and pH remained constant at about 6.5 (Table 1.4). Seasonal variability was observed with highest iron removal in summer and lowest in spring. It was consistent with the lowest hydraulic loading in the summer, as opposed to high hydraulic loading in spring due to spring runoff. The wetland improved in treatment efficiency over time, which was attributed to the decreased toxicity of MME (lower metal concentrations and acidity) and increased density of vegetative cover.

In Western Pennsylvania, a constructed sulfate-reducing bioreactor was built to treat MME from an underground coal mine abandoned since the 1950's. The substrate consisted of 50 %

wood chips, 30 % limestone, 10 % cow manure, and 10 % hay. Since its construction in 2002, it has been successful in increasing pH from 3 to 6.6 and decreasing aluminum, copper, iron, nickel, and zinc to levels that meet water quality standards (Gusek, 2005). Similarly to the Simco wetland, manganese concentration remained unchanged.

Two anaerobic bioreactors followed by three plant cells and a pond were built to treat leachate from a landfill at the lead zinc smelter in Trail, B.C. (Duncan et al., 2004). To prevent short-circuiting in the anaerobic bioreactors (vertical subsurface flow wetlands), the cells are constructed in three layers separated by waterproof liner, which forces the flow in a serpentine pattern up through the cell. The composition of organic substrate is 60 % kraft pulp mill biosolids, 35 % sand and 5 % cow manure. The system treats 12-15,000 L/day of leachate containing an average of 355 mg/L of zinc, 69.5 mg/L of arsenic and 5.1 mg/L of cadmium (Table 1.2). The removal efficiencies in the anaerobic bioreactor of arsenic, cadmium and zinc were 92.8 %, 98 % and 84.4 %, respectively (Duncan et al., 2004).

In many cases, the treatment efficiency of constructed wetlands declines as the system ages. For example, the iron and sulfate removal in a permeable reactive barrier made of municipal compost, leaf compost, and wood chips built to remediate effluent from Nickel Rim mine in Ontario declined after three years of operation by approximately 50 % and 30 %, respectively (Blowes et al., 2000). The decline in efficiency has been attributed to the depletion of the reactive portion of organic material as well as to the channeling of groundwater flow. Improvement in flow distribution and increased thickness of the reactive barrier were suggested to recover the initial performance.

Webb (1998) compared the activity of SRB isolated from two natural and one constructed wetland in Cornwall, U.K. The natural wetlands were covered with vegetation, such as *Juncus*, *Phragmites*, willow, grass, and reed. The substrate in the constructed wetland treating AMD consisted of a mixture of straw and manure. Results from laboratory microcosms showed that the isolates from the natural wetlands produced more sulfide and removed more zinc than the isolate from the wetland. On the other hand, iron was removed at a similar rate in isolates from all three wetlands and manganese remained in the solution regardless of the isolate. This study showed that the differences between various organic substrates and ecology of the wetlands were reflected in SRB species composition and treatment efficiencies. However, the factors that contributed to the changes in sulfide production and metal removal were not identified.

Generally, factors affecting the performance of passive treatment systems have not been well characterized. Specifically, the activity of SRB in natural environments is mostly limited by

the availability of suitable electron donors and competition with other bacteria (Johnson and Hallberg, 2003; Neculita et al., 2007). The concentration of sulfate before and after passive MME treatment often remains constant or decreases only slightly (Table 1.4), suggesting that sulfate reduction by SRB is not a dominant process. Assuming that SRB are limited by the rate of decomposition of complex carbon, there is a lack of understanding of the microbial species involved in various steps of organic substrate degradation and the dynamics of carbon mineralization. Therefore, it is difficult to assess and control the performance and sustainability of the passive treatment wetlands.

Mine pits lakes have also been considered for use as treatment systems of mine metal effluents. The characteristics of pit lakes differ greatly from natural lakes. While natural lake's relative depth (defined as maximum depth divided by width) is typically around 2 %, mine pit lakes reach values as high as 40 % (Castro et al., 2000). This often results in vertical stratification and enables the use of anaerobic microbial processes to remediate MME. The Island Copper Mine pit lake near Port Hardy, British Columbia, was flooded with seawater and capped with freshwater. The lake was fertilized with nitrate and phosphate to increase the primary productivity at the surface and thus provide sufficient organic carbon and nutrients for the SRB in the anoxic water. Even after 4 years of continuous nutrient additions the anoxia could not be established in the intermediate layer (Fisher and Lawrence, 2006). However, sufficient metal removal occurred in the system. Further studies on the factors affecting the remediation efficiency by both microbial and physico-chemical processes need to be carried out to confidently predict the system's behavior.

1.2.3 Choice of a Suitable Organic Substrate

The majority of known SRB strains utilize simple carbon molecules for their growth (see Chapter 1.2.5). Amendment of these organic compounds directly to MME passive treatment systems is economically unfavorable. Since MMEs usually contain only low concentrations of dissolved organic carbon (<10 $mg.L^{-1}$) (Kolmert and Johnson, 2001), an organic substrate must be added to provide enough nutrients for microbial growth. Several laboratory-scale studies assessed the suitability of waste and agricultural by-products for SRB growth. Generally, a mixture of materials, containing both rapidly degradable and more recalcitrant materials, performed better than a single material (Waybrant et al., 1998; Cocos et al., 2002; Zagury et al.,

2006). Multiple factorial design with different combinations of wood chips, leaf compost, poultry manure, oxidized mine tailings, and silica sand conducted over 40 days showed that increased content of poultry manure correlated with an increase in sulfate reduction rate (SRR) (Cocos et al., 2002). On the other hand, no sulfate reduction conditions were developed in a reactor containing only sheep manure after a 3-week batch reactor study (Waybrant et al., 1998). The outcomes from different experiments are often contradictory, and largely depend on the length of the study, temperature, pH, and properties of particular materials used. In practice, manure is added to passive treatment systems only in small amounts (less than 20 %) as its main role is to provide inoculum. Since cow manure consists of very little degradable material, it is seldom used alone in passive treatment systems, although some sources of manure contain bedding material consisting of wood chips and sawdust, which are materials that can provide carbon sources for SRB. Organics commonly used in passive treatment systems include wood chips, sawdust, hay, alfalfa, compost, biosolids from pulp and paper mills or from municipal wastewater treatment, leaf litter, natural vegetation, plus food processing and agricultural wastes. Usually local availability dictates what organics are used and little is known about how the nature of the organic material affects microbial communities and specifically sulfate reduction.

There have been attempts in the literature to correlate the reactivity of various materials used in passive sulfate reduction systems with their chemical characteristics, such as total organic carbon (TOC), dissolved organic carbon (DOC), carbon to nitrogen ratio (C/N), portion of easily degradable material (EDM), and amounts of lignin and cellulose (Prasad et al., 1999; Gibert et al., 2004; Zagury et al., 2006). However, the consensus is that these properties alone are not sufficient to determine how efficient the materials are in promoting sulfate reduction. Moreover, the variability of chemical parameters of the same materials often observed, suggests that they cannot be used solely to characterize the properties and suitability of a specific material.

1.2.4 Geochemistry and Microbial Interactions in Sulfate-Rich Environments

Besides the availability of carbon sources, there are geochemical factors that influence biological sulfate reduction. Anaerobic processes in aquatic environments are usually restricted to the sediment layer, with the oxic-anoxic interface around the sediment surface (Brune et al., 2000). However, the shift of this interface upwards into the water column can be observed in some water bodies due to high primary production or stagnant conditions (Konovalov et al., 2005;

Pawlowicz et al., 2006). Below this interface, vertical gradients are set up, in which various electron acceptors are consumed, usually in the order of decreasing redox potentials. An example of vertical gradient distributions in sulfate-rich environment is outlined in Figure 1.1.

Due to its high concentration, sulfate is the dominant electron acceptor in anaerobic zones of sulfate-rich systems. However, nitrate is a more thermodynamically favorable electron acceptor than sulfate and is taken up by many bacterial species (e.g., denitrifiers). The zones of manganese (Mn^{2+}) and iron (Fe^{2+}) reduction typically occur below the nitrate reduction zone. In many cases where sulfate reduction occurs, the dissolved iron is depleted due to reaction with sulfide forming precipitates. While sulfate reduction is predominant process in most marine sediments, CO_2 reduction to methane dominates in the low-sulfate freshwater.

1.2.5 Sulfur Cycle

The major processes within the sulfur cycle in aquatic environments, together with microbial species involved in each step are illustrated in Figure 1.2. Plants and most microorganisms perform assimilatory sulfate reduction in order to incorporate sulfur into amino acids and other organic molecules containing sulfur. However, SRB perform dissimilatory sulfate reduction under anaerobic conditions where sulfate is used as a terminal electron acceptor. Reduced sulfur is oxidized either under strictly aerobic conditions by chemotrophic bacteria, or under anaerobic conditions by phototrophic bacteria. Chemotrophs, namely colorless sulfide bacteria (*Beggiatoa*, *Thiobacillus*, *Thioploca, Thiothrix*, etc.) mostly oxidize sulfide to elemental sulfur, which they then deposit inside the cell in sulfur granules. However, some strains of *Thiobacillus* can oxidize elemental sulfur into sulfate. They require both oxygen and sulfide, therefore are mainly found in the oxic-anoxic interface. Phototrophs, such as green and purple sulfur bacteria use light as their energy source and oxidize sulfide under anaerobic conditions.

Sulfate-reducing and sulfide-oxidizing bacteria can often be found existing together near the oxic-anoxic interface. An example is large *Thioploca* filaments covered by filamentous SRB *Desulfonema* (Jorgensen and Gallardo, 1999). *Thioploca*, migrating in its own sheaths, takes up nitrate from the surface layers and sulfide from the bottom layers. This way, it can oxidize sulfide with nitrate as an electron acceptor. *Desulfonema* can benefit from availability of oxidized sulfur intermediates. Mixed cultures of *Desulfovibrio* and *Thiobacillus* were also able to coexist under limited O_2 concentrations (van den Ende et al., 1997). Because of the insufficient

oxygen supply, *Thiobacillus* does not oxidize sulfide completely to sulfate but to sulfur intermediates, which are more energetically favorable to *Desulfovibrio* than sulfate. *Desulfuromonas* was also found in co-culture with green sulfur bacteria *Chlorobium* in marine environments (Warthmann et al., 1992).

1.2.6 Background on the Sulfate-Reducing Bacteria

There are three basic groups of SRB based on their morphological characteristics: gram-negative Bacteria, gram-positive Bacteria and Archaea. General properties of most common strains are given in Table 1.5. SRB are often divided, regarding the organic matter utilized, into lactate oxidizers (lactate is incompletely oxidized to acetate), and acetate oxidizers. Lactate oxidizers, such as *Desulfovibrio* and *Desulfotomaculum*, do not possess enzymes necessary to oxidize acetate. *Desulfovibrio* is the most frequently studied genus of lactate oxidizers, as it is relatively easy to isolate in pure cultures (Postgate, 1984). They are curved or sigmoid in shape, polarly flagellated mesophilic SRB widespread in aquatic habitats. Acetate oxidizers, such as *Desulfobacter, Desulfococcus, Desulfosarcina* and *Desulfobacterium*, oxidize their substrate completely to carbon dioxide. Overall, members of the second group grow more slowly than lactate oxidizers, but are nutritionally more versatile. Besides acetate and lactate, propionate, pyruvate, fumarate, butyrate, and ethanol are suitable electron donors for SRB. Many SRB are able to use H_2 as their electron donor. Also, some complex carbon compounds, such as benzoate, toluene, etc., have been successfully used for SRB growth. Although they typically require organic carbon source, growth on H_2/CO_2 has been successfully applied in industrial bioreactors (van Houten et al., 1994).

1.2.7 Rates of Sulfate Reduction and SRB Distribution

There are many factors affecting SRB activity. Availability of sulfate and suitable electron donor, temperature and pH are among the most significant ones (Ravenschlag et al., 2000; Karnachuk et al., 2005). Table 1.6 summarizes sulfate reduction rates and related parameters in various environments.

The concentration of sulfate in freshwater ranges from ~10 to >500 μM, which is much lower than in seawater (ca. 28 mM) (Holmer and Storkholm, 2001). Lakes that are enriched with

sulfate from MME, disposal of wastewater or saltwater intrusions, may attain a very high concentration (>1000 μM), similar to that found in brackish environments (Wellsbury et al., 1996). Because of its low concentration, sulfate usually penetrates only to less than 10 cm into freshwater sediments, in contrast to marine sediments, where sulfate reduction can be found to depths of several meters.

Sulfate in the sediment of Lake Anna receiving acidic MME decreased from a surface concentration of 1270 μM to 6 μM 6 cm down (Herlihy and Mills, 1985). Rapid decrease in sulfate concentration from 4000 μM to 1000 μM within the first 20 cm of the sediment layer was also observed in an acidic lake in Argentina (Koschorreck et al., 2003). The relationship between sulfate concentration and SRR often exhibits exponential Monod-type kinetics with half-saturation constant (K_M) between 5-30 μmol/L (Fig. 1.3).

Typically, SRRs reach high values during the spring or summer (Table 1.6). Optimal growth temperature for SRB cultures is 35-40 °C, which is much higher than typical sediment temperatures. However, surprisingly Fortin et al. (2000) found the largest SRB population (10^{10} colony-forming units per gram of sediment) in the sediments of a constructed wetland in December, when the temperature was around 1°C. Another study on sulfate reduction in sediments of Arctic Ocean suggested that low temperatures might not inhibit bacterial growth, but rather decrease the affinity of bacteria to substrates (Sagemann et al., 1998). Seasonal variations of SRR in stratified lakes tend to follow a pattern, with a relatively high rate in the spring and summer, a decline at the end of the stratification period (late summer) and a relatively high rate in the winter. In spring and summer, SRRs are stimulated by increased temperature and sedimentation of organic matter from the spring bloom. In winter, the anoxic layer mixes with overlying oxic water leading to an increased oxidation of reduced sulfur, thus increasing sulfate concentration. This offsets the decrease in SRR due to lower temperatures (Li et al., 1999a).

The zone with the highest SRR is found most often in the top 10 cm of the sediment (Table 1.6). There is a strong correlation between the highest SRR and the presence of SRB in all types of sediment. SRB numbers in freshwater sediments, such as in Lake Stechlin, are several orders of magnitude lower than in marine or ARD affected sediments (Sass et al., 1997). The lack of an electron acceptor (sulfate) is usually the reason for low SRB population in freshwater, however, the culture-based most probable number technique (MPN) used in the Lake Stechlin study could have underestimated the SRB population.

Another factor influencing SRB activity and SRR is pH. Growth of SRB is severely inhibited at a pH below 4.5 or above 9.5, and negligible sulfate reduction is already observed outside the range from 6.5 to 8.5 (Postgate, 1984; Wu et al., 1991). Although SRB activity and SRRs of up to 26 nmol cm^{-3} d^{-1} were observed in acidic environments where the pH was below 3, cultures isolated from such habitats were inhibited below the pH of 6 (Widdel, 1988; Koschorreck et al., 2003). This indicates that SRB can exist in acidic environments, but are most likely present in the microniches with higher pH.

Even though SRB are considered to be strict anaerobes, they can survive long exposures to oxygen and can again become active under anaerobic conditions (Cypionka et al., 1985). Marschall et al. (1993) even reported aerobic respiration by SRB, although they were not able to double more than once and their motility and viability was decreasing after 50 h. Minz et al. (1999) reported that SRB, particularly *Desulfonema* and *Desulfococcus* occupied the oxic part of a microbial mat with 160 % air saturation during the day.

SRB require a reduced environment, with redox potential from -150 mV to -200 mV for maximum activity. Among the growth inhibitors of SRB are heavy metals like cadmium, copper, zinc, lead and nickel, although their toxicity limits greatly depend on factors like pH, temperature and sulfide concentration and their values vary. Selenate ion is a competitive antagonist of sulfate reduction and thus is one of the growth inhibitors of SRB. The second one is molybdate ion, which depletes the bacteria's ATP pool.

Iron-reducing bacteria together with methanogens are the major competitors for SRB. Iron reduction is more thermodynamically favorable than sulfate reduction, therefore it could be assumed that iron reducers would outcompete SRB. Edenborn et al. (2001) and Meier et al. (2004) suggested that in the presence of Fe^{3+}, sulfate reduction and methane production are inhibited, because iron reducers have greater affinity to H_2 and acetate. However, this is not always the case since, despite high concentrations of Fe^{3+} in the acidic Lake Brandenburg, SRB outcompeted iron reducers (Blodau et al., 1998).

1.2.8 Mathematical Modeling of SRR

Several mathematical models have been developed to determine the rate of sulfate reduction in natural systems. Benner et al. (2000) and Amos et al. (2004) used a simple model to determine the rate of sulfate reduction in a permeable reactive barrier, in which a constant SRR was

multiplied by a Monod-type hyperbolic term accounting for sulfate limitation. Bourdeau and Westrich (1984) considered aerobic degradation of seawater plankton as a precursor to sulfate reduction, suggesting first-order kinetics with respect to carbon as a model for the rates of plankton degradation and sulfate reduction. Drury (2000) assumed the SRR in solid-substrate bioreactors to be proportional to the rate of decomposition of biodegradable solid substrate initially added to the reactor. He used first-order kinetics with declining rate coefficient that reflects decreasing availability of reactive carbon molecules over time. However, none of these models account for SRB growth and decay, dynamics of bacterial populations (fermentative bacteria, SRB), or the availability of dissolved organic substrates formed by fermentation. Hemsi et al. (2005) developed a model including polysaccharide decomposition using Contois kinetics (Eqn. 6), sulfate reduction using Monod kinetics (Eqn. 7), precipitation of ferrous sulfide (Eqn. 8) and partial volatization of hydrogen sulfide (Eqn. 9).

$$\frac{d[CE_i]}{dt} = -k_{c,i}[X_{d,i}]\frac{\left([CE_i]/[X_{d,i}]\right)}{K_{c,i} + \left([CE_i]/[X_{d,i}]\right)} \qquad \frac{d[LA]}{dt} = Y_{LA/CE}\sum_{i=1}^{n}\left(\frac{d[CE_i]}{dt}\right) \tag{6}$$

$$\frac{d[X_{SRB}]}{dt} = \mu_{SRB}[X_{SRB}]\left(\frac{[LA]}{K_V + [LA]}\right)\left(\frac{[SO]}{K_{SO} + [SO]}\right) - d[X_{SRB}] \tag{7}$$

$$\frac{d[H_2S]}{dt} = -k_{vol}[H_2S] \tag{8}$$

$$r_{p,k}^{net} = k_{p,k}[M_k][H_2S] - k_{d,k}[H^+]^2 \tag{9}$$

where $[CE_i]$ = decomposable polysaccharide concentration (i=1…n), n = total number of polysaccharide materials in the system, $k_{c,i}$ = Contois rate coefficient for decomposition of polysaccharide 'i', $[X_{d,i}]$ = biomass concentration of decomposer bacteria (i=1…n), $K_{c,i}$ = Contois half saturation coefficient for decomposition of polysaccharide 'i', [LA] = concentration of lactic acid, $Y_{LA/CE}$ = mass yield coefficient for lactic acid, $[X_{SRB}]$ = biomass concentration of SRB, μ_{SRB} = Monod maximum specific growth rate of SRB, K_V = Monod half-saturation coefficient for lactic acid in sulfate reduction, [SO] = concentration of sulfate, K_{SO} = Monod half-saturation coefficient for sulfate in sulfate reduction, $r_{p,k}^{net}$ = net rate of precipitation of heavy metal sulfides (MS_k), $k_{p,k}$ = precipitation rate coefficient, $k_{d,k}$ = dissolution rate coefficient,

$[M_k]$ = concentration of any divalent heavy metal, $[H_2S]$ = concentration of hydrogen sulfide in solution, $[H^+]$ = concentration of hydrogen ions, and k_{vol} = rate coefficient of H_2S volatization.

The simulations of sulfate, ferrous ion and hydrogen sulfide versus time correlated well with experimental data from four batch-test experiments reported by Waybrant et al.(1998). The model also contained simulations of decomposable organic materials, dissolved organic substrates, and different populations of bacteria, but could not be verified since no such experimental results were reported. In addition, this model only considers that SRB consume lactate, which is not necessarily true in a complex carbon environment.

1.2.9 Organic Matter Degradation in Passive Treatment Systems

The lack of a suitable carbon compound as an electron donor for SRB is assumed to be the limiting factor of their activity in many cases (Waybrant et al., 1998; Castro et al., 1999; Gibert et al., 2004). SRB are dependent on other bacterial species that conduct extracellular hydrolysis of high molecular weight polymers (proteins, lipids, polysaccharides and nucleic acids) and ferment their respective monomers to CO_2, H_2, acetate and other organic acids and alcohols that can be utilized by SRB (Fig. 1.4). The activity of SRB is expected to be directly related to the presence and activity of these bacterial species, even though this relationship has not been extensively studied. Koschorreck et al. (2003) reported an abundance of short chain fatty acids and acetate (380 μmol C L^{-1}) in the sediments where sulfate reduction occurred. Edenborn et al. (2002) also suggested that SRB utilize mostly acetate or H_2 over lactate. On the other hand, the results from laboratory-scale tests with different substrates showed that lactate was superior to acetate, glucose or ethanol (Fauville et al., 2004; Logan et al., 2005; Martins et al., 2009).

Due to the complexity of these systems, it is difficult to identify individual reactions and their kinetics. As well, the processes that occur *in situ* do not correspond to simulated processes in the laboratory. Only a small fraction of all bacteria can be cultured, therefore the microbial community in a laboratory set-up would be fundamentally different from the original environment. One indirect approach is to use molecular tools to determine and quantify dominant microorganisms from the environment and correlate them with metabolic processes. A review of the most common molecular techniques used to assess microbial community structure is described in the next section.

1.2.10 Molecular Techniques Used in Microbial Ecology

Prior to the development of molecular tools to detect microorganisms in natural environments, isolation of pure cultures was required in order to identify the microbial species and their functions. These culturing techniques have identified many important and revealing biochemical processes (Madsen, 2005). However, since the number of cultivable cells from natural samples is assumed to be less than 1 % (Amann et al., 1995), other techniques were needed to identify ecologically significant organisms. Nowadays, a number of molecular-based methods have been developed that enable us to study the abundance and activity of microbial communities. Methods used for microbial identification and quantification of aquatic and sediment bacteria include quantitative polymerase chain reaction (qPCR), phylogenetic analysis using clone libraries, fluorescent in situ hybridization (FISH), and denaturing gradient gel electrophoresis (DGGE).

- Quantitative PCR (qPCR)

  QPCR is based on an enzymatic reaction with a fluorescent dye that uses a DNA polymerase enzyme to copy a target DNA sequence repeatedly during a series of 25-35 cycles. During each cycle, the amount of target DNA is doubled, resulting in an exponential increase of DNA, which makes it particularly useful for environmental samples where the initial amount of DNA is often very small. A fluorescent signal, which is proportional to the amount of PCR product, enables quantification using standards with known gene copy number. The reaction can be designed to target the conserved sequences that are common for e.g. all bacteria, such as some regions of SSU rRNA (small ribosomal subunit RNA), or specific for certain groups, such as dissimilatory sulfite reductase (*dsr*) found solely in SRB.

- Clone libraries

  This technique is used to estimate microbial diversity by PCR amplification, followed by cloning, sequencing and subsequent phylogenetic analysis. To assess the overall bacterial diversity, the small subunit of ribosomal DNA (SSU rRNA) is amplified and further cloned onto *E. coli* cells. A large number of individual inserts is sequenced and compared to a database with the sequences obtained from both cultured species and environmental samples. To date, around 1 million of good-quality full-length sequences are available in the public database (Genbank). Recent advances in data collection and analysis, such as pyrotag sequencing, enable detailed description of the microbial communities. In addition

to overall diversity, specific genes can also be sequenced to focus on a particular bacterial species or metabolic process.

- Denaturing gradient gel electrophoresis

  This method was first used in microbial ecology studies in 1993 (Muyzer et al., 1993). PCR-amplified SSU rRNA fragments, which are essentially the same size, can be separated into discrete bands during DGGE in a gel containing denaturing agents. This separation is based on the decreased electrophoretic mobility of partially denaturized DNA molecule in the gel. Each sequence denatures at different denaturant concentration on the gel and forms discrete bands. These bands can be excised from the gel and the sequences obtained can be compared with known SSU rRNA sequences in order to identify the organisms present. It is particularly effective when there is a need to study the same bacterial community over time, as the band profile can be compared without the need for sequencing.

- Fluorescent in situ hybridization

  This is a microscopic technique that targets specific groups of microorganisms using a fluorescent probe. The probe binds to DNA and the fluorescent signal can be then visualized. Therefore, it cannot be used to estimate overall diversity, but rather determine if particular group is present. However, this is a powerful technique for examining microbes in their in situ environment, without the biases of DNA extraction and PCR.

1.3 RESEARCH OBJECTIVES

The overall objective of this thesis is to identify the microbial communities and fundamental metabolic processes existing in natural and constructed high sulfate environments with a particular interest in the presence and activity of sulfate-reducing bacteria. The understanding of these processes will lead to improving the design and monitoring of MME passive treatment systems. The research can be divided into following areas:

- To demonstrate how molecular techniques, particularly qPCR and phylogenetic analysis can be adapted for use in MME passive treatment systems.

- To characterize dominant bacterial species and metabolic processes and determine their spatial and seasonal variability in a natural high-sulfate stratified environment, and relate the findings to the potential for sulfate reduction in analogous aqueous passive treatment systems.

- To determine the sulfate reduction potential in sediments from a high sulfate environment upon amendment with simple and complex carbon substrates in laboratory-scale batch reactors.

- To evaluate whether complex carbon substrates immersed in an anaerobic bioreactor treating MME differ in the numbers of SRB that they support and to elucidate correlations between properties of these substrates, sulfate reducing potential, and bacterial species diversity and distribution.

1.4 RESEARCH HYPOTHESES

Following central hypotheses will be tested in the course of this dissertation:

Chapters 2 and 3:

- Molecular tools can be used to provide information on dominant biogeochemical regimes in anaerobic environments of MME passive treatment systems

- Stratified aquatic environments high in sulfate, such as anoxic basins and mine pit lakes exhibit high SRB population and activity in the anoxic water column and sediments

Chapter 4:

- The choice of organic substrate for MME passive treatment systems affects the phylogenetic structure of the microbial community

- Phylogenetic diversity in organic substrates can be correlated with organic substrate chemical properties and related to treatment efficiency

- Higher amount and diversity of SRB in an organic substrate sample correlates with improved remediation performance of passive treatment systems

1.5 THESIS OUTLINE

Passive treatment of metal mining effluents and other environmentally hazardous leachates containing heavy metals and sulfate using biological sulfate reduction has shown to be promising technology. Currently, the lack of understanding of the microbial communities and their biochemical processes prevents further improvements of design and performance of these systems. A brief outline of chapters 2 to 4, where these issues are addressed, is presented.

In Chapter 2, a detailed survey of chemical regimes associated with microbial processes is performed in a permanently stratified fjord Nitinat Lake in B.C. Although this is a natural system not receiving any MME, this environment was chosen due to its ease of access and sampling compared to the stratified mine pit lakes. Furthermore, we consider the stability of the stratification and high sulfate levels analogous to desired conditions in engineered mine pit lakes. The assumption for mine pit lakes design that high SRB activity develops in an anaerobic sulfate-rich water column is tested. Molecular techniques are modified and tested for suitability to provide information on sulfate reduction processes. Phylogenetic analysis of SSU rRNA gene sequences from the transition and anoxic zones of the water column in Nitinat Lake is investigated. In addition, novel primers for qPCR targeting dominant bacteria are developed. The changes between spring and summer regimes of two sampling stations are also discussed.

Following the results from Chapter 2, phylogenetic analysis of SSU rRNA and *dsr* gene sequences in Nitinat Lake's sediments is presented in Chapter 3. SRB quantification and activity is also assessed. Further, a laboratory-scale batch reactor experiment is set up with two simple and a complex carbon source to determine the rate of carbon utilization and its effect on sulfate reduction. The implications of these results on treatment efficiency of stratified mine pit lakes are discussed.

In Chapter 4, an *in situ* experiment with five suitable carbon waste materials is described in a treatment system near Teck zinc and lead smelter in Trail, B.C. After 5 months of *in situ* incubation, the materials were analyzed for major bacterial species, SRB distribution, and carbon degradation. A tentative carbon degradation pathway is proposed. The hypothesis that different

organic materials develop different bacterial communities, and thus affect SRB activity, is evaluated.

Chapter 5 discusses the research outcomes, and their relevance for the field of study. Potential applications, suggestions for future research directions, and recommendations for the MME treatment systems stemming from this work are also proposed.

1.6 TABLES AND FIGURES

Table 1.1: Guidelines for metal concentration released as mining effluents

Substance	Maximum concentration*
Arsenic	0.5 mg/L
Copper	0.3 mg/L
Cyanide	1 mg/L
Lead	0.2 mg/L
Nickel	0.5 mg/L
Zinc	0.5 mg/L

*maximum monthly mean concentration

Table 1.2: Characteristics of MME from B.C. mines

Site	Source of ARD	pH	Metals (mg/L)	Reference
Duthie	base metal tailings	1.9	Arsenic 5.06 Copper 138 Iron 13,300 Zinc 1,360	(Bechard, 1994)
Equity Silver	waste rock	2.4	Copper 791 Zinc 350	(Bechard, 1994)
Britannia	leachate from mine shafts	3.5	Zinc 25 Copper 30	(O'Hara, 2004)
Teck	leachate from smelter waste material deposits	n/a	Arsenic 69.5 Cadmium 5.1 Zinc 355 Lead 0.056	(Duncan et al., 2004)

Table 1.3: MME treatment systems

Type of process	Technology	Target analytes	Effectiveness	Cost	Limitations
Active - Chemical	Addition of neutralizing agent	acidity, divalent metals, sulfate (up to 2 g L^{-1})	most proven to treat MME, best for highly acidic MME, depends on types of metals	requires engineered systems	frequent monitoring, waste sludge storage and treatment
Passive - Chemical	Limestone channels	acidity, metals	variable depending on aluminum and iron amounts	low cost and low maintenance	armoring, low limestone solubility, management of sludge
Passive - Chemical	Anoxic limestone drains	acidity, metals	effective for coal drainage, longer residence times increases efficiency	low cost and low maintenance	maintaining of anoxic conditions, armoring
Active - Biological	Sulfidogenic bioreactors	metals, sulfate	highly efficient when SRB active, ideal for pH > 5 and moderate metal loading	initial construction costs, maintenance of bioreactor, substrate and SRB culture	dependent on carbon availability, risk of clogging by metal precipitates
Passive - Biological	Anaerobic wetlands	metals, sulfate, small amounts of acidity	sensitive to environmental factors, variable performance overtime, good for low flows and low metal loading	low cost and low maintenance	difficult to control and predict, insufficient understanding of remediation processes inside the system
Passive - Biological	Permeable reactive barriers	metals, sulfate, small amounts of acidity	decreased performance overtime, pilot-scale studies required prior implementation	cost of reactive material	periodic replenishment of reactive material required, uncertain lifetime, low SRR
Passive - Biological	Aerobic wetlands	Fe, Mn, As	neutral pH required, effective for treatment of coal mine drainage	low cost and low maintenance	large area needed, periodic removal of precipitates and wetland reestablishment
Passive - Biological	Mine pit lakes	Heavy metals, sulfate	increased efficiency after carbon addition, long-term performance unknown	High cost of carbon and nutrients additions	large areas, dependent on permanent stratification

Table 1.4: Operational parameters of full-scale passive treatment systems

Site	Location	Organic substrate	Area (m2)	Construction costs (USD)	Flow rate (L/min)	Residence time (d)	Fe (mg/L) in/out	Mn (mg/L) in/out	Cu (mg/L) in/out	Ni (mg/L) in/out	Zn (mg/L) in/out	As (mg/L) in/out	Sulfate (mg/L) in/out	pH in/out
Simco wetland	Coshocton, Ohio	spent mushroom compost	3000	$12,510.60	607	2	69.5/13.5	1.34/1.45					863/824	6.5/6.5
Yellow Creek 2B	Indiana Co., PA	wood chips, limestone, cow manure, hay	1300	$158,000.00	38	18	45/0.5	2.6/2.3	0.1/0.009	0.32/0.002	0.86/0.06		n/a	3/6.6
Nature Works bioreactor 1	Trail, BC	pulp mill biosolids, sand, manure	278	n/a	9	40					395.9/226	39.4/9.2	910/910	5.13/6.76
Wheal Jane wetland	Cornwall, UK	sawdust, manure	765.6	$1,700,000.00 (3 systems)	36	14.8	3.8/17.3				36.4/16.3		245/205	3/6
Nickel Rim PRB	Sudbury, ON	municipal compost, leaf mulch, wood chips, gravel	n/a (PRB was 4 m thick)	$30,000.00	n/a	90	250-1350/80		3/<0.01	0.12-30/<0.1	1/<0.015		2500-5200/840	2.8-5.9/6.7

Table 1.5: Classification of sulfate-reducing bacteria

Group	Strain	Form	Motility	Spores	Major e^- donors
Gram-negative eubacteria	***Desulfovibrio***				
	africanus	vibrio	+	-	lactate, pyruvate, malate
	desulfuricans	vibrio	+	-	lactate, pyruvate, ethanol
	furfuralis	vibrio	+	-	furfural, lactate, ethanol
	gigas	spirilloid	+	-	lactate, pyruvate
	halophilus	vibrio	+	-	lactate, formate, ethanol
	longus	flexible rod	+	-	lactate
	salexigens	vibrio	+	-	lactate, pyruvate, malate
	simplex	vibrio	+	-	lactate, pyruvate, glucose
	sulfodismutants	vibrio	+	-	lactate, ethanol, propanol
	vulgaris	vibrio	+	-	lactate, pyruvate, ethanol
	Desulfobacter				
	curvatus	vibrio	+	-	acetate, ethanol
	hydrogenophilus	rod	-	-	acetate, H_2
	latus	large oval rod	-	-	acetate
	postgatei	ellipsoidal rod	-	-	acetate
	Desulfobacterium				
	anilini	oval	-	-	acetate, formate
	autotrophicum	oval	+	-	ethanol. Formate, H_2
	catecholicum	lemon	-	-	catechol, formate
	indolicum	oval rod	+	-	acetate, formate, indole
	macestii	rod	+	-	lactate, ethanol
	phenolicum	oval/curved rod	+	-	acetate, phenol
	vacuolatum	oval/sphere	-	-	lactate, formate, H_2
	Desulfobulbus				
	elongatus	rod	-	-	propionate, lactate, H_2
	propionicus	lemon	-	-	propionate, lactate, pyruvate
	Desulfococcus				
	biacutus	lemon	-	-	acetate, ethanol
	multivorans	sphere	-	-	lactate, ethanol, pyruvate
	Desulfomonas				
	pigra	rod	-	-	ethanol, pyruvate
	Desulfomonile				
	tiedjei	rod	-	-	formate, H_2
	Desulfonema				
	limicola	filament	+	-	acetate, benzoate, pyruvate
	magnum	filament	+	-	acetate, malate, benzoate
	Desulfosarcina				
	variabilis	irregular packs	-	-	acetate, H_2, ethanol
	Desulfomicrobium				
	aspheronum	rod	+	-	lactate, pyruvate, ethanol
	baculatus	short rod	+	-	lactate, formate
	Thermodesulfobacterium				
	commune	rod	-	-	lactate, pyruvate, H_2
Gram-positive eubacteria	***Desulfotomaculum***				
	acetoxidans	straight/curved rod	+	+	acetate, butyrate
	guttoideum	rod	+	+	lactate, H_2
	kuznetsovii	rod	+	+	acetate, lactate, ethanol
	nigrificans	rod	+	+	lactate, pyruvate
	orientis	straight/curved rod	+	+	lactate, pyruvate
	ruminis	rod	+	+	lactate, pyruvate
	sapomandens	rod	+	+	ethanol, higher fatty acids
Archaebacteria	***Archaeoglobus***				
	fulgidus	round	+	-	lactate, pyruvate

Table 1.6: Sulfate reduction in sediments

	Site	Highest SRR (nmol/cm^3/d)	Sediment depth of highest SRR	Sulfate concentration at highest SRR (mM)	Sampling time	Temperature (°C)	pH	Highest SRB concentration (cells/cm^3)	Sediment depth of highest SRB concentration	SRB quantification method	Reference
Freshwater	Lake Stechlin	3.4	15 cm	0.48	October	10	7.2-7.4	7.10^4	15 cm	MPN	(Sass et al., 1997)
	Little Rock Lake	75	9 cm	0.01	October	n/a	4.7-5.6	n/a	n/a	n/a	(Urban et al., 1994)
	Lake Kizaki	13	0-3 cm	0.032	July	6	n/a	n/a	0-3 cm	oligonucleotide probes	(Li et al., 1999a)
Seawater	River Colne	193.3	n/a	n/a	May	13	n/a	7.10^7	n/a	competitive PCR	(Kondo et al., 2004)
	Great Ouse	290	9-10 cm	14	spring	n/a	n/a	n/a	9-10 cm	oligonucleotide probes	(Trimmer et al., 1997)
	River Seine	158	4-6 cm	n/a	n/a	n/a	n/a	$2.34.10^8$	4-6 cm	competitive PCR	(Leloup et al., 2004)
	Svalbard	17	2-8 cm	n/a	July	0	n/a	$5.2.10^8$	2.25 cm	FISH	(Ravenschlag et al., 2000)
ARD	Mine Schlabendorf	40	4-5 cm	5	April	7	7.0	2.10^7	2-3 cm	MPN	(Meier et al., 2004)
	Lake Anna	200	0-1 cm	1.15	August	26	6.5	n/a	n/a	n/a	(Herlihy and Mills, 1985)
	Lake Caviahue	26	5-6 cm	3.8	February	4.5	3.0	n/a	n/a	n/a	(Koschorreck et al., 2003)
	Norilsk	30	10 cm	4.6	n/a	9.6	7.4	1.10^7	n/a	MPN	(Karnachuk et al., 2005)

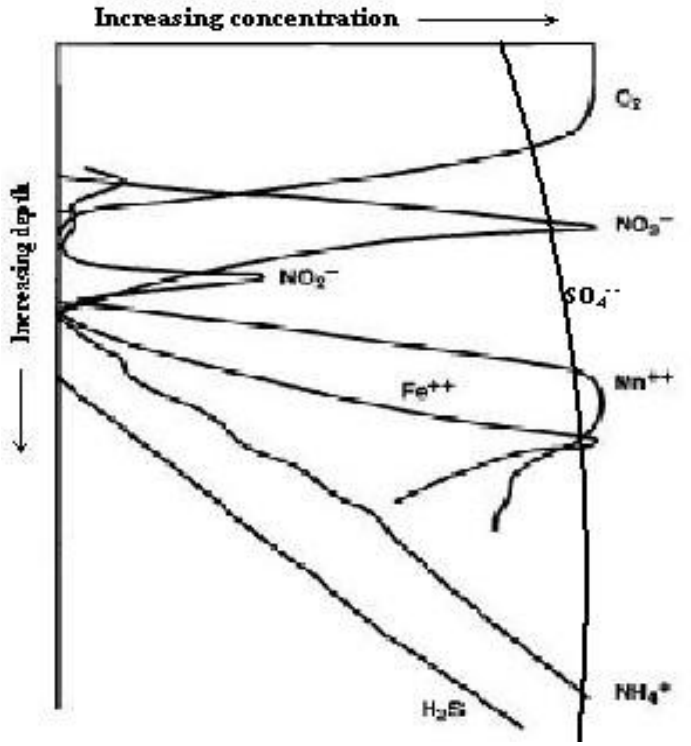

Figure 1.1: Typical vertical gradients in sulfate-rich environment (modified from Nealson(1997)).

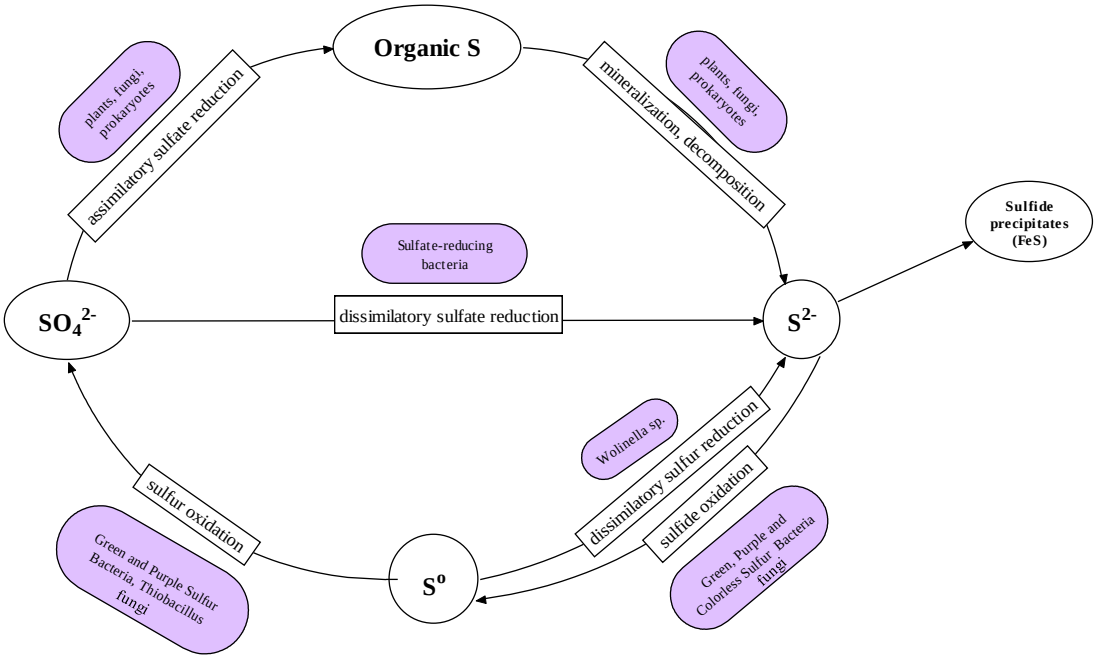

Figure 1.2: Biological sulfur cycle.

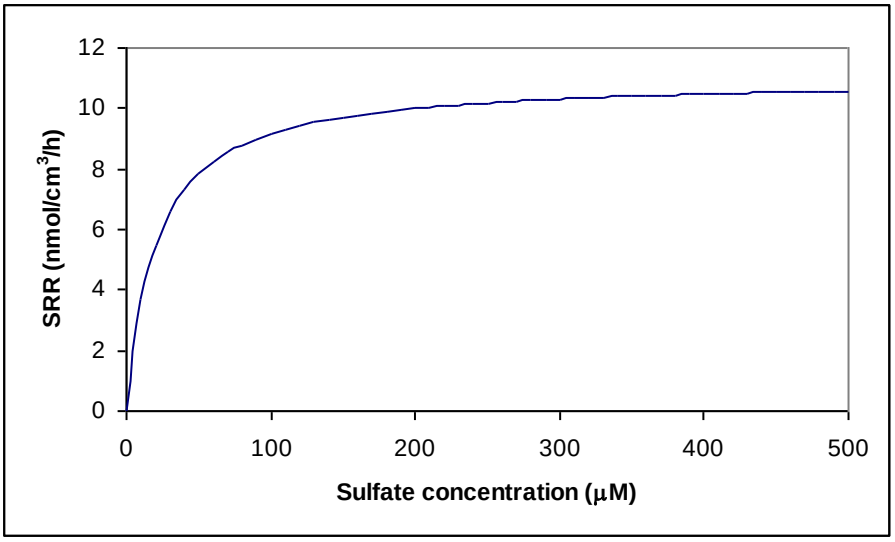

Figure 1.3: Monod-type kinetics of SRB from freshwater sediment slurry with Km = 20 µM and maximum SRR = 11 nmol cm^{-3} h^{-1} (Urban et al., 1994).

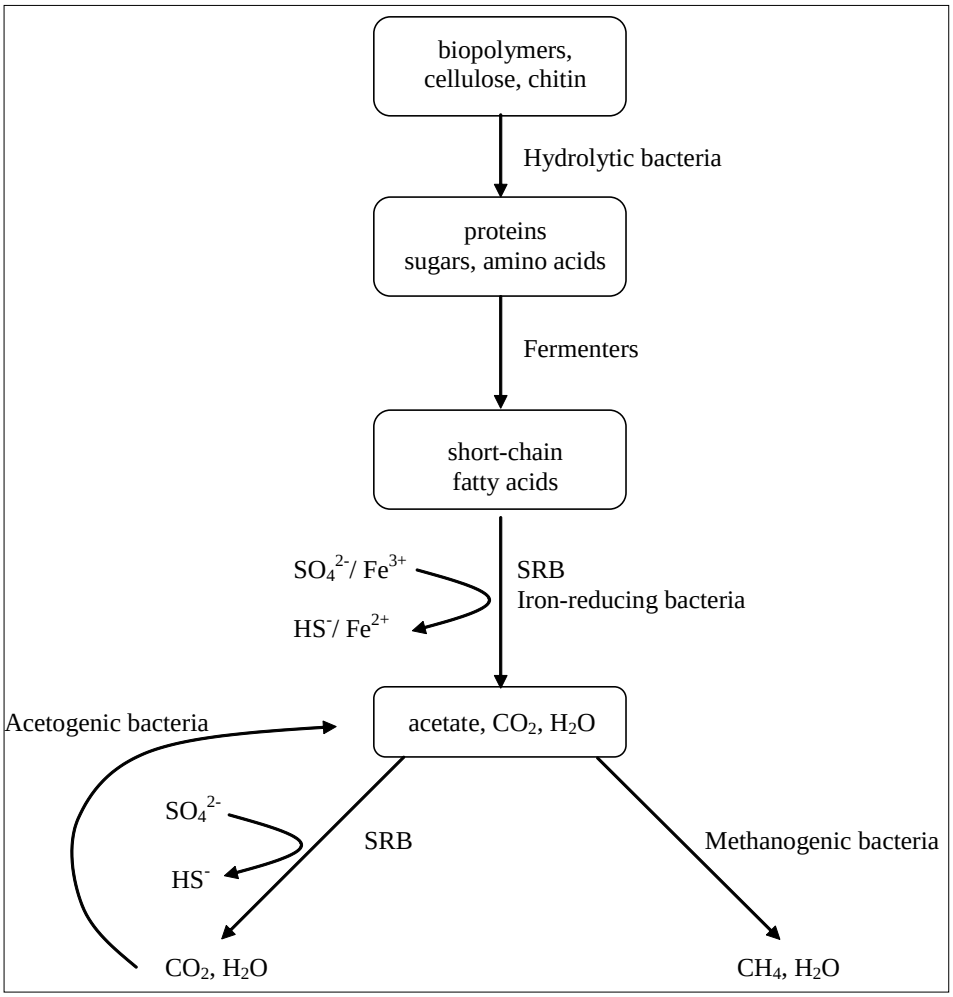

Figure 1.4: Pathways of anaerobic organic matter decomposition.

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CHAPTER 2

PHYLOGENETIC DIVERSITY OF TRANSITION AND ANOXIC ZONE BACTERIAL COMMUNITIES WITHIN A NEAR-SHORE ANOXIC BASIN: NITINAT LAKE

2.1 INTRODUCTION

Hypoxic and anaerobic zones that shift in extent and location seasonally characterize many coastal basins, fjords and estuaries. Since these zones are devoid of oxygen and sulfide is often present, they are uninhabitable by many economically and ecologically important aquatic species. This is the case in Nitinat Lake, an anoxic basin or fjord situated on the western coast of Vancouver Island, British Columbia, Canada. Restricted seawater circulation into the fjord combined with high surface productivity in summer are among the factors contributing to the formation of an anoxic region below depths of 10 to 40 m. This close proximity of hypoxia and sulfide to the surface waters threatens management of commercial fish stocks as evidenced by periodic die-offs in the fjord and at the Nitinat River Hatchery, the largest salmon hatchery in Canada, located at the terrestrial end of the fjord. Chemical and biological characteristics contributing to the distribution of sulfide in Nitinat Lake are therefore of great importance in predicting and preventing sulfide toxification events at the Hatchery.

In some cases, stratified lakes with minimal mixing are purposefully created. These include neutral pH mine pit lakes where the intent is to form anaerobic zones that do not mix with the oxygenated surface waters. These pits are flooded so as to prevent oxidation and acid production from waste rock or as bioreactors for metal removal from mine drainage (McCullough et al., 2008). Since many effectively remediated mine pit lakes are low in metals but have high sulfate concentrations, if the ore mined contained sulfide minerals, their eventual chemistry and biology can be expected to mimic natural seawater-fed lakes. Thus, there also is interest in studying Nitinat Lake as an analog for these types of mine pit lakes.

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Observational studies of Nitinat Lake in the 1960's and 1970's described many of the basin's key physical and chemical features, including its sharp pycnocline at 10 m, slow circulation associated with extended periods of anoxia, and high sulfide concentrations at depth (Pickard, 1963; Northcote et al., 1964; Richards, 1965; Ozretich, 1975). More recent analyses (Pawlowicz et al., 2006) using continuous *in situ* monitoring instrumentation have revealed a more dynamic ecosystem with horizontal transport and weak mixing events contributing to dissolved gas and nutrient cycling within the basin at different times of year. During winter and early spring, the chemical profile of the basin appears relatively uniform. During summer months, distinct chemical regimes form across the intermediate layer of the basin. At the seaward end, more dense seawater enters the fjord and sinks to deeper levels, creating an intermediate layer with both oxygen and sulfide present. A suboxic zone forms in the middle of the fjord, and the anoxic zone expands upwards, leaving only a shallow oxic layer at the river end (Pawlowicz et al., 2006). Seasonal measurements of particulate organic carbon, nitrogen, and DNA concentrations revealed at least two zones of biological activity in the vertical water column; a primary peak associated with phytoplankton activity near the surface, and a secondary peak close to the anoxic boundary layer. Due to the high concentrations of sulfate ions and sulfide in the anoxic zone, we expect many microbial groups in the transition and anoxic zone to be involved in sulfur cycling, similarly to other anoxic basins. For example, abundance of *ε-Proteobacteria*, and phototrophic and autotrophic *γ-Proteobacteria* in the transition zone, and *ε-*, and *δ-Proteobacteria* in the anoxic zone was found in Lake Cadagno, the Black Sea, Cariaco Basin and Mariager Fjord using various molecular techniques (Bosshard et al., 2000; Madrid et al., 2001; Vetriani et al., 2003). However, detailed surveys of microbial community structure or functional gene variation through the depth continuum at discrete seasonal time points or physical locations were not undertaken in Nitinat Lake. Therefore, this study characterizes the phylogenetic composition of ecologically important bacterial communities that correlate with specific chemical zones. We originally hypothesized that sulfate-reducing bacteria (SRB) would comprise a significant proportion of the anoxic and anaerobic communities due to the presence of high sulfate and sulfide concentrations in the fjord. These bacteria are of special interest due to their potential bioremediation capabilities in treatment of discontinued or abandoned open mine pits, where their presence is postulated to help prevent acid sulfate conditions with high amounts of dissolved metals. However, in contrast, the most abundant phylogenetic lineages, as identified in the phylogenetic classification, were associated with anaerobic sulfur oxidation. To

obtain a more quantitative characterization of the important microbial groups, SRB were quantified using functional dissimilatory sulfite reductase (*dsr*AB) gene amplification, thiotrophic symbionts of the SUP05 group, and a highly represented novel *Acrobacter* sp. related group, NITEP5, were quantified using quantitative polymerase chain reaction (qPCR).

2.2 MATERIALS AND METHODS

2.2.1 Lake Characteristics, Sample Collection, and Chemical Analyses

Nitinat Lake is about 23 km long with an average width of 1.2 km and a maximal depth of 200 m. The basin itself is divided into two domains of roughly equivalent size separated at Windy Point by a sill at 117 m in depth. A shallow channel called the Narrows (as small as 60 m wide and 2-5 m deep at low tide) separates the mouth of Nitinat Lake from the Pacific Ocean (Fig. 2.1).

Water samples were collected on 10 April 2006 and 25 August 2006 at one station located close to the ocean, S02 (N 48°42'181", W 124°47'847"), and another located closer to the Nitinat river inlet, S05 (N 48°46'306", W 124°43'834") (Fig. 2.1). Water was collected using standard 5-liter Niskin bottles in 2-m depth intervals. From each bottle, waters were subsampled to measure pH, temperature, and concentrations of dissolved oxygen, ammonia, nitrate, phosphate, sulfide, sulfate, particulate organic carbon (POC) and nitrogen (PN), and sulfate reduction rate (SRR). Salinity was measured with conductivity-temperature-depth (CTD) meter Seabird SBE-19s (Seabird Electronics, Bellevue, WA). pH and temperature were measured immediately after the bottle cast using a SympHony SB20 (VWR Scientific, West Chester, PA). Dissolved oxygen and ammonia were also measured immediately using Chemets^{TM} kits and procedures (CHEMetrics, Inc., Calverton, VA). All other samples were either filtered or stabilized and transported to Vancouver on ice. Aliquots (15 ml) for total dissolved sulfide analysis were stabilized immediately by addition of 0.5 mL of 20 % zinc acetate and later analyzed using the modified methylene blue method of Cline (Cline, 1969). Approximately 200 mL of each sample was filtered through a pre-combusted glass fiber filter to measure POC and PN using the method by Verardo et al. (1990). Remaining water (1-2 L) was filtered through 0.22 μm membrane filters (Millipore, Billerica, MA) and stored in ethanol-phosphate-buffered saline solution at –20°C until DNA extraction. The filtrate was used to measure nitrate and

phosphate using the respective LaMotte analysis kits and procedures (LaMotte Company, Chestertown, MD), and sulfate using the barium chloride precipitation method (Clesceri et al., 1998). All samples were processed in triplicate. The SRR was measured using the radiotracer $^{35}SO_4^{2-}$ method. Water samples were transferred without contact with oxygen to 40 mL glass vials and sealed. 2.5 μCi of $Na^{35}SO_4^{2-}$ was injected within 4 hours of sampling, and incubated at *in situ* temperature in the dark for 20 and 44 hours. The reaction was stopped by adding 1 mL of 20 % Zn-Ac and freezing. One-step cold distillation method with HCl was used to recover acid-soluble sulfides (Jorgensen, 1978). Radioactivity was determined using liquid scintillation counting.

2.2.2 DNA Extraction

Environmental DNA was extracted from archived membrane filters using the MoBio® DNA extraction kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions with the following modifications: alternative protocol for maximum yields was used; 160 μL of solution S1 was added to bead solution; the spin column was rinsed twice with 300 μL of solution S4; and finally DNA was eluted in 100 μL of solution S5. Total nucleic acid concentration and purity were measured spectrophotometrically with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm.

2.2.3 Quantitative PCR (qPCR)

Based on the multiple alignments of *dsrA* genes from both cultured SRB and environmental sequences from GenBank (app. 200 sequences), conserved regions of the gene were selected as primers for SRB quantification. The forward primer DSR1F' (5'-ACSCACTGGAAGCACGGC-3') was modified from previously published primer DSR1F (Wagner et al., 1998). A degenerate reverse primer DSR210R (5'-CGGTGGMRCCRTGCATRTT-3') was designed to match the highest number of SRB species and yield a target product of ca. 200 bp. Appendix A shows the details of the *dsr* primers. The primers were tested by amplification of several pure SRB strains: *Desulfobacterium autotrophicum* (DSM 3382), *Desulfobacter curvatus* (DSM 3379), *Desulfosarcina variabilis* (DSM 2060), and *Desulfovibrio desulfuricans* subsp. *desulfuricans*

(DSM 1926). *ε-Proteobacterial* primers were developed based on the regions of SSU rRNA sequences specific to *ε-Proteobacteria* obtained from the anoxic clone libraries. After ensuring that all primer parameters are suitable with Primer Express software (Applied Biosystems, Foster City, CA), a forward primer eps490F (5'-TGCCAGCAGCCGCG-3') and reverse primer eps540R (5'-CAGTGATTCCGAGTAACGCTTG-3') were chosen. Both primer pairs' specificity was analyzed by BLAST search for the NCBI database (Altschul et al., 1990). Total eubacterial primers used were 27F (Lane, 1991) and degenerate 519R (5'-GNTTTACCGCGGCKGCTG-3'). SUP05 group specific SSU rRNA gene copy number was determined using primers and methods described in Zaikova et al. (Zaikova et al., 2009).

QPCR of SRB and *ε-Proteobacteria* was performed on the ABI PRISM® 7000 (Applied Biosystems). The reaction mixture (12 μl) contained iTaq^{TM} SYBR$^{®}$ Green Supermix with ROX (Biorad, Hercules, CA), each primer at a final concentration 300 nM, nanopure water and template DNA. MicroAmp 96-well reaction plates (Applied Biosystems) were used. The amplification conditions were as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 94°C followed by 1 min at 60°C. Each sample was amplified in triplicates, and several samples were chosen and the reaction was repeated, yielding 6 replicates. The external standard curve for *dsr* quantification was constructed with a total extracted genomic DNA of *Desulfobacterium autotrophicum* (DSM 3382). The detection limit was 100 *dsr* copies per reaction, the efficiency ($E=10^{(-1/slope)}$; where 2 indicates an exact doubling per cycle) was between 1.87 and 1.95, and R^2=0.987-0.991. To calculate the number of SSU rRNA *ε-Proteobacterial* copies, a plasmid with the insert of SSU rRNA gene that represents an *ε-Proteobacterial* sequence obtained from the anoxic zone's clone library was used. Plasmid DNA was extracted from *E. coli* glycerol stock with Qiagen Plasmid mini kit (Qiagen, Mississauga, ON). The original copy number of the plasmid DNA was determined spectrophotometrically. To construct a standard curve, it was diluted from 30 to 3.10^6 copies. The efficiency was 2.02-2.09, and R^2=0.998-0.999. Concentrations of the samples were extrapolated from the standard curve using ABI Prism 7000 SDS Software (Version 1.0, Applied Biosystems).

QPCR of total bacteria and SUP05 group was performed on the Miniopticon system (Biorad, Hercules, CA). The reaction mixture (25 μl) contained iTaq^{TM} SYBR$^{®}$ Green Supermix (Biorad), each primer at a final concentration 300 nM, nanopure water and template DNA. The amplification conditions were as follows: 3 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 55°C and 63°C for total bacteria and SUP05, respectively, and 30 s at 72°C. The external

standard curve was constructed from a plasmid with the insert of bacterial or SUP05-specific SSU rRNA gene. The gene copy number was diluted from 100 to 1.10^8 copies.

2.2.4 Clone Library Construction

PCR amplification of SSU rRNA genes from environmental extracts was carried out on an iCycler® (Biorad) using universal bacterial primers 27f and 1492f (Lane, 1991). TaqPlus DNA polymerase (Stratagene, La Jolla, CA) was used and the following reaction conditions were applied: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 40 s, 55°C for 1.5 min, 72°C for 2 min; 1 cycle at 72°C for 10 min. Products were further purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions.

Purified PCR products were ligated into the pCR®2.1-TOPO® vector as described in the protocol of TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). Ligation reaction mixtures were transformed into One Shot® TOP10 competent *E. coli* cells (Invitrogen). Transformants were selected by blue and white screening. 192 white colonies were randomly selected from each sample and stored in a glycerol stock solution in 96-well culture plates at -80°C. Plasmid inserts from colonies stored in glycerol stock were amplified by direct PCR using standard M13F and M13R primers and confirmed with agarose electrophoresis. PCR-amplified inserts of one 96-well plate per sample were digested with the 4-base restriction endonuclease RsaI (Invitrogen). Each 10.5-μL RFLP reaction mixture contained 1.05 μL of supplied buffer 10xReact1, 0.25 μL of enzyme, 2.5 μL of PCR product and 6.7 μL of H_2O. The mixture was digested at 37°C for 3 h. The fragments were visualized by electrophoresis on a 2.5% agarose gel.

2.2.5 Sequencing and Phylogenetic Analysis

Bacterial SSU rRNA clones exhibiting unique RFLP patterns from the first 96-well plate per sample, and all clones from the second 96-well plate per sample were reamplified from glycerol stocks using M13F and M13R primer pair, purified with QIAquick® PCR purification kit and sequenced bidirectionally by the NAPS Unit (www.michaelsmith.ubc.ca/services/NAPS/), or the Sequencing platform at the McGill University and Genome Quebec Innovation Centre (www.genomequebec.mcgill.ca/services/sequencing.php) using M13F and M13R primers. Both facilities use automated sequencers from Applied Biosystems. Assembled and trimmed

sequences (Sequencher; Gene Codes, Ann Arbor, MI) were imported and aligned with the ARB phylogeny computer program (Ludwig et al., 2004). All sequences were checked for chimeras with Ribosomal Database Project II chimera check program (Cole et al., 2003). Sequences with higher than 97% similarity were combined into operational taxonomy units (OTUs) using the PHYLIP version 3.68 (Felsenstein, 2005) and DOTUR (Schloss and Handelsman, 2005). The closest phylogenetic neighbors were found using BLAST search for the NCBI database (Altschul et al., 1990). The phylogenetic tree and the bootstrap analysis (100 replicates) were constructed with the PhyML software (Guindon and Gascuel, 2003) by using the maximum likelihood method. Good's coverage was calculated by using the following formula: $C = (1 - (n_1/N)) \times 100$, where n_1 is the number of clones that occurred only once in the clone library and N is the total number of clones analyzed (Mullins et al., 1995). Shannon-Weiner index, Chao1, and Morisita-Horn similarity index were calculated based on 97 % similarity of OTUs using EstimateS (version 8.0, R. K. Colwell, http://purl.oclc.org/estimates).

2.2.6 Nucleotide Sequence Accession Numbers

The sequences of bacterial SSU rRNA clones identified in this study were deposited in GenBank under accession numbers EU265929 to EU266021, EU570831 to EU570913, and FJ628173 to FJ628355.

2.3 RESULTS

2.3.1 Physicochemical Characteristics of the Water Column

First, we located chemical regimes within the water column by charting vertical profiles of potential electron acceptors, electron donors and nutrients. Since the most recent study showed Nitinat Lake to be a more dynamic system than previously thought (Pawlowicz et al., 2006), samples were taken in two different months (April (Spring) and August (Summer)) to study how chemical and biological vertical stratification varies between seasons. As the sill at Windy Point divides the fjord into two basins (Fig. 2.1) with very different inputs (oceanic versus terrestrial) chemical and biological characteristics of Nitinat Lake were expected to be quite different at the

two sampling sites: S02 (ocean-side) and S05 (river-side). Indeed, vertical profiles of dissolved oxygen and sulfide showed significant differences between April (Fig. 2.2A) and August (Fig. 2.2B). A sharp oxic-anoxic interface with simultaneous disappearance of oxygen and appearance of sulfide could be seen in April at both ocean-side (S02) and river-side (S05) stations at the fairly equivalent depths of 26 m and 23 m, respectively. However, in August, although the oxygen concentration at S02 decreased rapidly from 62.5 μM at the surface to 9.4 μM at 37.5 m, it continued to be detected below this depth even though sulfide was present, albeit at lower concentrations than in April (40 versus 70 mM, respectively). In contrast to chemical observations made in April, an oxygen concentration of 4.7 μM was still present at a depth of 55 m. Thus, the line separating the transition and anoxic zone in Figure 2.2B was drawn at a depth where sulfide appeared and nitrate disappeared. At the river-side station, S05, in August oxygen was consumed more rapidly within a much shallower layer of about 11 m. Below this was a suboxic zone from 11 to 15 m, where neither O_2 nor sulfide was detected in measurable amounts, which was not seen in April.

Nitrate in all cases decreased with depth until the oxic-anoxic interface, where it disappeared completely. However, at S05 in August, a slight increase in nitrate was observed just below the suboxic zone. Ammonium showed a typical opposing gradient to nitrate with amounts increasing in the anoxic waters. In August at the inshore station S05, there was a small peak in ammonium concentration (14.2 μM) just above the suboxic zone. Nitrite was not detected at any depth. There was little indication of simultaneous detection of nitrate and ammonium, except for possibly at S02 at 26 m or at S02 at 35 m, although concentrations of ammonium at these locations were close to the detection limit. The phosphate profile was similar for both locations in April. It reached minimum values at the lower oxycline and then gradually increased to a maximum around the interface, while remaining constant at 7-9 μM throughout the anoxic zone. Whereas in August at S02, phosphate concentrations were much lower at 4 μM, although still fairly constant from just above the interface to 55 m depth. In contrast, at the river-side station (S05), although phosphate concentrations were in a similar range to S02 (3-5 μM) just above and in the suboxic zone, they increased to 8 μM in the anaerobic zone. The peaks in POC and PN concentrations, suggesting the presence of biological activity, occurred at 18 m during the April sampling at both stations. A second, smaller peak also occurred just at the interface at S02. Similarly, increase in POC and PN values were also observed at the oxic-anoxic interface at S02 and oxic-suboxic interface at S05 in August. The values remained relatively constant with depth

in the anoxic water with the highest concentration of POC in anoxia (41 μM) measured in August at S02. POC to PN ratio followed the Redfield stoichiometric ratio for C:N of 106:16 (Redfield et al., 1963) throughout the water column in all samples except for station S02 in August, where the ratio of C:N was slightly lower than the Redfield ratio (Fig. 2.2B). Since peaks in POC and PN concentrations are believed to coincide with increased biological activity, we chose to sample the depths in the oxic-anoxic transition zones where these peaks occurred for our biological characterization using phylogenetic analysis.

2.3.2 Small Subunit Ribosomal RNA Gene Library Construction and Analysis

To determine vertical distribution of bacteria in the water column, small subunit ribosomal RNA (SSU rRNA) gene clone libraries were constructed from S02 April 2006 samples from the oxic-anoxic interface (26 m) and deep anoxic layer (50 m), S02 August 2006 samples from the interface (37.5 m) and the deep anoxic layer (50 m), S05 April 2006 samples from the oxic-anoxic interface (22 m) and deep anoxic layer (50 m), and S05 August 2006 samples from the suboxic zone (13 m) and anoxic layer (28 m). One 96-well plate for each sample was first screened with RFLP, and unique patterns were sequenced (Fig. C.1). Additionally, a second 96-well plate per sample was sequenced without prior RFLP analysis. We did not find significant differences between these two approaches, as the distribution and taxonomic classification did not change between the plate first screened with RFLP and the plate that was directly sequenced. To avoid microdiversity and to account for possible errors during PCR and sequencing, we considered sequences with similarity higher than 97 % as the same operational taxonomic unit (OTU). A total of 1306 good quality sequences were analyzed, yielding 314 OTUs (Table 2.1, Table B.1, Supplementary material). Figures 2.3A and 2.3B show a maximum likelihood phylogenetic tree constructed with sequences from the most abundant classes: *γ–* and *ε–Proteobacteria*, and *δ–Proteobacteria* and *Chlorobia*, respectively. Some sequence clusters were related (> 95 % sequence similarity) to known genera or families of cultured species. Clusters whose most closely related sequences came from uncultured environmental samples were grouped into orders or environmental clades, however, many sequences had no close relatives in the current NCBI database and therefore remain unclassified (Figs. 2.3, 2.6 and Table B.1).

2.3.3 Bacteria in the Transition Zone

Overall, members of *ε-, γ-Proteobacteria* and *Chlorobia* classes were most abundant in the SSU rRNA clone libraries from the transition zone (Fig. 2.4). In many cases, individual OTUs were conserved among and between libraries although relative abundance varied widely according to station and season. Both libraries from station S02 were dominated by the *ε-Proteobacteria* (37.4 and 48.2 %), most of them related to thiotrophic endosymbionts of marine invertebrates that clustered together with *Sulfurimonas* sp. (Figs. 2.3A, 2.6). Remaining sequences were distantly related to environmental sequences from marine sediments and hydrothermal vents (unclassified Campylobacteriales), and *Arcobacter* sp., all of which are associated with oxidation of sulfur compounds. No *ε-Proteobacteria* appeared in the S05 library in April, and only 8 sequences (4.7 %) were detected in August. Instead, *Chlorobia* sp. dominated the interface library at S05 in April (72.6 %) including two highly represented clusters: one related to the environmental clone *Chlorobium* sp. Mog 4 (EF149015) from Lake Mogilnoe (Lunina et al., 2005) and the other related to *Chlorobium phaeobacteriodes* BS1 (CP001101) isolated from the Black Sea chemocline (Manske et al., 2005). The same clusters also appeared to a lesser extent in the remaining interface libraries, but were completely absent from deep anoxic samples. *γ-Proteobacteria* were prevalent in both interface libraries at S02 and dominated the suboxic zone library from S05 in August (39.8 %). Many of these sequences were affiliated with the environmental group SUP05 that includes the thiotrophic gill symbionts of clams and mussels (Figs. 2.3A, 2.6) (Sunamura et al., 2004). However, a number of *γ-Proteobacteria* were distantly related to unclassified environmental sequences or not related to any other sequences in the current database. Two sequences were related to methanotrophic endosymbiont of *Bathymodiolus* sp., and several others clustered together within the *Methylococcales* (Fig. 2.6). Three clones were assigned to the *Legionellales*, which includes the agg47 clade. Interestingly, within the *δ-Proteobacteria*, all but 3 of the 33 sequences were found in transition zone libraries, representing 1.8-12.2 % of those libraries' population. Sixteen sequences clustered together with incomplete-oxidizing SRB from the *Desulfobulbaceae* family. A second cluster comprised of 14 sequences related to complete-oxidizing SRB from the *Desulfobacteraceae* family (Fig. 2.3B). In August at both stations, increased occurrence of sequences assigned to *α-Proteobacteria* and the *Cytophaga-Flavobacter-Bacteroidetes* (CFB) group was observed. Most of the *α-Proteobacteria* sequences grouped into the *Rhodobacteriales* and *Rickettsiales*, and one environmental clade,

SAR-11 (Fig. 2.6). Within the *Bacteroidetes*, *Flavobacteria* were found in most transition zone libraries. Sequences related to *Sphingobacteria* were found at S02 in August and S05 in April. *β-proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Verrucomicrobiae*, *Planctomycetia* and Candidate division OD1 were also represented at low abundance in the clone libraries, typically as singletons (Fig. 2.6 and Table B.1).

2.3.4 Bacteria in the Anoxic Zone

The SSU rRNA gene libraries from the anoxic zones appeared surprisingly uniform with the majority of sequences affiliated with the *ε-Proteobacteria* (Fig. 2.4). For example, at station S02 in April, over 97 % of all sequences belonged to *ε-Proteobacteria*. Moreover, with the exception of S02 in August, a single OTU (Nit2A0620_5) related to *Arcobacter* sp. dominated all anoxic libraries (89 % in S02 in April, 78 % in S05 in April, and 86 % in S05 in August), The S02 library contained a number of sequences related to *Sulfurovum* and *Sulfurimonas* sp., (also abundant in transition zone libraries) as well as sequences affiliated with the SUP05 clade. Overall, community structure in the anoxic zone of S02 in August resembled that of transition zone libraries. This could be attributed to the flow of seawater into the layer, resulting in detectable oxygen concentrations and lower sulfide levels.

2.3.5 Diversity Analyses and Comparisons of SSU rRNA Gene Libraries

To determine the diversity of Nitinat Lake SSU rRNA gene libraries and compare our results with other studies, we calculated several commonly used statistical parameters such as Good's coverage value, Shannon-Weiner index, and Chao1 (Hill et al., 2003; Kemp and Aller, 2004). Good's coverage values, estimating the percentage of OTUs included in the library compared to the total number of OTUs in a given sample, ranged from 53-92 % (Table 2.1). The lowest coverage, indicating the highest diversity, was found in the suboxic zone at S05 in August. In general, the coverage values in the anoxic zone were higher than those at or above the oxycline. Shannon-Weiner index ranged from 2.83 to 4.23 in the transition samples, and from 0.59 to 2.92 in the anoxic samples, which confirmed the low library richness in the anoxia. Non-parametric Chao1, which is a suitable parameter for environmental samples, especially those exhibiting high

diversity, was used as a richness estimator. Only 18-78 OTUs were estimated to be present at the deep sample in April at seaside station S02. On the other hand, the highest number of OTUs (210-600) is predicted in the suboxic zone at 13 m in August, which is in agreement with the other richness parameters. Rarefaction curve analysis supported the low OTU diversity of the bacterial community in anoxic zone libraries (Fig. A1).

A number of common OTUs, especially from the γ-, ε-*Proteobacteria*, and *Chlorobium* species were found among all libraries (Table B.1). The Morisita-Horn index, determining the similarity between two libraries, varied significantly between samples. Three libraries from the anoxic zone were highly similar (98.9-99.8 %; Fig. 2.5). The remaining libraries clustered together depending on the sampling location.

2.3.6 Quantification of Bacterial Groups

In addition to qualitative measures of bacterial distribution and community structure, we determined the quantitative abundance of several frequently occurring bacterial groups. Total bacterial cell numbers in April and August ranged from 1×10^3 to 1×10^4 and from 0.8×10^5 to 1.1×10^6, respectively. Since the initial focus of our study was to determine the contribution of biological sulfate reduction to the overall geochemical cycling in Nitinat Lake, we performed several molecular analyses to detect sulfate-reducing bacteria. Initial screening with PCR primers DSR1F and DSR4R from Wagner (Wagner et al., 1998) for a highly conserved 1.9 kb region of the *dsr*AB gene revealed a product at all depths below the oxycline (data not shown). Also, our phylogenetic analysis revealed sequences closely related to SRB, but their numbers were fewer than for other groups (Fig. 2.6) and they were found largely at the interface. Minor contribution of SRB to total bacterial abundance was also confirmed by qPCR of the dissimilatory sulfite reductase (*dsr*) gene. Primers were modified from previously published studies to match higher amounts of cultured and uncultured SRB species (Table A.1). With these primers we detected SRB in all transition and anoxic samples, although the amounts were low, comprising of only 0-3.2 % of total cell numbers (Fig. 2.4). The highest number of SRB, 795±30 cells mL^{-1} was found below the suboxic zone in S05 in August.

Given that sequences affiliated with a novel *Acrobacter* sp. group (NITEP5) dominated most of the anoxic SSU rRNA libraries, we wanted to estimate more accurately their numbers in these zones. Primers specific to this group, which is represented by clone Nit2A0620_5 and

relatives, were designed from SSU rRNA gene sequences obtained from the clone libraries. All OTUs that are targeted by the NITEP5 primers are highlighted in Table B.1. We found that the quantitative results compared well with the clone library distribution. Except for station S05 in August, cell counts just above the transition zone were lower (6.8-15.3 % of total SSU rRNA gene copies) than those at the oxic-anoxic interface and in the deep anoxic zone (Fig. 2.4). The quantification also confirmed the dominance of NITEP5 group in the anoxic samples, except for that from S02 in August where oxygen was detected even at 50 m. The counts at 50 m in S02 in April slightly exceeded the total cell count, which could be explained by varying PCR efficiencies and experimental errors between the methods. The highest number of NITEP5 was 2.8×10^5 cells mL^{-1} at a depth of 55 m at station S02 in August, even though they contributed only 23 % of the total cell count.

The SUP05 group was also targeted due to its prevalence in transition zone clone libraries using group-specific SSU rRNA primers (Zaikova et al., 2009). SUP05 SSU rRNA gene copy numbers up to 4.3×10^5 copies ml^{-1} were measured in August at the ocean-side station S02, where oxygenated and nutrient-rich seawater penetrated to the intermediate zone (Fig. 2.4B). SUP05 abundance in the remaining transition zone samples ranged between 4 to 40 % of total bacterial SSU rRNA gene copies. In most cases, for the samples taken close to the oxic-anoxic interface, the estimated total bacterial copy numbers largely exceeded the sum of the SUP05, SRB and NITEP5 cluster estimates indicating that other bacterial groups, *i.e. Chlorobia* as suggested by the SSU rRNA analysis, were also present in large numbers.

2.4 DISCUSSION

The chemical composition and microbial community structure of Nitinat Lake has been understudied when compared to enclosed anoxic basins or OMZs in other parts of the world, including the Black Sea, Mariager Fjord, Cariaco Basin, Mono Lake, Saanich Inlet, and Namibian and South Pacific upwelling areas (Ramsing et al., 1996; Humayoun et al., 2003; Lin et al., 2006; Woebken et al., 2007a; Stevens and Ulloa, 2008; Zaikova et al., 2009). Given the near surface oxic-anoxic interface, deep anoxia, and the natural partitioning of the basin into ocean- and river-facing domains, Nitinat Lake provides an ideal analog for studies focused on microbial communities that correlate with high sulfate and sulfide environments in deep lakes and fjords. Of particular interest is to compare the diversity of microbial communities within

vertical chemical and physical regimes across the chemocline and into anoxia, as well as within the ocean- and river-facing domains with their contrasting influences and the stability of these communities during seasonal fluctuations in nutrient concentrations. In this study, in addition to profiling the fjord chemistry, the application of culture-independent SSU rRNA gene clone library methods with RFLP screening and qPCR were used to characterize the bacterial community structure specifically in hypoxic and anoxic zones of Nitinat Lake. Although potential biases can arise from this approach, stemming from reduced primer efficiency, point mutation and chimeric sequences resulting from PCR amplification, variability in copy numbers of the ribosomal (*rrn*) operons among different taxa, or variable cloning efficiencies due to inherent variation in G+C composition of environmental DNA sequences, SSU rRNA gene screening provides one of the most powerful yet simple ways to survey microbial community structure as a prelude to more in-depth quantitative, functional or genomic studies. Moreover, although absolute gene copy numbers obtained using qPCR may be underestimations due to differing DNA extraction efficiencies and primer specificity, a number of interesting trends and conclusions can be drawn from these data, and hypotheses for downstream studies can be formulated (see below).

2.4.1 Physicochemical Properties of Nitinat Lake

One of the unusual physicochemical features observed was the formation of a broad suboxic layer, which occurred in August at the river-side station S05. A previous study on Nitinat Lake physicochemical regimes attributed the formation of this suboxic zone to horizontal fluxes of dense, oxygenated seawater from the ocean, analogous to deep water renewal events associated with deeper fjord systems along the B.C. coastline (Pawlowicz et al., 2006; Zaikova et al., 2009). This phenomenon has also been observed in other systems, including the Black Sea due to horizontal mixing of an oxygenated, high salinity plume from Bosporus Strait with a cold intermediate layer (Konovalov et al., 2005), and Mariager Fjord, where the suboxic zone is believed to be caused by a dense population of phototrophic sulfide-oxidizing bacteria, that utilize both sulfide and oxygen (Ramsing et al., 1996).

Sulfide concentrations in Nitinat Lake's anoxic layer are similar to Cariaco Basin sulfide maxima, but lower than other stratified systems, including the Black Sea and Mariager Fjord (less than half the amount). Total vertical flux of sulfide was calculated from the concentration

gradient as described by Zopfi et al. (2001). The vertical eddy diffusivity (K_z) was calculated using the empirical equation of Gargett (1984) for every spatial and seasonal sampling point. The sulfide flux averages at 0.65 mmol m^{-2} day^{-1}, which is more than 9 times lower than in Mariager Fjord. However, the sulfide gradient in Nitinat Lake is 2.7-8.2 μM m^{-1}, which is approximately 10 times higher than that observed in the Black Sea and 40 times higher than in the Cariaco Basin (Taylor et al., 2001), implying that the chemical gradient between oxic and anoxic water in Nitinat Lake follows a steep transition. In terms of spatial differences, we found the sulfide flux at S05 more than twice as high as S02. Likely, this can be attributed to intrusions of oxygenated water from the ocean to the bottom waters at S02. Seasonally, the flux in the summer was higher than in the spring for both stations. Both the largest flux (1.22 mmol m^{-2} day^{-1}) and the steepest gradient (8.2 μM m^{-1}) were found at S05 in August. However, it is still much lower than that found in an OMZ off the Namibian coast, where a particularly rapid sulfide flux (~8 mmol m^{-2} day^{-1}), close to fluxes measured in sediment, was found during a bloom of chemolithotrophic sulfide-oxidizing bacteria that included the SUP05 group (Lavik et al., 2009).

The stoichiometry of biochemical reactions in anoxic basins can be summarized by the following Redfield-ratio equations for oxic (Eq. 10) and anoxic (Eq. 11) waters (Richards, 1965):

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 138\ O_2 = 106\ CO_2 + 16\ HNO_3 + H_3PO_4 + 132\ H_2O \quad (10)$$

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 53\ SO_4^{2-} = 106\ HCO_3^- + 53\ H_2S + 16\ NH_3 + H_3PO_4 \quad (11)$$

C:N:P ratios in April are close to these theoretical values only in deep anoxia. The upper and intermediate layers are more dynamic, and therefore the chemical profiles are influenced, besides microbial processes, also by ocean and river horizontal fluxes and vertical mixing. In August near the ocean-end, there is a gradual increase of the N:P and decrease of C:N ratio with depth (Fig. B.2). Also, the ratio between ammonium (dominant nitrogen compound at this depth) and sulfide is much higher than predicted by stoichiometry alone (0.9-1 vs. 0.3). The same observation was made in Mariager Fjord, the Black Sea, and Framvaren Fjord, where biotic and abiotic mechanisms of sulfide elimination were proposed, such as direct incorporation into organic matter, oxidation of sulfide due to oxygen influx, precipitation of FeS_2, or volatilization of H_2S (Yao and Millero, 1995; Ramsing et al., 1996; Konovalov and Murray, 2001). The formation of iron sulfides is unlikely in Nitinat Lake. Although oxygen influx coming from the dense ocean water into deeper anoxic waters has been detected especially in the summer (Pawlowicz et al., 2006), this process is somewhat restricted by the shallow sill that separates

Nitinat Lake from the ocean and the second sill that divides the fjord into ocean-side and river-side domains and is probable only at station S02. Besides possible partial H_2S volatization, the disappearance of sulfide at S05 is likely due to biological processes.

2.4.2 Correlation Between Chemical Regimes and Bacterial Population

Overall, significant shifts in bacterial community structures from the transition to the anoxic zones are observed. First of all, there is marked transition from much greater diversity in the transition zone SSU rRNA gene libraries to virtually complete dominance of *ε-Proteobacteria* in the deep anoxic samples. This is in contrast to findings from some other fjords such as Cariaco basin, where, despite similarities to Nitinat Lake in chemical profile, diversity increased with increasing depth and anoxia (Madrid et al., 2001). Nevertheless, many features of the distribution of OTUs in Nitinat Lake are similar to those found in other anoxic basins as well as marine and terrestrial environments where sulfide is produced biologically or geochemically. Bacterial diversity is often richest in the transition zones of these environments due the availability of many alternate electron acceptors (Stevens and Ulloa, 2008). By examining what is known about the metabolisms of cultured organisms closely related to highly represented OTUs in Nitinat Lake, the shifts in bacterial communities around the interface can be attributed to the gradients of oxygen, nitrate, and sulfide. For instance, high occurrence of *ε-Proteobacteria*, *γ-Proteobacteria*, and green sulfur bacteria (*Chlorobia*) OTUs, which are related to organisms known to perform microaerophilic or anoxygenic sulfide oxidation, is characteristic for the transition zone which was also the case in the transition zones of the Black Sea, Lake Kaiike and Cariaco Basin (Madrid et al., 2001; Koizumi et al., 2004b; Manske et al., 2005). In Nitinat Lake, sequences related to the environmental *γ-Proteobacteria* group SUP05 are represented especially at the ocean-side sampling station just above the oxic-anoxic interface in April and in both transition and anoxic zones in August as well as in the upper suboxic zone at the river-side station in August. The SUP05 group, which encompasses thiotrophic endosymbionts of clams and mussels, was first identified near the Suiyo Seamount, a deep-sea hydrothermal vent (Sunamura et al., 2004). Since then, closely related free-living SUP05 sequences have been found in other ecotypes such as marine hypoxic regions where they are associated with sulfide oxidation coupled with nitrate reduction such as in the Namibian OMZ (Lavik et al., 2009) and Saanich Inlet (Zaikova et al., 2009; Walsh et al., Science 2009, submitted). Chemical gradients observed

in association with SUP05 *i.e.* nitrate and sulfide depletion zone, support a similar hypothesis (except for S02 in August) that these bacteria are responsible for sulfide removal as well as nitrate reduction. The SUP05 associated sequences were distributed throughout all sampled depths at S02 in August since, at that time, conditions were similar to those in OMZs such as off the coasts of Chile (Stevens and Ulloa, 2008) and Namibia (Woebken et al., 2007a). Although no nitrate was detected below the depth of 37 m in Nitinat Lake, it may have been transported in with the horizontal influx of seawater and completely consumed within the transition zone. Whole genome sequencing of a hydrothermal vent *Calyptogena magnifica* symbiont closely related to the Nitinat Lake SUP05-like group, *Candidatus Ruthia magnifica,* further reveals that besides sulfide, these *γ-Proteobacteria* can also use thiosulfate or elemental sulfur to obtain energy for autotrophic carbon assimilation (Newton et al., 2007). These partially oxidized sulfur compounds are likely to accumulate in the transition zone of Nitinat Lake where they become available as electron donors, although this still needs to be confirmed with further sampling. While most studied *γ-proteobacteria* fix CO_2 through the Calvin-Benson-Bassham cycle, metabolic versatility and rapid adaptation are characteristics typical of microbial groups found in habitats with geochemical gradients. For example, a proteomics approach to determine the pathways present in a deep-sea endosymbiont of *Riftia pachyptila* showed that besides the Calvin cycle, these species were able to fix carbon by a less energy-requiring reductive tricarboxylic acid cycle (rTCA) (Markert et al., 2007). More evidence is accumulating to indicate that chemolithoautotrophic groups of *ε-proteobacteria*, in contrast to many *γ-proteobacteria*, use the rTCA cycle for carbon fixation (Campbell et al., 2006). This may be one reason for the shift to *ε-proteobacteria* in the anoxic zones of Nitinat Lake where more efficient carbon fixation is advantageous for surviving under high sulfide concentrations, limited carbon availability, and microaerobic or anaerobic conditions.

Sulfur-oxidizing *ε-Proteobacterial* sequences related to both *Sulfurimonas* sp. and *Arcobacter* sp. are also found in the transition zones of Nitinat Lake with *Arcobacter* sp. related sequences becoming the most predominant feature of the deeper hypoxic zone (except for S02 in August). In the transition zones, more *ε-Proteobacteria* are present at S02 in both April and August than at the river-side station (S05). Although both *Arcobacter* (NITEP5) and *Sulfurimonas*-related sequences were found in similar numbers of clones at S02 in April, mostly *Sulfurimonas*-related clones were found in the transition zone of S02 in August (Fig. 2.6). These *Sulfurimonas*-related OTUs seem to prefer the transition zone of Nitinat Lake where sulfide and

oxygen meet since they were not found in appreciable numbers in the anoxic zones. Their presence at 50 m at S02 in August coincides with detectable amounts of oxygen. Predominance of *Sulfurimonas* sp. related groups at redoxclines was also observed in the Baltic and Black Seas where subgroup GD17 comprised as much as 70 % and up to 100 % of chemoautotrophic cells, respectively (Grote et al., 2008). Several *Sulfurimonas* species have been isolated and characterized from sulfidogenic environments. One such bacterium, *Sulfurimonas paralvinellae*, was facultatively anaerobic capable of growing autotrophically using hydrogen and sulfur or thiosulfate as electron donor with either oxygen or nitrate as an electron acceptor (Takai et al., 2006). This bacterium grows under microaerobic conditions preferring oxygen concentrations less than 10 $\%_{sat}$. It is not known if H_2 gas is produced in Nitinat Lake and would be an available energy source. Other *Sulfurimonas* sp. such as *S. denitrificans*, have been isolated from marine oxic-anoxic interfaces and oil wells (Campbell et al., 2006). Genome sequencing of *S. denitrificans* revealed genes encoding for hydrogen, reduced sulfur compound and formate oxidation as well as for reduction of nitrate and oxygen (Sievert et al., 2008). This microbe grows under very low oxygen concentrations below 0.5 $\%_{sat}$ (Takai et al., 2006). In summary, *Sulfurimonas* associated OTUs in Nitinat Lake appear to prefer transitions zones or other habitats where sulfide and microaerobic conditions coexist.

The unique feature of this study is the dominance of novel *ε-proteobacteria* affiliated with *Arcobacter* sp. in the anoxic clone libraries. The NITEP5 group dominates all deep-water libraries except for the 50 m sample taken from station S02 in August where detectable oxygen concentrations persisted. Therefore, we propose that this new *Arcobacter* sp. group is particularly adapted to the highly sulfidic conditions associated with deep Nitinat Lake waters. SSU rRNA gene sequences related to *Arcobacter* sp. were found also in Lake Kaiike's sediments and chemocline (Koizumi et al., 2004a). In Cariaco Basin, where oxygen is absent below depths of 240-310 m, *ε-Proteobacteria* sequences also dominated the SSU rRNA clones found at 320 m (Madrid et al., 2001), but unlike this study, their abundance decreased further into the anoxic zone. The first *Arcobacter* species characterized were pathogens associated with animals and humans (Miller et al., 2007). However, more recently, free-living *Arcobacter* sp. have been found in terrestrial and aquatic environments. The sequences found thus far cluster according to ecotype (Campbell et al., 2006). A marine sulfide-oxidizing autotrophic bacterium, *Arcobacter sulfidicus*, was isolated from a sulfide and oxygen mixing zone near a hydrothermal vent (Wirsen et al., 2002). The optimum sulfide concentrations (1-2 mM) preferred by this bacterium are

higher than those reported for other sulfur oxidizing bacteria, which explains their proliferation and dominance in high sulfide environments (Sievert et al., 2007a). The same authors also found CO_2 fixation rates of *A. sulfidicus* to be high compared with other sulfur oxidizers, which again gives them a metabolic advantage in deep anoxic and sulfidogenic environments. In laboratory cultures, *A. sulfidicus* was able to grow under oxygen concentrations as low as 1-10 µM (Sievert et al., 2007b). The NITEP5 *Arcobacter* group identified in Nitinat Lake likely possesses similar attributes of tolerance of high sulfide concentrations and very low oxygen levels together with efficient autotrophic growth via the rTCA cycle, which is why it dominates the anoxic libraries.

While groups from the *ε-* and *γ-Proteobacteria* are likely key players in dark CO_2 fixation at the interface, green sulfur bacteria (*Chlorobia*), found in high amounts especially in April at the riverside station S05, also contribute to CO_2 assimilation phototrophically. Green sulfur bacteria are anoxygenic phototrophic bacteria, and therefore the balance between light availability, absence of oxygen and presence of sulfide determines their vertical position in the water column. In Nitinat Lake, they are found mostly at the interface and the number of *Chlorobium* sp. related sequences in the SSU rRNA libraries is higher at the river-side station S05 compared with the ocean-side station for both seasons. This could be caused by several factors, such as by the light intensity at the interface, which was closer to the surface at S05 in both seasons, or by the composition of the light spectrum at the river-side station, which could favor the brown-colored green sulfur bacteria found in the libraries (Overmann, 2008). In Nitinat Lake, light penetration is not significant at the interface depths, except for S05 in August, as previous observations found only 1 % light intensity level at 10-15 m depth (Pawlowicz et al., 2006). However, the nearest relative, *Chlorobium phaeobacteriodes* BS1, which is 99 % identical to Nitinat clone Nit2A0626_57 (found 55 times in the libraries), was found between 90 and 120 m in the Black Sea where there is only 0.0007 % of surface light intensity. These species are adapted to low light by increased concentration of light-harvesting pigments and twofold size increase of chlorosomes (Overmann, 2008). Although it was found that they can grow at light intensities that are extraordinarily low, they do so extremely slowly with a doubling time of several years (Manske et al., 2005). Nitinat Lake and the Black Sea share the similar characteristics, such as bacteriochlorophyll (BChl) e peaks coincident with the highest density of *Chlorobium* sp. and the fact that these species were the only phototrophs characterized in the clone libraries constructed from both environments. Thus, in Nitinat Lake we would expect to

find *Chlorobia* where oxygen is absent and sulfide present at the position closest to the surface where there is sufficient light for growth to ecologically relevant numbers.

Besides these previously described sulfur-oxidizing bacterial groups, several clones from the *γ-Proteobacteria* class related to methylotrophic bacteria suggest that metabolism of C1 compounds including methane, methylamines or methylsulfides also occurs at the interface. Unlike the transition zones in the Black Sea (Kuypers et al., 2003) or Golfo Dulce (Dalsgaard et al., 2003), neither the SSU rRNA gene analysis nor the chemical profiles suggest that significant anammox occurs in Nitinat Lake. Admittedly, it is possible that bias against anammox-affiliated sequences by the bacterial primers, which were used to create clone libraries, could have prevented them from occurring in the libraries. We did however obtain several planctomycete sequences not affiliated with anammox from the oxic-anoxic interface, including clone Nit5A0622_532 that was 95 % identical to sequences recovered from the Namibian upwelling system (Woebken et al., 2007b). Although we have identified the dominant bacterial players in the transition zone, our qualitative and quantitative findings reveal that more small-scale diversity is likely to be discovered.

2.4.3 Sulfur Cycling in the Anoxic Water Column

Sulfate-reducing bacteria were expected in the transition and anoxic zones of Nitinat Lake, as they are typically found in saline, anoxic environments, where they play a major role in sulfur and carbon cycling (Scholten et al., 2005; Foti et al., 2007; Leloup et al., 2007). In other anoxic lakes and fjords such as Mariager Fjord, Lake Cadagno and Lake Pavin high densities of SRB were detected in the water column peaking around the chemocline (Teske et al., 1996; Tonolla et al., 2000; Lehours et al., 2005). In contrast, in Nitinat Lake both the SSU rRNA gene survey and *dsr*AB quantification detected few SRB in the transition and almost no SRB in the anoxic zone. As well, attempts to measure *in situ* sulfate reduction were not successful in any transition and anoxic samples when using the radiotracer method, although the measurement could have been biased by several factors, such as insufficient method sensitivity or oxygen contamination during the water sampling. However, the potential for sulfate reduction in the water column does exist since water removed from the fjord at 50 m when inoculated into Postgate B medium resulted in sulfate reduction (data not shown). The amount of SRB cells found in Nitinat Lake was comparable with those found in the Black Sea, where a maximum of 630 copies mL^{-1} was found

just below the suboxic zone, but, unlike in Nitinat Lake, SRB in the Black Sea were also detected throughout the anoxic water column (Neretin et al., 2007). Sulfate reduction rates in the water column also could not be measured in Lake Kaiike, although SSU rRNA gene sequences related to SRB were recovered (Koizumi et al., 2004b). Therefore, given the low abundance of SRB in the water column, we believe that significant biogenesis of sulfide is restricted to the basin-floor sediment interval.

Up to 40 % of bacterial SSU rRNA gene copies at the oxic-anoxic interfaces belonged to *γ-Proteobacteria* related to SUP05. These chemolithoautotrophic bacteria are believed to play an important role in oxidation of reduced sulfur compounds and inorganic carbon fixation at the Nitinat Lake interface, and they are widely distributed in other oxygen-deficient marine environments, such as in an OMZ off the coast of Chile (Stevens and Ulloa, 2008), the Namibian upwelling system (Woebken et al., 2007a; Lavik et al., 2009), the Black Sea (Vetriani et al., 2003), Cariaco Trench (Madrid et al., 2001), and Saanich Inlet (Zaikova et al., 2009; Walsh et al., Science 2009, submitted). Similarly to other studies, we did not see any potential host organisms when collecting and filtering the water samples, thus we hypothesize that these bacteria are free-living. SUP05 peak at the interface where opposing gradients of nitrate and sulfide meet except at S02 in April where they were found in increasing numbers at the greater depths due to oxygen penetration. The other important group likely involved in sulfur cycling is the *ε-Proteobacteria*. Specifically, bacteria related to *Sulfurimonas* and *Thiomicrospira* sp. (such as GD17 group found in Baltic and Black Seas, (Grote et al., 2008)) are expected to play important roles in concomitant turnover of carbon, nitrogen, and sulfur, and greatly contribute to denitrification and dark CO_2 fixation at the interface (Brettar et al., 2006; Campbell et al., 2006; Grote et al., 2007). An *in vitro* study of Baltic seawater from the transition zone by Labrenz *et* al. (Labrenz et al., 2005) showed that maximal dark CO_2 fixation was achieved when amended with thiosulfate. Due to the similarity of chemical conditions, and quantity and identity of SSU rRNA gene sequences, we can hypothesize that similar processes occur also in Nitinat Lake's transition zone. Also in the transition but especially in the anoxic zones (where 10^3-10^5 copies mL^{-1} were found) *ε-Proteobacteria* related to *Arcobacter* sp. (NITEP5) may be important players in sulfur cycling. It is interesting to note that although both *γ-* and *ε-Proteobacterial* sequences in this study are associated with the same metabolic pathways (microaerobic sulfide oxidation possibly coupled with nitrate reduction and dark CO_2 fixation), a clear shift from a mixed community in the transition zone to absolute dominance of one particular *Arcobacter* related *ε-Proteobacteria*

in the anoxic zone is observed. We believe that this is due to the more efficient growth of NITEP5 group under high sulfide and extremely low oxygen conditions, which is, in part, attributable to the more efficient CO_2 fixation with the reverse TCA cycle (Campbell et al., 2006). Since rTCA requires four less ATPs to synthesize one triose phosphate molecule, *ε-Proteobacteria* could have the advantage over *γ-Proteobacteria* in oxygen-depleted deep waters. The NITEP5 group likely has additional advantages over other *ε-Proteobacteria*, such as tolerance to high sulfide concentrations. Still, the Nitinat Lake environment is unique in that although *Arcobacter*-related groups have been found in other environments, they have always been associated with the oxic-anoxic interface. It would be surprising that microaerobic conditions are present in the deep waters of Nitinat Lake where the NITEP5 group is found, especially at the river-side station where O_2 renewal is restricted due to the shallow sill midway along the fjord. Therefore, it will be necessary to investigate further the metabolic potential of this group through culturing or metagenomic analyses to better constrain the metabolic pathways for growth under the conditions of Nitinat Lake anoxia.

2.5 CONCLUSIONS

This study used cultivation-independent methods to explore bacterial community structure across the oxycline of a stratified anoxic basin at two different times of year. The expansion of stratified basins and OMZs due to global climate changes makes the understanding of microbial activities in these areas increasingly important for understanding elemental and nutrient cycling within marine environments. We found that the anoxic waters in Nitinat Lake were surprisingly uniform, dominated by a single *ε-Proteobacterial* OTU likely involved in sulfur cycling. In general, the majority of recovered clones from all depths were associated with characterized groups implicated in the oxidation of reduced and partially oxidized sulfur compounds. We found many similarities with other anoxic basins around the globe, but several features, such as the NITEP5 group dominance in the anoxic layer and the lack of SRB have not been observed in any other system. Further efforts to identify the metabolic pathways of the abundant bacterial species will be necessary to fully understand their impact on the overall biogeochemical cycling within Nitinat Lake and beyond.

2.6 ACKNOWLEDGMENTS

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2.7 TABLES AND FIGURES

Table 2.1: Statistical parameters of SSU rRNA libraries from Nitinat Lake samples

	S02				S05			
	April		August		April		August	
	26 m	50 m	37.5 m	50 m	22 m	50 m	13 m	28 m
Number of clones in library	123	172	166	159	167	168	171	180
Number of sequenced clones	105	101	104	86	101	99	109	99
Number of OTUs[a]	69	16	71	48	63	33	104	22
Good's coverage (%)	54	92	69	82	73	83	53	90
Shannon-Weiner index	3.45	0.59	3.56	2.92	2.83	1.19	4.23	0.82
Chao1 richness estimator	88-213	18-78	120-367	60-155	100-364	55-254	210-600	37-196

[a] OTUs defined at ≥97% sequence identity

Figure 2.1: Map of Nitinat Lake. The location of the Lake is indicated by the box at the bottom right-hand corner. The Narrows channel, which separated the Lake from the ocean, the Nitinat River and the sampling stations S02 and S05 are marked.

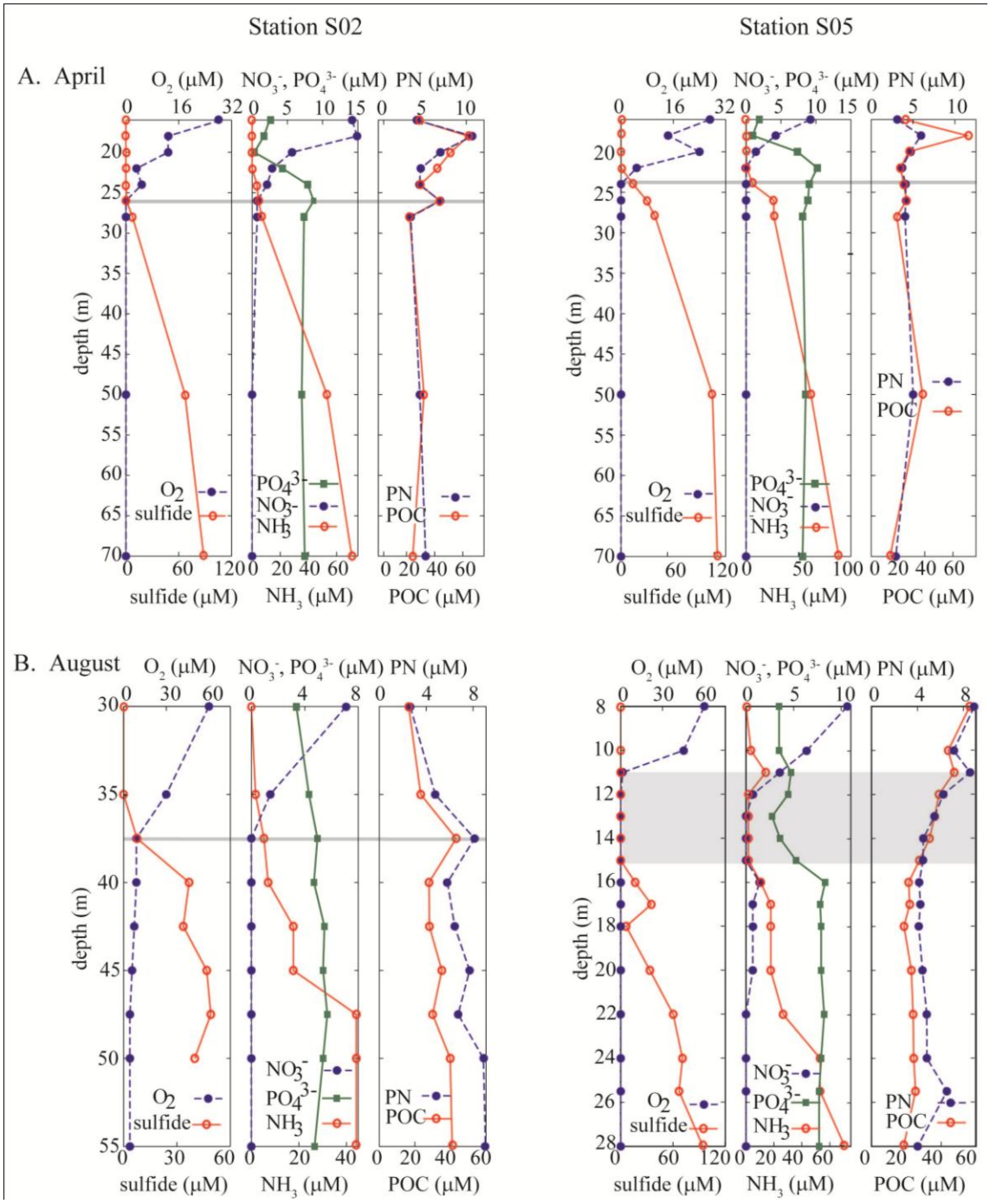
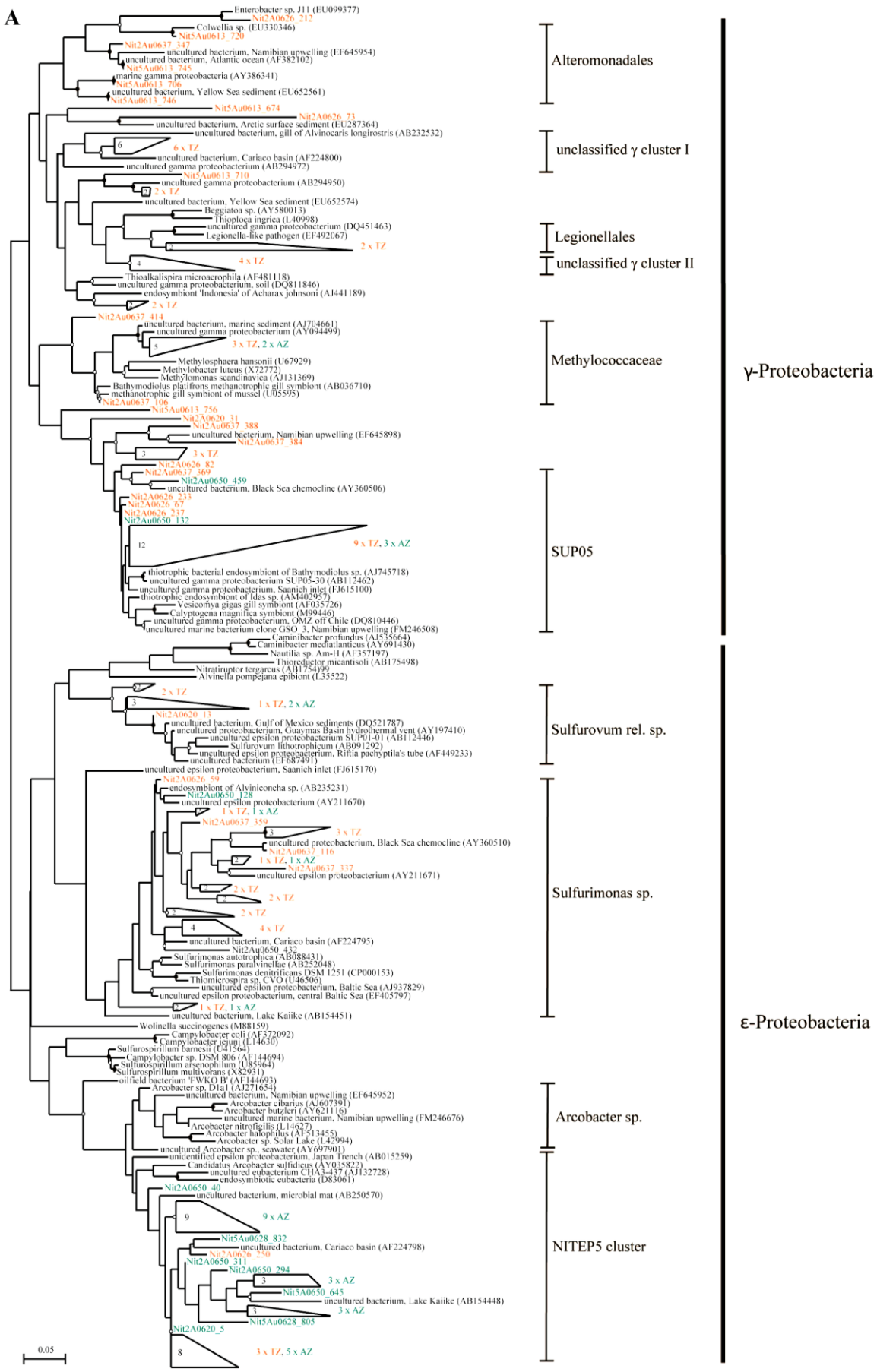


Figure 2.2: Vertical profiles of physicochemical and biological parameters. (A) April 2006 depth profiles between 16-70 m at stations S02 and S05. Oxic-anoxic boundaries are shaded. (B) August 2006 depth profiles at the intermediate layer (between 30-55 m at station S02 and 8-28 m at station S05). Oxic-anoxic boundary at S02 and suboxic zone at S05 are shaded. Note that PN scale is related to POC scale by 106-16 ratio.


A
Enterobacter sp. J11 (EU099377)
Nit2A0626_212
Colwellia sp. (EU330346)
Nit5Au0613_720
Nit2Au0637_347
uncultured bacterium, Namibian upwelling (EF645954)
uncultured bacterium, Atlantic ocean (AF382102)
Nit5Au0613_745
marine gamma proteobacteria (AY386341)
Nit5Au0613_706
uncultured bacterium, Yellow Sea sediment (EU652561)
Nit5Au0613_746
Nit5Au0613_674
Nit2A0626_73
uncultured bacterium, Arctic surface sediment (EU287364)
uncultured bacterium, gill of Alvinocaris longirostris (AB232532)
6
6 x TZ
uncultured bacterium, Cariaco basin (AF224800)
uncultured gamma proteobacterium (AB294972)
Nit5Au0613_710
uncultured gamma proteobacterium (AB294950)
2 x TZ
uncultured bacterium, Yellow Sea sediment (EU652574)
Beggiatoa sp. (AY580013)
Thioploca ingrica (L40998)
uncultured gamma proteobacterium (DQ451463)
Legionella-like pathogen (EF492067)
2
2 x TZ
4
4 x TZ
Thioalkalispira microaerophila (AF481118)
uncultured gamma proteobacterium, soil (DQ811846)
endosymbiont 'Indonesia' of Acharax johnsoni (AJ441189)
2 x TZ
Nit2Au0637_414
uncultured bacterium, marine sediment (AJ704661)
uncultured gamma proteobacterium (AY094499)
3 x TZ, 2 x AZ
Methylosphaera hansonii (U67929)
Methylobacter luteus (X72772)
Methylomonas scandinavica (AJ131369)
Bathymodiolus platifrons methanotrophic gill symbiont (AB036710)
methanotrophic gill symbiont of mussel (U05595)
Nit2Au0637_106
Nit5Au0613_756
Nit2A0620_31
Nit2Au0637_388
uncultured bacterium, Namibian upwelling (EF645898)
Nit2Au0637_384
3
3 x TZ
Nit2A0626_82
Nit2Au0637_369
Nit2Au0650_459
uncultured bacterium, Black Sea chemocline (AY360506)
Nit2A0626_233
Nit2A0626_67
Nit2A0626_237
Nit2Au0650_132
12
9 x TZ, 3 x AZ
thiotrophic bacterial endosymbiont of Bathymodiolus sp. (AJ745718)
uncultured gamma proteobacterium SUP05-30 (AB112462)
uncultured gamma proteobacterium, Saanich inlet (FJ615100)
thiotrophic endosymbiont of Idas sp. (AM402957)
Vesicomya gigas gill symbiont (AF035726)
Calyptogena magnifica symbiont (M99446)
uncultured gamma proteobacterium, OMZ off Chile (DQ810446)
uncultured marine bacterium clone GSO_3, Namibian upwelling (FM246508)
Caminibacter profundus (AJ535664)
Caminibacter mediatlanticus (AY691430)
Nautilia sp. Am-H (AF357197)
Thioreductor micantisoli (AB175498)
Nitratiruptor tergarcus (AB175499)
Alvinella pompejana epibiont (L35522)
2
2 x TZ
3
1 x TZ, 2 x AZ
Nit2A0620_13
uncultured bacterium, Gulf of Mexico sediments (DQ521787)
uncultured proteobacterium, Guaymas Basin hydrothermal vent (AY197410)
uncultured epsilon proteobacterium SUP01-01 (AB112446)
Sulfurovum lithotrophicum (AB091292)
uncultured epsilon proteobacterium, Riftia pachyptila's tube (AF449233)
uncultured bacterium (EF687491)
uncultured epsilon proteobacterium, Saanich inlet (FJ615170)
Nit2A0626_59
endosymbiont of Alviniconcha sp. (AB235231)
Nit2Au0650_128
uncultured epsilon proteobacterium (AY211670)
1 x TZ, 1 x AZ
Nit2Au0637_359
3
3 x TZ
uncultured proteobacterium, Black Sea chemocline (AY360510)
Nit2Au0637_116
2
1 x TZ, 1 x AZ
Nit2Au0637_337
uncultured epsilon proteobacterium (AY211671)
2 x TZ
2
2 x TZ
2
2 x TZ
4
4 x TZ
uncultured bacterium, Cariaco basin (AF224795)
Nit2Au0650_432
Sulfurimonas autotrophica (AB088431)
Sulfurimonas paralvinellae (AB252048)
Sulfurimonas denitrificans DSM 1251 (CP000153)
Thiomicrospira sp. CVO (U46506)
uncultured epsilon proteobacterium, Baltic Sea (AJ937829)
uncultured epsilon proteobacterium, central Baltic Sea (EF405797)
1 x TZ, 1 x AZ
uncultured bacterium, Lake Kaiike (AB154451)
Wolinella succinogenes (M88159)
Campylobacter coli (AF372092)
Campylobacter jejuni (L14630)
Sulfurospirillum barnesii (U41564)
Campylobacter sp. DSM 806 (AF144694)
Sulfurospirillum arsenophilum (U85964)
Sulfurospirillum multivorans (X82931)
oilfield bacterium TWKO B (AF144693)
Arcobacter sp. D1a1 (AJ271654)
uncultured bacterium, Namibian upwelling (EF645952)
Arcobacter cibarius (AJ607391)
Arcobacter butzleri (AY621116)
uncultured marine bacterium, Namibian upwelling (FM246676)
Arcobacter nitrofigilis (L14627)
Arcobacter halophilus (AF513455)
Arcobacter sp. Solar Lake (L42994)
uncultured Arcobacter sp., seawater (AY697901)
unidentified epsilon proteobacterium, Japan Trench (AB015259)
Candidatus Arcobacter sulfidicus (AY035822)
uncultured eubacterium CHA3-437 (AJ132728)
endosymbiotic eubacteria (D83061)
Nit2A0650_40
uncultured bacterium, microbial mat (AB250570)
9
9 x AZ
Nit5Au0628_832
uncultured bacterium, Cariaco basin (AF224798)
Nit2A0626_250
Nit2A0650_311
Nit2A0650_294
3
3 x AZ
Nit5A0650_645
uncultured bacterium, Lake Kaiike (AB154448)
3
3 x AZ
Nit5Au0628_805
Nit2A0620_5
8
3 x TZ, 5 x AZ
0.05
Alteromonadales
unclassified γ cluster I
Legionellales
unclassified γ cluster II
Methylococcaceae
SUP05
γ-Proteobacteria
Sulfurovum rel. sp.
Sulfurimonas sp.
ε-Proteobacteria
Arcobacter sp.
NITEP5 cluster

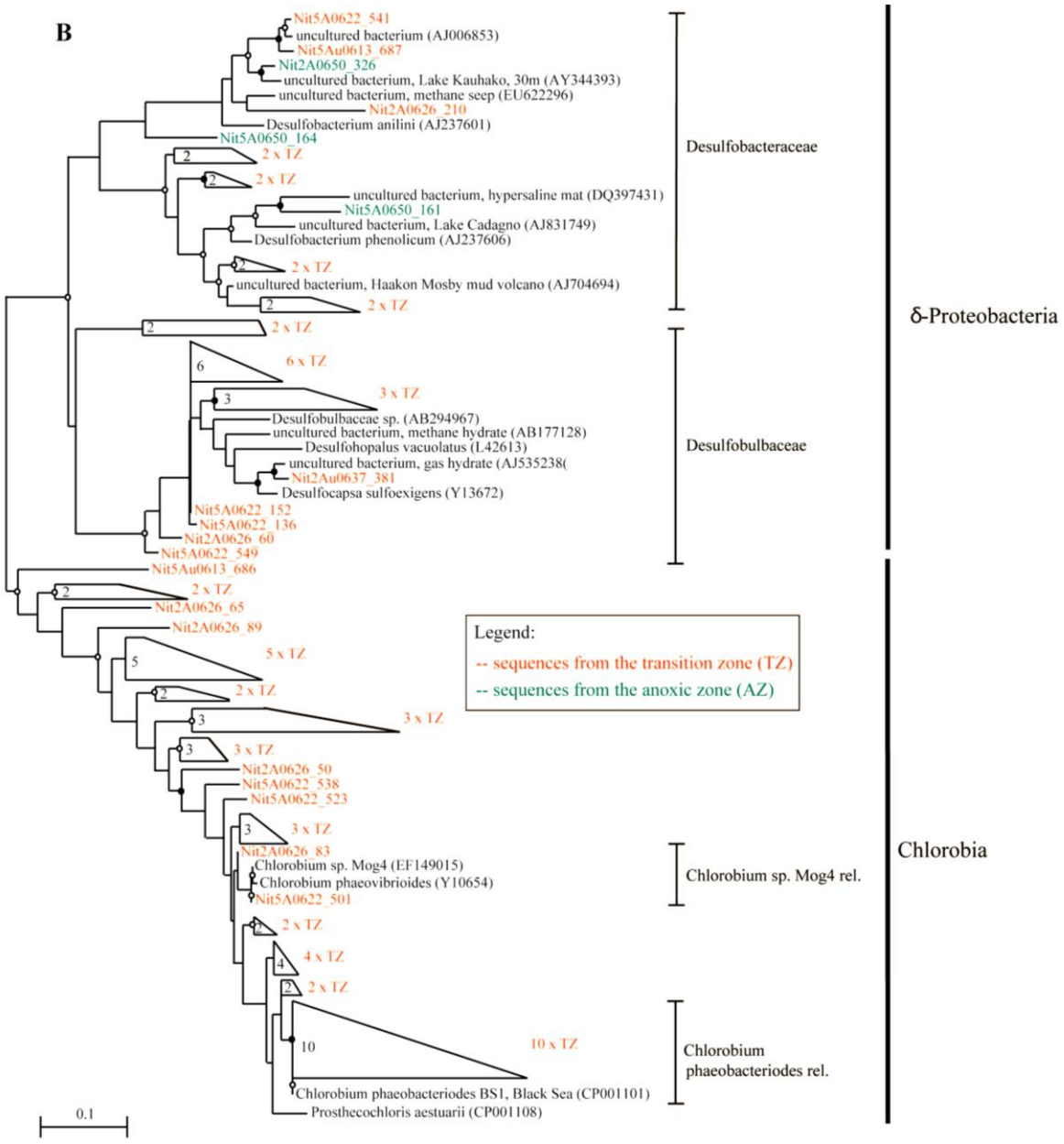


Figure 2.3: Unrooted maximum likelihood phylogenetic tree showing SSU rRNA gene sequences from Nitinat Lake and related sequences from Silva database release 96 (http://www.arb-silva.de/) and Genbank; A, *γ–* and *ε–Proteobacteria*; B, *δ–Proteobacteria* and *Chlorobia*. Clones from this study are coded as follows, with Nit2A0620_1 as an example: Nit, Nitinat Lake; 2, station S02; A, April (Au, August); 06, 2006; 20, sample depth in meters; and 1, number assigned to the clone. Bootstrap values are shown with following symbols: open circles for >50 % and closed circles for >90 % (for 100 iterations). Values below 50 % are not shown.

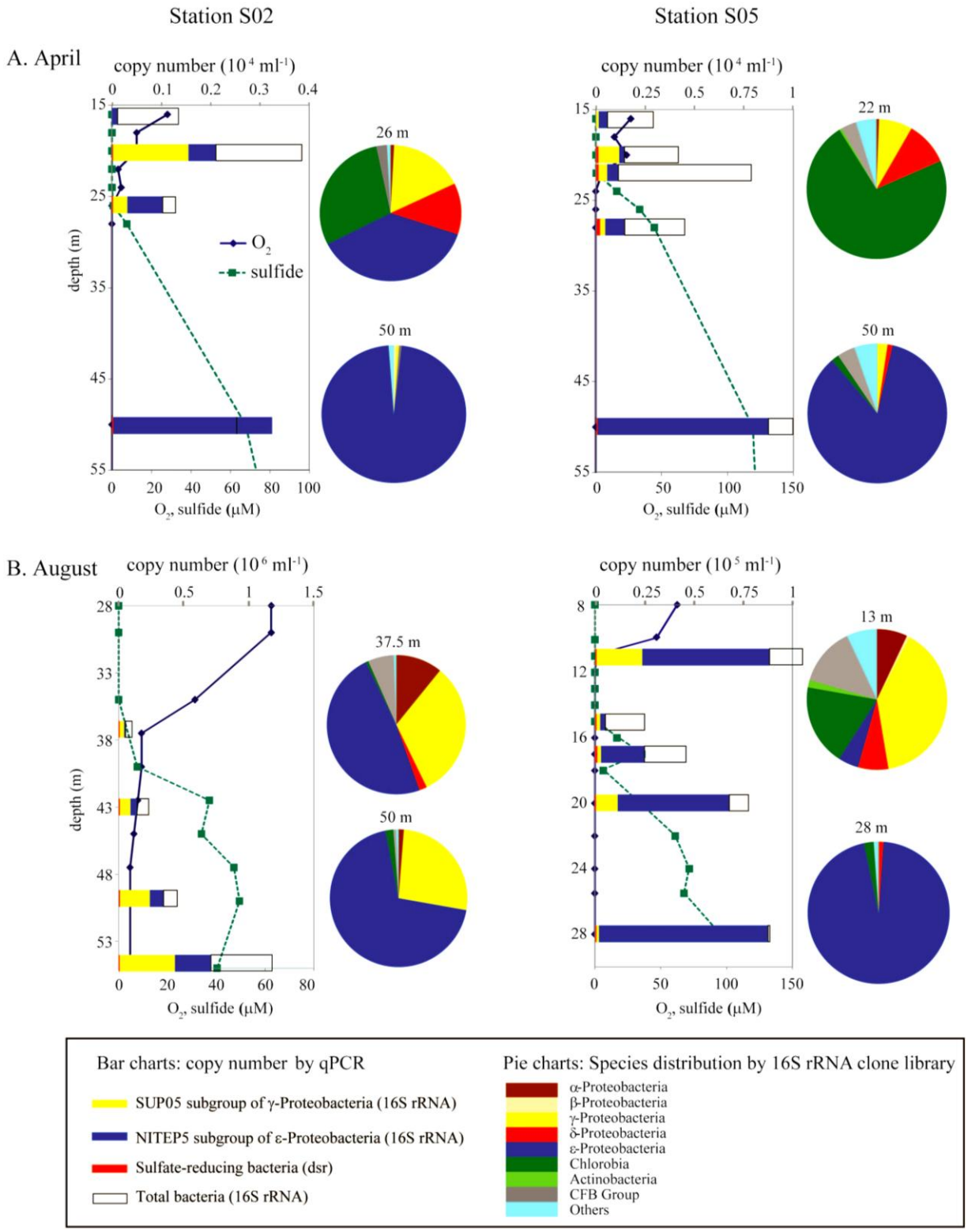


Figure 2.4: Bar charts showing the amounts of bacterial groups as determined by qPCR. Profiles of O_2 and sulfide are also shown. Pie charts showing the frequencies of major bacterial groups as determined by SSU rRNA analysis.

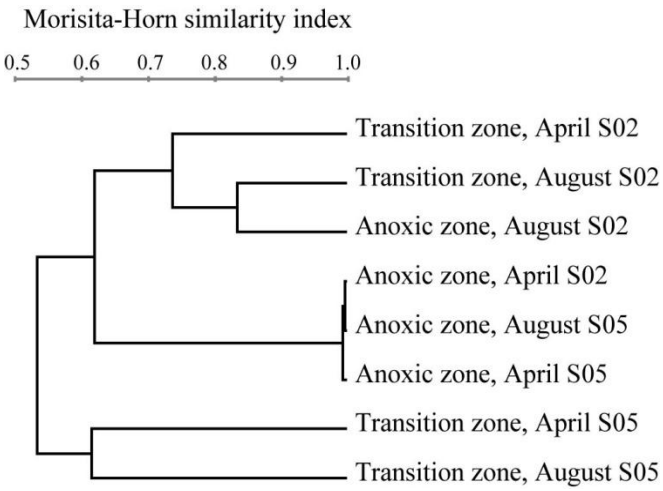


Figure 2.5: Cluster diagram using neighbor-joining algorithm showing Morisita-Horn similarity of individual clone libraries.

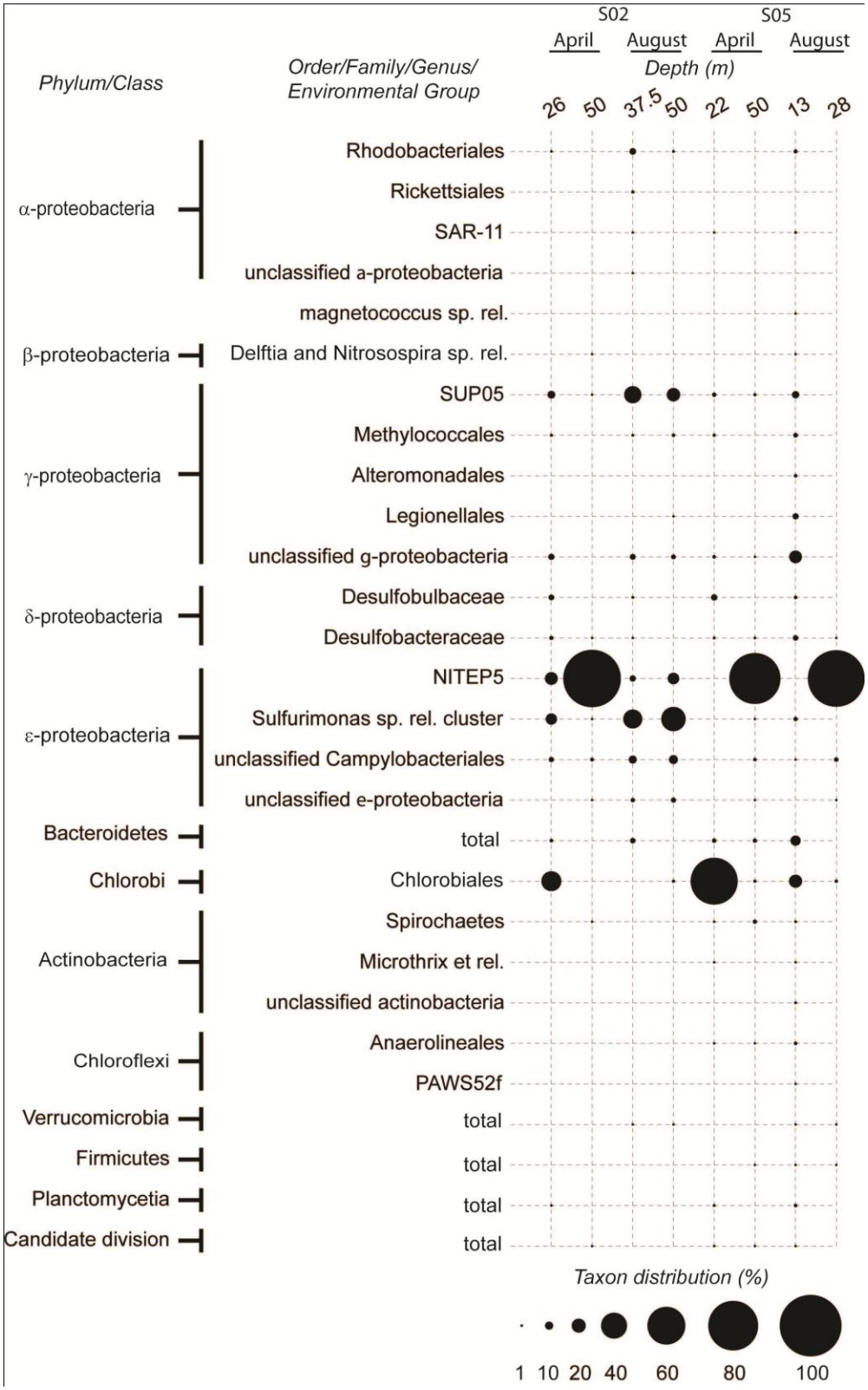


Figure 2.6: Dot representation of bacterial diversity in Nitinat Lake libraries based on phylogenetic proximity to relevant reference groups and environmental sequences. The circumference of closed circles determines the percentage of clones falling within certain group.

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CHAPTER 3

NATURAL AND STIMULATED SULFATE REDUCTION RATES IN FJORD SEDIMENTS

3.1 INTRODUCTION

Sulfate reduction in marine and other high sulfate-containing sediments is an important terminal process that contributes significantly to the decomposition and mineralization of organic matter (Jorgensen, 1982). Majority of the sulfate-reducing bacteria (SRB) belong to the *δ-Proteobacterial* class and grow heterotrophically by oxidizing a variety of low molecular weight organic molecules with sulfate as a terminal electron acceptor under anaerobic or microaerophilic conditions (Widdel, 1988). They are divided into two groups; complete-oxidizing, which can degrade organic substrate all the way to CO_2, and incomplete-oxidizing, for which the end product is acetate. Besides their important role for global sulfur and carbon turnover, SRB's ability to produce sulfide and alkalinity makes them also suitable for remediation of waters containing high sulfate concentrations, such as drainage from waste rock piles or pits remaining after closure of mining operations that can become acidic and laden with high concentrations of metals (McCullough, 2008). In a favorable anaerobic environment, SRB can effectively prevent acidity production by generating alkalinity, as well as indirectly remove dissolved metals by generating sulfide, which rapidly forms metal sulfide precipitates.

In British Columbia, Canada, several remediation strategies have been applied where open mine pits were flooded with water to create pit lakes with the intention of establishing an anaerobic environment for SRB growth. For instance, the Island Copper Mine pit was flooded with seawater to create an anoxic bottom layer where ideally a SRB community could be established (Fisher and Lawrence, 2006). As well, pit lakes at the Equity Silver Mine became effective in metal removal after they were amended with nutrients (N, P) and organic carbon (ethanol) to promote primary production at the surface and sulfate reduction at the bottom of the

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pit (McNee et al., 2003). Permanent anoxic conditions and sufficient suitable organic carbon availability are the most important requirements for establishment of SRB population. To date, few studies have addressed the factors that influence the sulfate reduction rates in such complex stratified lake systems. Specifically, the availability of electron donors for SRB is likely the limiting step of the process, as it has been shown in many laboratory-scale studies with SRB (Neculita et al., 2007).

Naturally occurring anoxic basins, where sulfate reduction is a dominant process, such as meromictic lakes or permanently stratified anoxic fjords, can serve as analog systems to determine what conditions drive the sulfur cycle in stratified water bodies. Nitinat Lake is a permanently anoxic fjord on the west coast of Vancouver Island, British Columbia, Canada. This fjord is long (23 km), narrow (1.2 km), deep (maximum of 200 m) and, like many mine pit lakes, meromictic, which is in part due to seasonal influxes of saline oceanic water combined with freshwater inputs from the terrestrial end of the fjord. Unlike mine pit lakes, there is no influent plume containing toxic levels of metals such as copper or zinc, but, due to Nitinat Lake's stratified nature and high concentrations of sulfate and sulfide, we chose this fjord as an analog system to use molecular tools in hopes to determine the contribution of SRB to the overall biogeochemical cycling. Moreover, the accessibility of Nitinat Lake when compared to mine pit lakes in the Northern B.C. has contributed to the choice of the study site. Our previous results from a survey of the chemistry and bacterial phylogenetic diversity of Nitinat Lake (Schmidtova et al., 2009) suggest that, despite reducing conditions and sulfate availability, no significant sulfate reduction occurs in the anoxic sulfidic water column. Very few *δ–Proteobacterial* sequences related to SRB were found in the water column and low numbers of SRB were detected with quantitative polymerase chain reaction (qPCR) with dissimilatory sulfite reductase gene (*dsr*) specific primers (maximum of ~10^3 gene copies mL^{-1}). Thus, it appears that formation of sulfide occurs below the water column in the sediments. This observation is crucial for further application of mine pit lakes treatment systems, as it denies their core assumption that SRB can remove metals from the anoxic zone. Therefore, the present study focuses on the upper sediment layer of Nitinat Lake, which we hypothesize is the preferred habitat for SRB and the likely source of sulfide formation. Phylogenetic analysis of both the overall bacterial community with SSU rRNA, as well as SRB with specific functional gene dissimilatory sulfite reductase (*dsr*) clone libraries, were used to identify key microbial populations. *In situ* sulfate-reduction rates and *dsr* gene quantification were used to estimate the activity of SRB in the sediments.

Furthermore, to explore the potential for enhancing SRR in lakes through C addition, sediments from Nitinat Lake were amended in batch microcosms in the laboratory with a range of pure and complex carbon sources (acetate, lactate, and a mixture of compost, silage and molasses). While most SRB utilize simple organic molecules, addition of complex waste materials was shown to promote SRR in many bioreactors and passive systems (Neculita et al., 2007). The objective of this batch setup was to evaluate the effect of two simple versus a complex carbon source on SRR and determine the kinetics of carbon degradation, which has not been well studied during carbon amendment in mine pit lakes and other passive MME treatment systems. During this 190-day experiment, SRR and volatile fatty acids (VFA) uptake rates were monitored.

3.2 MATERIALS AND METHODS

3.2.1 Sediment and Water Collection

Two sediment cores (~ 40 cm in length, 10 cm in diameter) were collected using a gravity corer (Rigosha, Tokyo, Japan) from Nitinat Lake's sampling station S5 (N 48° 46' 306", W 124° 43' 834") on July 3rd, 2008. The cores were immediately placed on ice and transported to the laboratory in Vancouver. Subsamples of the top 20 cm were taken under anaerobic conditions in an anaerobic chamber (Coy, Grass Lake, MI) for radiolabelled sulfate reduction rate measurement. The remaining upper 20 cm of the sediment from both cores were mixed together and used for chemical analyses, DNA extraction and the carbon amendment study. Nitinat Lake water from 50 m depth was collected using standard 5 L Niskin bottles. This water was transferred to 2 L plastic bottles with minimum oxygen exposure and kept on ice during transportation to the laboratory where it was filtered through 0.22 μm membrane filter paper to remove all particulates including planktonic bacteria so that it could be used for the batch-growth carbon amendment study.

3.2.2 Sulfate Reduction Rate (SRR) Measurement

Six subcores per large core were taken from the upper 20 cm of sediment in an anaerobic chamber with a 10 mL syringe that had the tip cut off, and transferred to 25 mL brown glass vials. The vials were sealed with septa screw caps and incubated for 1 h at 11°C to bring them

back to the *in situ* temperature. After 1 h, 40 μL of 3.7 MBq/mL of $Na_2{}^{35}SO_4$ (PerkinEmler, Waltham, MA) was added and the vials were incubated at *in situ* temperature in the dark for 15, 19, and 36 h. The reaction was stopped by adding 1 mL of 20 % Zn-Ac and freezing. One-step cold distillation method with HCl and chromium was used to recover sulfides in zinc-acetate traps as described by Fossing and Joergensen (1989). Samples were placed into 3-neck round-bottom flasks purged with N_2. Five mL of 6 N HCl and 5 mL of Cr^{2+} as $CrCl_2$ were added with a syringe through a stopper under continuous N_2 flow. The reaction ran for 1 h under constant N_2 flow (~2 bubbles/s) and sulfide was collected in two sets of 10 mL vial traps with 4 mL of 5 % zinc-acetate. Radioactivity was determined using liquid scintillation counting. The SRR was calculated from the equation

$$SRR = \frac{[SO_4^{2-}].A_r.\alpha}{A_t.t},$$ where $[SO_4{}^{2-}]$ is the actual sulfate concentration in nmol.cm^{-3}; A_r is the radioactivity of the reduced ^{35}S-compounds per unit volume (dpm cm^{-3}); A_t is the total radioactivity of ^{35}S injected per unit volume (dpm cm^{-3}); α is the isotope fractionation factor (0.6), and t is the incubation time in days.

3.2.3 DNA Extraction

Genomic DNA was extracted from thawed sediment sample using the MoBio® PowerSoil DNA extraction kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions with the following modifications: alternative protocol for maximum yields was used; the spin column was rinsed twice with 300 μL of solution C4; and finally DNA was eluted in 100 μL of 10 mM Tris. Total nucleic acid concentration and purity were measured spectrophotometrically with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm.

3.2.4 Quantitative Polymerase Chain Reaction (qPCR)

SRB in DNA from upper 20 cm of Nitinat Lake sediment were quantified with previously designed dissimilatory sulfite reductase (*dsr*) forward primer DSR1F' (5'-ACSCACTGGAAGCACGGC-3') and reverse primer DSR210R (5'-CGGTGGMRCCRTGCATRTT-3') (Schmidtova et al., 2009). Total bacteria were also

quantified with primers 27F and degenerate 519R (5'-GNTTTACCGCGGCKGCTG-3') (Lane, 1991). Quantitative PCR of SRB was performed on the ABI PRISM® 7000 (Applied Biosystems). The reaction mixture (12 μl) contained iTaqTM SYBR® Green Supermix with ROX (Biorad), each primer at a final concentration 300 nM, nanopure water and template DNA. MicroAmp 96-well reaction plates (Applied Biosystems) were used. The amplification conditions were as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 94°C followed by 1 min at 60°C. Each sample was amplified in triplicates, and several samples were chosen and the reaction was repeated, yielding 6 replicates. The external standard curve for *dsr* quantification was constructed with a total extracted genomic DNA of *Desulfobacterium autotrophicum* (DSM 3382). The detection limit was 100 *dsr* copies per reaction, the efficiency ($E=10^{(-1/slope)}$; where 2 indicates an exact doubling per cycle) was 1.97, and $R^2=0.95$. Concentrations of the samples were extrapolated from the standard curve using ABI Prism 7000 SDS Software (Version 1.2.3, Applied Biosystems). Q-PCR of total eubacteria was performed as described in Zaikova et al. (Zaikova et al., 2009).

3.2.5 Clone Library Construction

The same genomic DNA as used in q-PCR was also used to create clone libraries of SSU rRNA and *dsr*. PCR amplification of SSU rRNA genes was carried out on an iCycler® (Biorad) using universal bacterial primers 27f and 1492f (Lane, 1991). Taq DNA polymerase (Invitrogen) was used and the following reaction conditions were applied: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 40 s, 55°C for 1.5 min, 72°C for 2 min; 1 cycle at 72°C for 10 min. PCR amplification of *dsr* genes was carried out using primers DSR1F and DSR4R (Wagner et al., 1998). The same conditions applied except for melting temperature, which was 60°C in this case. Products were further purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were ligated into the pCR®2.1-TOPO® vector as described in the protocol of TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). Ligation reaction mixtures were transformed into One Shot® TOP10 competent E. coli cells (Invitrogen). Transformants were selected by blue and white screening. 192 white colonies were randomly selected for both SSU rRNA and *dsr* libraries, and stored in a glycerol stock solution in 96-well culture plates at -80°C. Several plasmid inserts from colonies stored in glycerol stock

were checked by direct PCR using standard M13F and M13R primers and confirmed with agarose electrophoresis.

3.2.6 Sequencing and Phylogenetic Analysis

Clones were purified with QIAquick® PCR purification kit and sequenced bidirectionally by the Canadian Michael Smith Genome Sciences Centre (www.bcgsc.ca) using M13F and M13R primers. Assembled and trimmed sequences of SSU rRNA inserts (Sequencher; Gene Codes, Ann Arbor, MI) were imported and aligned with the ARB phylogeny computer program (Ludwig et al., 2004). All sequences were checked for chimeras with Ribosomal Database Project II chimera check program (Cole et al., 2003). Sequences with higher than 97 % similarity were combined into OTUs using PHYLIP version 3.68 (Felsenstein, 2005) and DOTUR (Schloss and Handelsman, 2005). The closest phylogenetic neighbors were found using BLAST search for the NCBI database (Altschul et al., 1990). The phylogenetic tree and the bootstrap analysis (100 replicates) were constructed with the PhyML software (Guindon and Gascuel, 2003) by using the maximum likelihood method. Trimmed *dsr*A sequences were compared to database and phylogenetic neighbors were found using BLAST search for the NCBI database. The OTUs and phylogenetic tree was constructed with closest relatives and additional cultured SRB species using methods described above.

3.2.7 Batch Experiment

Sediment from Nitinat Lake was incubated with three different carbon materials and sulfate reduction rates were measured in batch reactors during a 196-day study. Acetate, lactate, and a mixture of complex waste materials: compost (University of British Columbia, Vancouver, BC), alfalfa silage (Poundmaker Agriventures, Lanikan, SK), and dried molasses of sugar beet pulp (Westway Feed Products, Tomball, TX) in 1:1:1 (w/w/w) ratio were used. The batch reactor constituents are outlined in Table 3.1. The batch reactors were set up in the anaerobic chamber as follows: 500 mL glass bottles with butyl-rubber septa screw caps were filled with 50 mL of homogenized sediment, carbon source (3 g L^{-1} lactate or acetate, 6 g L^{-1} complex mixture), and filled to the rim with filtered 50 m fjord water. Additionally, one set of bottles contained nutrients (0.5 g L^{-1} $NaNO_3$, 0.025 g L^{-1} KH_2PO_4, 0.025 g L^{-1} K_2HPO_4). These specific nutrients

amounts are used in SRB growth medium Postgate B. A fourth set of bottles was set up with sodium molybdate (4 g L^{-1}), which inhibits the activity of SRB. The bottles were kept sealed in the dark at the *in situ* Nitinat Lake water temperature of 11°C. Water samples were taken periodically from the bottles in the anaerobic chamber to prevent oxygen contamination.

3.2.8 Chemical Analyses

Ammonia, sulfite, and thiosulfate in the water immediately above the sediment were measured immediately after collecting the samples on the boat using Chemets™ kits and procedures (CHEMetrics, Inc., Calverton, VA). Sulfate in the sediment porewater was analyzed using the barium chloride precipitation method (Clesceri et al., 1998). Porewater for sulfide analysis was stabilized immediately by addition of 20 % zinc acetate and later analyzed using the modified methylene blue method of Cline (Cline, 1969). Total carbon, total organic carbon (TOC), and total nitrogen were analyzed by ALS Laboratory Group (Vancouver, BC). Samples from the carbon-amendment batch experiment were immediately filtered through 0.22 μm syringe filters and aliquots were used to measure sulfate and sulfide as described above. The remaining filtered samples were used for volatile fatty acids (VFA) analysis using an HPLC system by Waters (Milford, MA). The HPLC mobile phase for the IC-Pak ion exclusion column (Waters; 7.8 mm diameter, 30 mm length) was 13 mM H_2SO_4 prepared with nanopure water, filtered and degassed. The flow rate was 1 ml min^{-1} and absorbance at 214 nm was measured with UV detector (Waters). Standard solutions of acetate, lactate, propionate, and formate, prepared from HPLC-grade reagents, were used for calibration curves. All analyses were performed in triplicate and the results are presented as average values with error bars as standard deviation.

3.3 RESULTS

3.3.1 Presence and Activity of SRB in Nitinat Lake Sediment

The sediment core collected from Nitinat Lake was uniform in color (dark brown) with fine particle size and strong sulfide odor. It contained 4.7 % (w/w) of total carbon, of which 91 % was organic. Only the top 20 cm layer was used for both phylogenetic analysis and the

amendment experiment, as it was shown in previous studies that SRR typically peaks just below the sediment surface and decreases with depth, as the sulfate becomes depleted (Lehours et al., 2005; Leloup et al., 2007; Harrison et al., 2009). Within the upper 20 cm of the sediment, the sulfate reduction rate measured using radiolabelled sulfate was 250 ± 60 nmol SO_4^{2-} cm^{-3} d^{-1}. There were no significant differences between means of two cores and three incubation times (as determined by t-test), therefore the final SRR is presented as arithmetic mean with standard deviation. As well, quantitative bacterial counts support the high sulfate reduction activity with estimated 15 % of all bacteria, i.e. 5.8 ± 1.1 x 10^7 copies of *dsr* gene cm^{-3}, belonging to the SRB group (Table 3.2).

3.3.2 Phylogenetic Diversity of the Bacterial Community in Nitinat Lake Sediment

Total bacterial community was assessed by phylogenetic analysis of a SSU rRNA clone library. A total of 159 good quality sequences were grouped into 112 operational taxonomic units (OTUs), defined by ≥ 97 % sequence identity. High occurrence of singletons, as well as high Shannon-Wiener index (4.5) suggests that the bacterial population in the upper sediment is fairly diverse. This is despite the fact that, unlike in the majority of sediments at the bottom of shallower waters, the water overlaying Nitinat Lake sediment is permanently anoxic and therefore no steep oxygen and nitrate gradients can develop in the upper sediment layer. Thus, the only major electron acceptor is sulfate. The sequences from the SSU rRNA library fell into eight major phyla. *Proteobacteria* were the most frequently occurring phylum with 30.8 % of total clones (Fig. 3.1). As expected, over 75 % of *Proteobacterial* sequences belonged to the *δ-Proteobacteria* class that contains SRB. Second most abundant group was candidate division JS1 with 23.9 % of clones, followed by *Cyanobacteria* with 15.1 % of sequences. Other bacterial phyla that appeared multiple times in the SSU rRNA library were *Bacteroidetes*, candidate division OP8, *Acidobacteria*, *Planctomycetes*, *Chloroflexi*. Twelve sequences did not affiliate with any known bacterial group. The majority of the sequences were related to clones from similar environments such as marine and hypersaline sediments, but also hydrothermal vents and methane seeps (Fig. 3.2A, B).

The diversity and metabolic implications of SRB were the focus of the phylogenetic analysis. SSU rRNA species distribution confirmed our quantitative results that SRB constitute a significant part of the bacterial population. In addition to SSU rRNA library, the highly

conserved functional gene specific to SRB (*dsr*), was also used for identification of this diverse group. In general, the majority of sequences belonged to completely oxidizing SRB (Fig. 3.3). In both SSU rRNA and *dsr* libraries, most SRB sequences affiliated with the typically acetate-oxidizing *Desulfobacteraceae* family. The largest cluster in the SSU rRNA library (16 sequences), named NITSRB-16S, was closely related (95-98 %) to clone 8bav_D2 from sulfate-methane transition zone of Santa Barbara Basin sediments (Harrison et al., 2009) (Fig. 3.2B). In the *dsr* library, a cluster named NITSRB-DSR, comprising 39 out of the total 109 sequences (including 23 times occurring clone NSED1_4 and 9 times occurring NSED1_10), was also found abundant in the Black Sea sediment and other marine sediments (Fig. 3.3) (Leloup et al., 2007). Identically to the largest SSU rRNA cluster NITSRB-16S, *dsr* cluster NITSRB-DSR's nearest cultured representative is also *Desulfobacterium anilini*. We can assume that these SSU rRNA and *dsr* sequences belong to the same bacterial cells. Besides SRB from *δ-Proteobacterial* class, sequences distantly related to Gram-positive *Desulfotomaculum* sp. from the *Clostridia* class were also present in significant number (Fig. 3.3).

3.3.3 Sulfate Removal in Amended Batch Reactors

Changes in sulfate and sulfide concentration as well as volatile fatty acids (VFA) of sediment amended with acetate are shown in Figure 3.4, with lactate in Figure 3.5, and with complex carbon mixture in Figure 3.6. No significant differences (determined by F-test, α=0.05) between the duplicate bottles A1-A2, L1-L2, and C1-C2 were observed. All reactors supplemented with additional carbon without N, P and K nutrients were able to enhance sulfate reduction significantly, when compared to the control reactor, and reduced more than 93 % of sulfate in the 28-week experiment. During the first 33 d of the experiment, no significant SRB activity was observed in any of the reactors. During the second phase, between days 33 and 100, a rapid decrease in sulfate from 21.7 mM to 3.6 mM in acetate-amended A1, A2 and from 25 mM to 2 mM for lactate-amended L1, L2 occurred. Also, sulfide increased to 9 mM and 10-12 mM in acetate-amended and lactate-amended reactors, respectively. In reactors C1 and C2, more gradual sulfate reduction occurred, with around 50 % of sulfate reduced after 100 d (Fig. 3.6). Interestingly, the addition of nutrients (N, P and K) prolonged the SRB acclimatization phase in reactor AN and CN to ~80 d (Figs. 3.4, 3.6). This was not the case in lactate-amended reactor LN, where addition of nutrients did not affect the SRR significantly. After this extended

acclimation period in AN, sulfate reduced rapidly disappearing within 20 d. Similarly in LN, sulfate reduction exhibited a slightly steeper gradient than L1 and L2 when almost 70 % of sulfate was consumed between days 33 and 72 (Fig. 3.5). When complex carbon materials were added to the sediment, SRB again were stimulated but, as expected, their SRR was less than in the presence of the defined electron donors resulting in only 69 % of sulfate being reduced during the length of the experiment. The addition of molybdate inhibited the SRB activity in reactors with acetate (Amo) and lactate (Lmo) completely, but only decreased their activity in complex carbon reactor Cmo. No sulfate reduction occurred in the control reactor without an additional carbon source.

3.3.4 Volatile Fatty Acids (VFA) Uptake by SRB in Batch Reactors

In acetate-amended reactors, the trend in acetate utilization mirrored that of sulfate consumption. Initially, acetate concentration remained fairly constant at around 35-37 mM in reactors A1, A2, and then decreased rapidly to zero after 90 d. No acetate was consumed in reactor inhibited with molybdate (Fig. 3.4). In lactate-amended reactors, lactate was consumed in first 13 d at a rate of 2 mM d^{-1}. During this period, sulfate concentration remained constant, thus we argue that SRB did not utilize lactate. Also, acetate only appeared in day 9, and no other VFA were detected, so we can assume lactate did not break down into other VFA. After initial steep increase in acetate concentration between days 9 and 13, it kept accumulating, reached its peak of 5.65 mM on day 62, and then decreased rapidly and completely disappeared on day 80.

Many more carbon compounds were available to SRB in the complex carbon reactors, including complex molecules like cellulose (present in compost), sucrose (main component of molasses), but also easily degradable small molecules, like lactate (present in high amounts in silage). We were able to detect the following VFA: lactate, acetate, formate, and propionate. All but propionate were present at the beginning of the experiment, suggesting they were readily available from the added complex waste materials. Nevertheless, no significant sulfate reduction was measured during the first 33 d. During this initial period, lactate and formate completely disappeared. Propionate accumulated between days 5 and 13, and then remained constant until day 33. Interestingly, the reactor supplemented with molybdate exhibited a different pattern during the first 33 days (Fig. 3.6). Both lactate and formate remained in the solution and no propionate accumulated. It appears that SRB are responsible for breakdown of VFA with

electron acceptor other than sulfate or that molybdate inhibits the other microbes that are using lactate and formate. Between days 33 and 46, propionate was rapidly consumed at a rate of 0.19 mM d^{-1} in reactor C1. At the same time, acetate was produced at a rate of 0.5 mM d^{-1} and sulfate was consumed at a rate of 0.77 mM d^{-1}. After day 46, only acetate remained in the reactor and was quickly consumed. During this time, 1.11 mM was consumed per day whereas only 0.17 mM of sulfate was consumed, implicating that besides SRB, other bacteria were also utilizing acetate. No VFA were detected after day 90.

3.4 DISCUSSION

3.4.1 Sulfate Reduction in Nitinat Lake Sediments

Our previous research at Nitinat Lake showed that although the fjord is permanently anoxic below 11-30 m from the surface (its depth is in the range of 150-200 m), SRB make up only a very small fraction of the planktonic bacterial community and no significant sulfate reduction was detected in the anoxic water column (Schmidtova et al., 2009). Therefore, we conclude that the majority of sulfide that is present in the anoxic water is generated in the sediment and diffuses upwards, since biological sulfate reduction into sulfide is an important, if not often dominant, biological process in most sulfate-rich sediments (e.g., (Holmer and Storkholm, 2001; Purdy et al., 2002; Karnachuk et al., 2005)). The rate of sulfate reduction measured in the top layer of the Nitinat Lake sediment is high compared to other high-sulfate marine sediment (Fukui et al., 1997; Trimmer et al., 1997; Kondo et al., 2004). Using the SRB cell estimates determined by qPCR, a cell-specific sulfate reduction rate of 4.3 x 10^{-15} mol SO_4^{2-} $cell^{-1}$ d^{-1} was calculated. Cell-specific sulfate reduction rates of 10^{-17}-10^{-15} mol SO_4^{2-} $cell^{-1}$ d^{-1} were also found in sediments of Colne River estuary (Kondo et al., 2004). It is interesting to note, that those SRR were obtained from sediments at a temperature 24 °C, as compared to ~10.5°C in Nitinat Lake. Typically, SRR has a large positive correlation with temperature, with optimal growth temperature of SRB species averaging at 30ºC in culture studies [*e.g.*, (Nedwell and Abram, 1979)]. Cell-specific SRR in the coastal sediment of the Arctic Ocean was 0.14 x 10^{-15} mol SO_4^{2-} $cell^{-1}$ d^{-1}, which is 30 x less than in Nitinat Lake (Ravenschlag et al., 2000). In this case, the sediment depth was comparable with our study, but the temperature was 0ºC, which was likely the reason for lower SRB activity. In the Black Sea sediments, several orders of magnitude lower

SRR was measured in the sulfate zone, even though the SRB cell numbers were around 10 x higher than in the Nitinat Lake (Leloup et al., 2007). One significant difference between these two locations is the basin depth; whereas Nitinat Lake's sediment is at about 200 m, the Black Sea samples were taken from a depth of 1024 m. Therefore, it is possible that although the number of SRB cells in the Black Sea is higher, their activity in such extreme depth is significantly diminished compared to Nitinat Lake. On the contrary, sulfate reduction rate in shallow sediments (5-10 m deep) on the coast of Japan with intensive shellfish aquaculture averaged around 170 nmol SO_4^{2-} g^{-1} d^{-1} (Asami et al., 2005). This rate is already more than twice as high compared to a neighboring site without any shellfish, but still lower than a rate of 180 nmol SO_4^{2-} g^{-1} d^{-1} calculated for our study. Therefore, it appears that the SRB in Nitinat Lake sediments achieve high overall and cell-specific reduction rates compared to similar environments.

3.4.2 Bacterial Processes in Nitinat Lake Sediments

We identified major bacterial phyla in the upper layer of Nitinat sediment. Our findings showed a large variety of bacterial species, and more diversity is likely to be discovered with additional molecular techniques and increased sample size. The most frequently occurring groups are *δ-Proteobacteria* and candidate division JS1. The dominance of *δ-Proteobacterial* species together with quantitative results, confirms that sulfate reduction has a significant contribution to carbon mineralization in the sediments. The affiliates of SRB in both SSU rRNA and *dsr* libraries were mostly complete-oxidizing sequences from *Desulfobacteraceae* family, which are also found in many other marine sediments, such as the Black Sea, Guaymas Basin, or Santa Barbara Basin (Dhillon et al., 2003; Leloup et al., 2007; Harrison et al., 2009). Many *dsr* OTUs were also closely related to sequences from nearby Puget Sound, even though in this sediment, both oxygen and nitrate were still present in the top layers of the sediment (Tiquia, 2008). The dominant cluster of the *dsr* library NITSRB_DSR (also named Cluster D in Leloup *et* al.(2007)) was related to cultured *Desulfobacterium aniline*, which is able to degrade aromatic hydrocarbons and other organic substrates (Schnell et al., 1989). The ability of this organism to utilize a wide range of complex organic compounds is likely the reason why they are abundant in Nitinat Lake's sediments. Many *dsr* sequences, especially from the deep-branching non-*Proteobacterial* cluster, were related to sequences obtained from Puget Sound, which is in close

proximity to Nitinat Lake. Interestingly, these two environments exhibit different physico-chemical conditions, where Puget Sound sediment samples are at a depth of 3-10 m and oxygen is present at concentrations of 85-250 μM (Tiquia, 2008). The ability of these species to adapt to various challenging environmental conditions by spore formation and extensive electron donor utilization enables them to thrive in these environments.

It is interesting to note that sequences from *ε-Proteobacteria* most likely related to sulfur oxidation, which entirely dominated throughout the anoxic water column (Schmidtova et al., 2009), were completely absent in the sediment. Even though a similar distribution of chemical species can be seen in the upper sediment and overlaying anoxic water, bacterial processes, as determined by bacterial sequence distribution, differ greatly. Also unlike similar marine coastal sediments, common phyla such as *Chloroflexi* and *Planctomycetes* were not abundant in our samples. A number of sequences affiliate with sequences from methane-rich environments (Fig. 3.2A, B), suggesting that besides sulfate reduction, methanogenesis and/or anaerobic methane oxidation is also substantial in Nitinat Lake sediments. For example, *Bacteroidetes* clone NSED2_32 is 93 % identical to a clone from methane-seep sediment in the Eel River Basin in California (Beal et al., 2009). This study used an enrichment experiment to show that anaerobic oxidation of methane (AOM) is possible with electron acceptors other than sulfate, such as manganese or iron. They also identified bacterial sequences likely responsible for metal-dependent AOM, in addition to previously found sulfate-dependent AOM archae.

Candidate divisions also occurred frequently in the clone library. The most dominant division JS, which does not have a cultured representative, was previously found in deep marine sediments, but also in methane-hydrate bearing sediments, near mud volcanoes and hydrothermal vents (Li et al., 1999b; Inagaki et al., 2003; Webster et al., 2007). Unfortunately, due to the lack of isolates, their exact role in carbon mineralization cannot be defined, although, they seem to occur frequently in organic-rich, sulfate-reducing or methane-discharging environments (Webster et al., 2007). A stable-isotope study found that JS1 bacteria were able to actively assimilate ^{13}C-labelled acetate and glucose under sulfate-reducing conditions (Webster et al., 2006), thus possibly competing for these electron donors with SRB. Surprisingly, many sequences belonged to *Cyanobacteria*/chloroplast group, specifically sequences similar to chloroplasts of diatom *Chaetoceros calcitrans*. It is very unlikely that light sufficient for photosynthesis is available in the sediment layer. Therefore, these sequences are most probably

obtained from dead algal cells that were deposited from the water surface layers during an algal bloom that usually occurs in March or April in Nitinat Lake.

3.4.3 Enhancement of Sulfate Reduction with Addition of Electron Donors

The differences in SRR between the reactors amended with different carbon sources are outlined in Figure 3.7. The lag phase observed in all reactors is slightly higher than other batch or continuous-flow reactor studies, where sulfate reduction was established after ca. 3 weeks (Waybrant et al., 1998; Cocos et al., 2002; Logan et al., 2005). The relatively long acclimatization phase was likely caused by the changes in environmental conditions during the transfer and set-up of the reactors, such as pressure and temperature changes, or possible oxygen contamination although steps were taken to prevent this. In addition, the lag phase in reactors AN and CN was even longer than in reactors that were not supplemented with N, P, K, which suggests that other bacterial communities benefitted from added nutrients and outcompeted SRB at the early phase. The addition of molybdate (4 g L^{-1}) had only partial effect on the SRR in complex carbon-amended reactor. This can be explained by the different structures of the carbon sources: Whereas acetate and lactate were added to the reactor in a liquid form, complex carbon material consisted of solid particles of compost, molasses, and silage, and thus some molybdate could have bound (physically or chemically) to the particles. Higher concentration would have likely completely inhibited the SRB.

The maximal SRR is calculated as the slope of sulfate reduction during the linear decrease phase. The highest overall SRR (240 nmol SO_4^{2-} cm^{-3} d^{-1}) was observed in acetate-containing reactor AN, followed by A1-A2 and lactate-containing reactors. This SRR is almost identical to the SRR measured *in situ* in the sediments using the radiotracer (Table 3.2). During the linear phase of sulfate reduction, SRR in reactor AN reached 1000 nmol SO_4^{2-} cm^{-3} d^{-1}, suggesting that once the SRB community is established, acetate in combination with nutrients N, P and K is the most favorable organic substrate for stimulation of SRR. Similar observations were made in other coastal sediments, such as in Ise Bay, Japan (Fukui et al., 1997), or Scottish estuaries (Parkes et al., 1989). On the other hand, acetate addition to lake sediment impacted with acid mine drainage did not result in decreased sulfate concentration (Fauville et al., 2004). The second highest SRR of 500 nmol SO_4^{2-} cm^{-3} d^{-1} was obtained from reactor LN, followed by reactors amended with only acetate and lactate. The SRR for the complex carbon reactors ranged

from 90 to 130 nmol SO_4^{2-} cm^{-3} d^{-1}. In a study by Gibert et al. (2004), SRR of 80 and 250 nmol SO_4^{2-} cm^{-3} d^{-1} was achieved when compost with poultry manure and oak leaf with sheep manure, respectively, was used. Significantly higher SRR (600-1800 nmol SO_4^{2-} cm^{-3} d^{-1}) was achieved in a column experiment with a mixture of wood shavings, manure, alfalfa pellets (Logan et al., 2005), and in a batch system with sewage sludge, leaf mulch, wood chips, manure, sawdust and cellulose (up to 1135 nmol SO_4^{2-} cm^{-3} d^{-1}) (Waybrant et al., 1998). Many factors can influence the SRR in laboratory-scale systems, such as the reactor type, amount and characteristics of added carbon source, SRB inoculum, initial sulfate concentration, temperature, length of the experiment, etc., and the results from different laboratory-scale studies are often contradictory (Neculita et al., 2007).

In terms of the organic substrate uptake, several conclusions can be drawn from comparing the experimental measurements with the theoretical stoichiometric values. Theoretically, 1 mol of acetate is needed for reduction of 1 mol of sulfate (Table 3.3). During the maximal SRR phase in reactors A1 and A2, 1.6 mol acetate is consumed per 1 mol of sulfate. Therefore, assuming that acetate is the sole electron donor for SRB, the remaining acetate must be taken up by other groups, like methanogens or candidate division JS1. It has been shown in other studies that methanogens compete with SRB for electron donors, especially acetate, although sulfate reduction with acetate is thermodynamically more favorable compared to methanogenesis (Raskin et al., 1996). Methane-producing microorganisms are typically archaea; therefore, we could not detect them in the bacterial clone library. Curiously, acetate concentration remained almost unchanged in reactor Amo with molybdate (Fig. 3.4). It appears that with SRB inhibited, the acetate is not utilized by other groups. However, a study by Patidar and Tare (2005) showed that methanogens can also be in some cases inhibited by molybdate, which would explain why acetate was not consumed in Amo. In lactate-amended reactors, lactate was consumed rapidly by processes other than sulfate reduction. One possibility is the fermentation of lactate into ethanol (Table 3.3). This was the case in sulfidogenic continuously stirred tank reactor (CSTR), where lactate was fermented to ethanol and then ethanol was utilized by SRB (Zhao et al., 2008). Ethanol can be oxidized by SRB either completely or incompletely to acetate. The stoichiometry dictates that for every 1 mol of sulfate reduced with ethanol, 2 mol of acetate should be produced. When looking into the results for reactors L1 and L2 during days 33-62, during which time acetate accumulated, we can calculate that for every 1 mol of reduced sulfate, only 0.26 mol of acetate was produced. This suggests that either the majority of ethanol was oxidized completely by SRB, or the produced acetate was immediately

utilized by other microbes. In the reactor with molybdate, lactate was also consumed in the same manner as in the remaining lactate-amended reactors. However, the concentration of accumulated acetate was approximately half of that in reactors L1, L2, and LN (Fig. 3.5). Accordingly, some acetate must have been produced by SRB. During the acetate consumption phase between days 60 and 80, the rate of acetate consumption was 314 nmol cm^{-3} d^{-1} and the rate of sulfate consumption was 318 nmol cm^{-3} d^{-1}. This is very close to stoichiometric ratio 1:1, thus strongly suggesting than SRB utilized solely acetate during this period. After 90 days acetate could not be detected in the reactor, sulfate reduction tapered off and completely stopped on day 100. These results again suggest that acetate and possibly ethanol are the favored electron donors for SRB in lactate-amended reactors.

For reactors with complex carbon materials addition, three VFAs could be detected in the reactor start-up. Between days 0 and 33, consumption of lactate correlated well with formation of acetate and propionate, suggesting that mixed-acid fermentation occurred in the reactors. Again, this was not the case for the SRB-inhibited reactor Cmo. One possible explanation is that other bacterial groups responsible for this reaction were also inhibited by molybdate. During the second phase between days 33 and 46, it is likely that propionate served as an electron donor for part of the reduced sulfate, with acetate as the end product. Although, other carbon molecules must have been taken up by SRB as well, as the amount of consumed propionate is lower than the stoichiometric ratio (Table 3.3). Interestingly, no VFAs could be detected in the later stages of the experiment, even though sulfate was still being reduced. Therefore, other electron donors, such as sugars, alcohols, H_2, or more complex carbon molecules, must have been consumed after this time. Further studies will be necessary to specify the electron donors for SRB in such a complex system. Also, unlike the reactors amended with acetate and lactate, additional bacteria were introduced into the reactor with the carbon source, thus further bacterial groups, for instance cellulose degrading bacteria, fermenters, acidogens, etc., not found in the original sediments, could have been enriched.

The laboratory-scale study of Nitinat Lake sediment's sulfate reducing potential enabled us to obtain kinetic rates of sulfate reduction correlated with carbon degradation. Understandably, the bacterial community structure of the *in situ* sediment and the enriched bench-scale reactors would be different, but overall trends can be still applicable. The 40-day lag phase suggests that significant acclimatization was necessary. This could have been due to the change in environmental factors, most importantly pressure, light and oxygen exposure, as well as addition of a single particular carbon material. However, the results indicating the preference

of acetate as SRB electron donor in the bench-scale reactors can be correlated with the phylogenetic analysis of the *in situ* sediment bacterial community, where the majority of SRB sequences are also associated with acetate-oxidizing SRB species (Fig. 3.2B, 3.3). Therefore, we can assume that addition of acetate to the Nitinat Lake sediment layer could increase sulfate reduction rate up to 4-fold, as determined by the laboratory experiment.

3.4.4 Implications of Nitinat Lake Study for Use of Mine Pit Lakes for Treating MME

One motivation for studying microbial processes in a permanently stratified water body such as Nitinat Lake was to determine the factors that influence sulfate and sulfide cycling, which is of great importance in mine pit lakes. The inaccessibility and sampling challenges were reasons why we chose to study a natural stratified system, Nitinat Lake, instead of an actual mine pit lake. Nevertheless, there are some key similarities, namely high sulfate concentrations and stratification that lead us to believe that the bacterial processes would be analogous between natural and man-made lakes of this type.

Mine pit lakes are used for MME remediation based on the assumption that after the flooded mine pit becomes stratified, sulfate-reducing activity by SRB in the anoxic layer will occur and remove dissolved metals (Castro and Moore, 2000). This was the case for The Island Copper Mine Pit near Port Hardy, B.C., which was flooded with seawater and capped with freshwater (creating a pycnocline similar to Nitinat Lake) in order to develop permanent stratification. Even 4 years after the flooding, the deep intermediate layer (from 10 m to 220 m below surface) receiving MME, was not completely anoxic and still contained 2 mg L^{-1} of dissolved oxygen (Fisher and Lawrence, 2006). Continuous fertilization of the surface waters over the course of the next 4 years resulted in increased primary productivity, which further decreased the dissolved oxygen. A simplified mass balance that considered the intermediate zone to be a continuously stirred reactor, and included actual metal concentrations and flow rates of MME, resulted in the estimation that a minimum of 10.2 tonnes of copper and zinc per year would have to be removed from the anoxic layer in order to meet the water quality standards. Assuming near-neutral pH, approximately 50 % of produced sulfide would be available for metal sulfide precipitation. Therefore, 318 kmol per year of sulfide would have to be produced (or sulfate reduced, assuming that sulfide is produced solely by SRB). However, the outcomes from

the current study show that significant sulfate reduction is restricted to the upper layer of sediment and does not occur in the anoxic water column. Assuming SRR determined from the Nitinat Lake sediment can be extended to the Island Copper Mine Pit Lake sediment, whose surface area is 1.72 km^2, 31.4 Mmol of sulfate could be reduced per year, thus providing enough sulfide to satisfy the metal removal requirements. However, it is important to note that all SRB activity is confined to the upper layer of the sediment, and diffusion limitations have to be taken into account, especially when MME is injected into the intermediate or upper layers. Considering the overall volume of the system, a sulfate reduction rate of 0.35 nmol cm^{-3} d^{-1} in The Island Copper Mine Pit Lake and 0.50 nmol cm^{-3} d^{-1} in Nitinat Lake is calculated. Also, less than 0.1 % of the system's volume is actively involved in the treatment process. The study of Nitinat Lake's sulfate reduction by combining molecular and chemical analyses showed that hypothesis assuming high rates of sulfate reduction in the anoxic water column, upon which the design of stratified mine pit lakes is based on, might not be accurate.

Therefore, one potential improvement in the mine pit lake design would be to increase the surface/volume ratio and thus the active layer of sulfate reduction. This can be done by designing a structured bottom surface of the mine pit, increasing the surface area where SRB community can be established. Also, the MME flow can be redirected to enter the pit lake at the bottom. For mine pit lakes currently in operation, the process can be optimized by the addition of easily available porous material that would sink to the bottom and increase the active depth of sulfide formation, mimicking the conditions in a constructed wetland. As determined by the bench-scale study with different electron donors for SRB, addition of complex organic material did not directly affect the SRR, but if added to the mine pit lake, it would rather provide solid media for SRB and contribute to the overall carbon budget. In order to increase SRR directly, acetate was found as preferred electron donor and could be added at the beginning for SRB establishment. However, continuous amendment with pure carbon source would increase costs and maintenance and is not feasible in passive treatment systems.

3.5 CONCLUSIONS

The following conclusions can be drawn from the present study:

- Sulfate reduction represents significant mineralization process in the upper 20 cm of Nitinat Lake sediment with an average sulfate reduction rate of 250 ± 60 nmol SO_4^{2-} cm^{-3} d^{-1}. Fifteen

percent of total bacteria ($5.8 \pm 1.1 \times 10^7$ copies cm^{-3}), as determined by quantitative PCR, belong to the SRB, which results in a cell-specific sulfate reduction rate of $4.3 \pm 1.0 \times 10^{-15}$ mol SO_4^{2-} $cell^{-1}$ d^{-1}.

- *δ-Proteobacteria*, candidate divisions JS1 and OP8, *Cyanobacteria*, and *Bacteroidetes* were most represented bacterial groups in the sediment's SSU rRNA clone library. Both SSU rRNA and *dsr* clone libraries identified *Desulfobacteraceae* the dominant SRB family.
- Supplemented organic carbon did enhance the SRB activity in batch experiments, with 93 % or higher percentage of initial sulfate consumed in any uninhibited reactor after 28 weeks. The reactor amended with acetate and nutrients exhibited the highest SRR of 1.0 μmol cm^{-3} d^{-1} during the maximum growth phase. In general, much faster sulfate consumption was observed in reactors with pure carbon materials (acetate and lactate) compared to the reactors with complex carbon materials. The addition of nutrients did not have significant effect on the SRB activity.
- VFA measurements from acetate- and lactate-amended reactors show that acetate is the preferred electron donor for SRB in this system. Lactate was not utilized by SRB, but rather by other bacterial groups present. The stoichiometric ratios suggest that other bacteria, for example methanogens, also utilize acetate. This agrees well with the phylogenetic findings, where mostly relatives of acetate-oxidizing SRB were found in the libraries.
- SRB in reactors amended with complex carbon mixture use also other electron donors besides VFA. We found that lactate and formate can be utilized by SRB without sulfate as electron acceptor. Propionate and acetate were electron donors for dissimilatory sulfate reduction. The use of complex carbon mixture as a sole carbon source in sulfate reduction reactors is promising alternative to pure substances due to slower substrate uptake rate and thus longer time period of active sulfate reduction, however, it would not have a significant effect on SRR in a natural stratified basin, where other carbon sources, such as algal blooms and primary productivity deposits, are available.
- The design of mine pit lakes for MME treatment should be optimized based on the rates and distribution of SRB found in Nitinat Lake. Models that are based on sulfide formation in the anoxic water column are perhaps not accurate, as this process occurs only below the sediment surface. The efficiency can be increased for example by increasing the sediment surface area and diverting the MME flow through the sediment layer. One-time addition of acetate would

be most effective in promoting the activity of SRB. Certainly more research on SRR in mine pit lakes is needed to accurately assess the treatment potential.

3.6 TABLES AND FIGURES

Table 3.1: Set-up of batch reactors for sulfate reducing experiment

Reactor	Organic carbon	Nutrients (N, P, K)	Molybdate
A1	acetate	no	no
A2	acetate	no	no
AN	acetate	yes	no
Amo	acetate	no	yes
L1	lactate	no	no
L2	lactate	no	no
LN	lactate	yes	no
Lmo	lactate	no	yes
C1	complex mixture	no	no
C2	complex mixture	no	no
CN	complex mixture	yes	no
Cmo	complex mixture	no	yes
Control	no	no	no

Table 3.2: Chemical and biological characteristics of Nitinat Lake sediment sample

	Station S5
Depth (m)	170
Water content (%)	78
Total carbon (C) (%)	4.7
TOC	4.3
Total nitrogen (N) (%)	0.29
C/N	16.2
Ammonium (mg/L)*	3.5
Sulfate (mg/L)	2000
Sulfide (mg/L)	1.73
Thiosulfate (mg/L)*	6
Sulfite (mg/L)*	5
SRR (nmol/cm^3/d)	248.6 ± 64.1
Total bacteria (10^7 copies/mL)	5.8 ± 1.1
SRB (10^6 copies/mL)	8.7 ± 0.7
SRB (%)	15

TOC, total organic carbon; C/N, molar ratio of total carbon to total nitrogen; SRR, sulfate reduction rate (measured at 11°C); total bacteria as determined by SSU rRNA qPCR amplification, SRB as determined by *dsr* gene qPCR amplification. * measured in the water column above the sediment.

Table 3.3: Stoichiometry of sulfate reduction

Electron donor	Reaction	Electron donor : acceptor stoichiometric ratio
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^-$	1:1
Lactate	$CH_3CHOHCOO^- + 3/2\ SO_4^{2-} \rightarrow 3\ HCO_3^- + 3/2\ HS^- + 1/2\ H^+$	3:2
Lactate	$CH_3CHOHCOO^- + 1/2\ SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 1/2\ HS^- + 1/2\ H^+$	2:1
Propionate	$CH_3CH_2COO^- + 3/4\ SO_4^{2-} \rightarrow 3/4\ HS^- + CH_3COO^- + HCO_3^- + 1/4\ H^+$	4:3
Formate	$HCOO^- + 1/4\ SO_4^{2-} + 1/4\ H^+ \rightarrow 1/4\ HS^- + HCO_3^-$	4:1
Ethanol	$CH_3CH_2OH + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^- + H^+ + H_2O$	1:1
Ethanol	$CH_3CH_2OH + 1/2\ SO_4^{2-} \rightarrow CH_3COO^- + 1/2\ HS^- + 1/2\ H^+ + H_2O$	2:1
Hydrogen	$H_2 + 1/4\ SO_4^{2-} + 1/4\ H^+ \rightarrow 1/4\ HS^- + H_2O$	4:1
Lactate	$CH_3CHOHCOO^- + H_2O \rightarrow CH_3CH_2OH$	——
Lactate	$3\ CH_3CHOHCOO^- \rightarrow 2\ CH_3CH_2COO^- + CH_3COO^- + CO_2$	——
Molasses (Sucrose)	$C_{12}H_{22}O_{11} + H_2O \rightarrow 4\ CH_3CHOHCOOH$	——

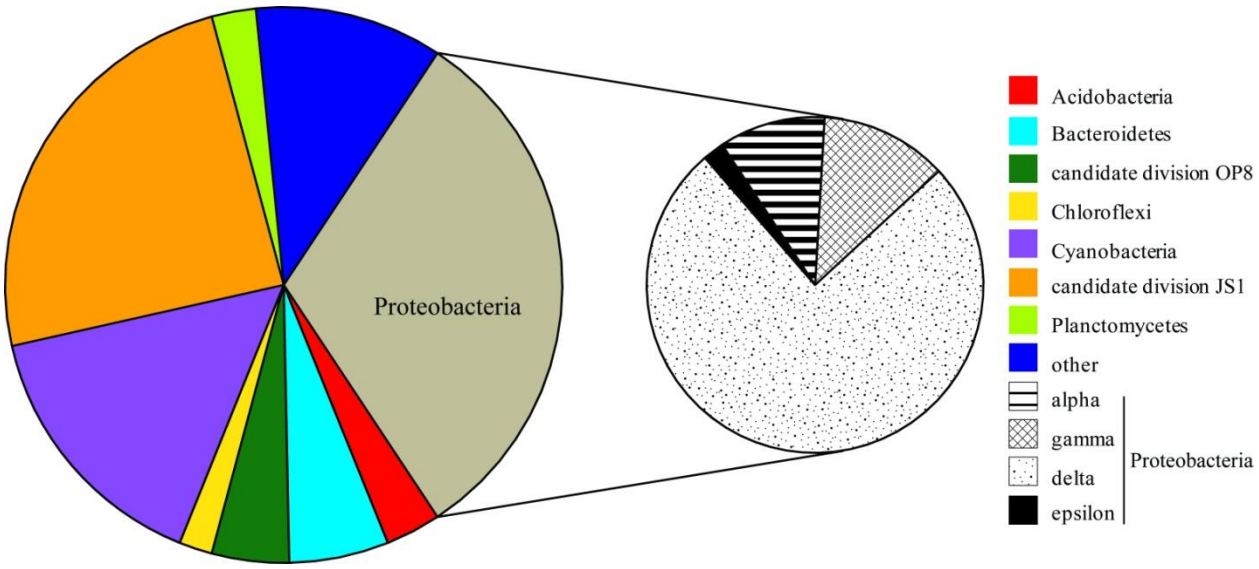

Figure 3.1: Distribution of major phylogenetic group as determined by the SSU rRNA clone library.


A
NSED2_30
Galbibacter mesophilus (AB255367)
Sphingobacterium sp. (AM411962)
clone BacA_056, soil (EU335290)
NSED2_19
NSED2_29
clone SSS8N, hypersaline sediment (EU592419)
NSED2_52 (2x)
Bacteria from anoxic bulk soil (AJ229237)
NSED2_31
clone C13S-108, marine sediment (EU617865)
Bacteroidetes bacterium, hydrothermal vent (AJ969457)
Cytophaga sp. Dex80-37 (AJ431253)
clone Mn3b-B31, methane seep sediment (FJ264576)
NSED2_32
clone 104B392, marine sediment (EF687321)
NSED2_33
clone CK_1C3_43, sediment (EU488021)
NSED2_34
clone Mn3b-B10, methane seep sediment (FJ264591)
Cytophaga sp., deep-sea sediment (AB015532)
Bacteroidetes
clone 5bav_F7_ARB, marine sediment (EU181500)
NSED2_16
NSED2_17
clone A8S-116, marine sediment (EU652668)
clone MD2902-B110, marine sediment (EU385913)
clone SPG12_213_223_B92, ocean sediment (FJ746241)
NSED2_15
NSED2_14
Pirellula sp. (X86393)
Planctomycetes
clone Er-LLAYS-77, sediment and soil slurry (EU542526)
NSED2_39 (2x)
clone Er-MS-24, sediment and soil slurry (EU542437)
NSED2_13
Chloroflexi
clone BS1-0-111, tidal flat (AY255001)
NSED2_1 (2x)
Prochlorales cyanobacterium EV-7 (DQ059300)
clone 051011_T3S4_W_T_SDP_322 (FJ349923)
NSED2_28
NSED2_92
Cyanothece sp. (AF132771)
clone 1533AA_41, South Pacific (EU188269)
NSED2_93
NSED2_98
NSED2_99
clone HH1516, Lake Cadagno chemocline (FJ502256)
clone TDNP_USbc97_2_47_65, upper sediment (FJ516953)
Chaetoceros calcitrans isolate (FJ002215)
NSED2_105
NSED2_102
NSED2_106 (2x)
clone s109, ocean water (EU919852)
Podosira stelligera isolate (FJ002172)
NSED2_108 (7x)
clone bOHTK-105, cold-seep sediment (FJ873346)
NSED2_117 (2x)
NSED2_115 (2x)
NSED2_100
clone Cc095, marine sponge (AY942770)
NSED2_101
NSED2_104
Cyanobacteria / chloroplasts
NSED2_11 (2x)
clone C90, volcanic deposit (FJ466150)
clone B76, sphagnum peat bog (AM162428)
clone HF_NC_23, soil (FJ625362)
NSED2_10
NSED2_9
Acidobacteria
Acidobacterium capsulatum (D26171)
clone MS12-2-A07, hydrothermal vent (AM712324)
NSED2_8
clone SSS85N, hypersaline sediment (EU592454)
NSED2_48
clone CK_2C6_20, sediment (EU488495)
NSED2_50 (2x)
clone CK_1C3_11, sediment (EU487989)
NSED2_56
clone NKBT8, deep-sea sediment (AB013270)
clone MD2896-B58, marine sediment (EU385679)
NSED2_57 (2x)
Candidate division OP8
clone 113B509, deep-sea sediment (EF687513)
NSED2_3
clone 5bav_H9, marine sediment (EU181511)
NSED2_4
NSED2_5 (2x)
Caldicellulosiruptor kronotskiensis (EF100909)
Moorella sp. (AY766035)
SJA-88 (AJ009479)
NSED2_20
clone B9D, hydrothermal field sediment (FJ205198)
NSED2_27
NSED2_54
NSED2_70 (2x)
NSED2_69
clone Z273MBM16 (FJ485343)
NSED2_61
NSED2_62
clone CK_1C1_18, sediment (EU487873)
clone MD2896-B27, marine sediment (EU048684)
clone MD2905-B10, marine sediment (EU386122)
NSED2_63
unclassified
NSED2_45
NSED2_46 (2x)
clone 09D2Z45, hypersalin mat (DQ329569)
NSED2_44
NSED2_41
clone CK_1C4_63, sediment (EU488092)
candidate division KSB1
NSED2_119
NSED2_120 (2x)
clone CK_1C2_54, sediment (EU487957)
NSED2_97
NSED2_130
NSED2_138
NSED2_129
NSED2_133
NSED2_132
NSED2_134 (2x)
NSED2_136 (2x)
candidate division JS1 bacterium, marine sediment (AM745156)
NSED2_140
candidate division JS1 bacterium, marine sediment (AM745153)
clone B103E10, marine sediment (FJ455886)
candidate division JS1 bacterium (AM746086)
NSED2_141 (2x)
NSED2_144 (4x)
NSED2_143
NSED2_147 (2x)
clone KZNMV-30-B24, Mediterranean Sea (FJ712605)
NSED2_142
NSED2_151 (7x)
NSED2_139
clone bOHTK-28, cold-seep sediment (FJ873257)
clone MD2902-B161, marine sediment (EU385947)
NSED2_126 (2x)
NSED2_128
NSED2_122
NSED2_131
clone, hydrocarbon seep sediment (DQ007538)
clone MD2896-B132, marine sediment (EU385733)
clone MA-A2-104, deep sediment (AY093459)
candidate division JS1
0.1

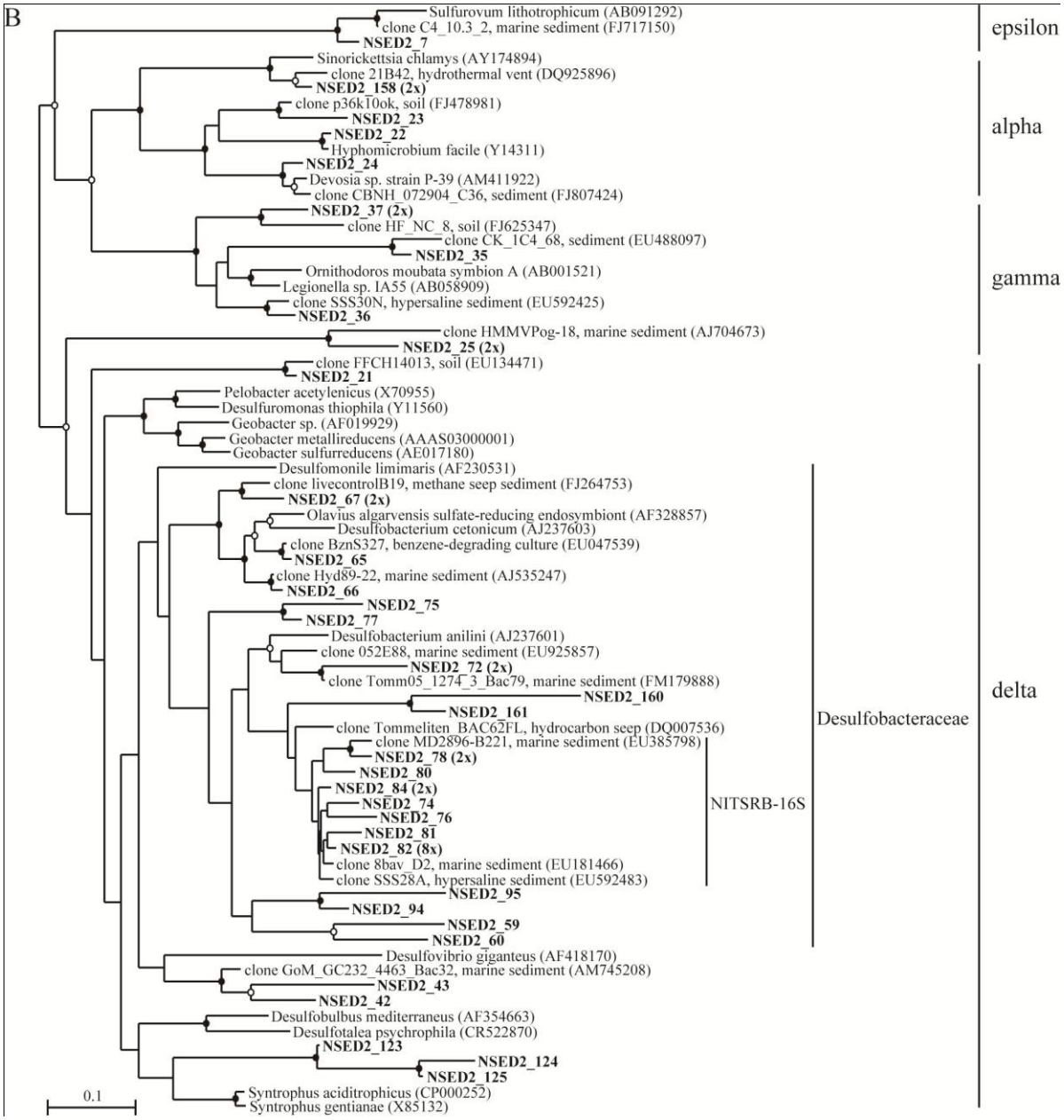

Figure 3.2: Unrooted maximum likelihood phylogenetic tree showing (A) other than *Proteobacterial* and (B) *Proteobacterial* SSU rRNA gene sequences from Nitinat Lake sediment and related sequences. Clones from this study are in bold and numbers in brackets represent frequency in the library. Bootstrap values are shown with following symbols: open circles for >50 % and closed circles for >90 % (for 100 iterations). Values below 50 % are not shown.

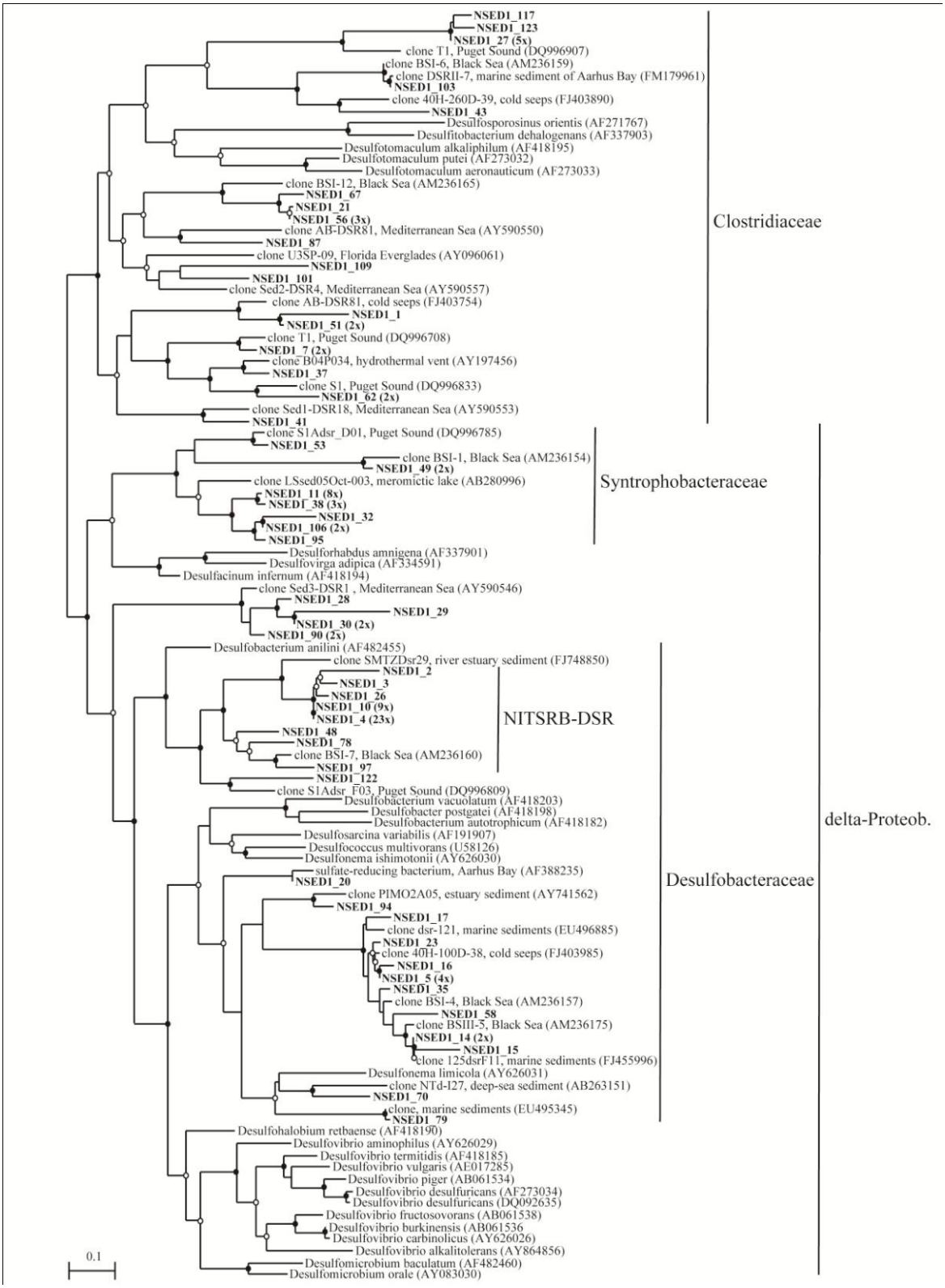

Figure 3.3: Unrooted maximum likelihood phylogenetic tree showing *dsr* gene sequences from Nitinat Lake sediment and related sequences. Clones from this study are in bold and numbers in brackets represent frequency in the library. Bootstrap values are shown with following symbols: open circles for >50 % and closed circles for >90 % (for 100 iterations). Values below 50 % are not show.

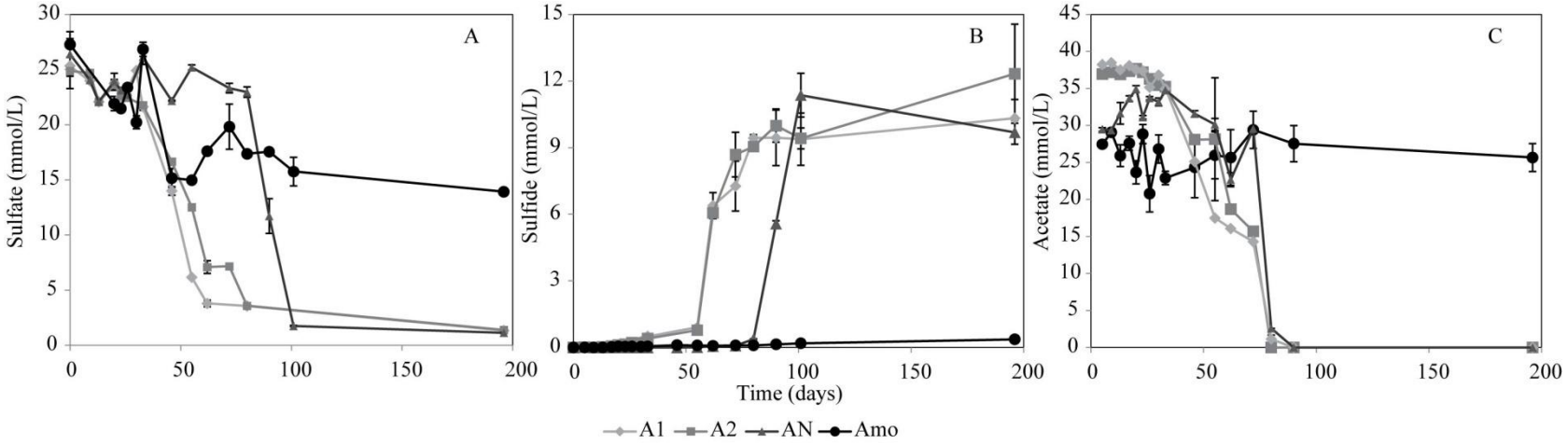

Figure 3.4: Profiles of sulfate (A), sulfide (B), and acetate (C) in batch reactors amended with acetate. Data are shown as average values of triplicate measurements and error bars represent standard deviation.

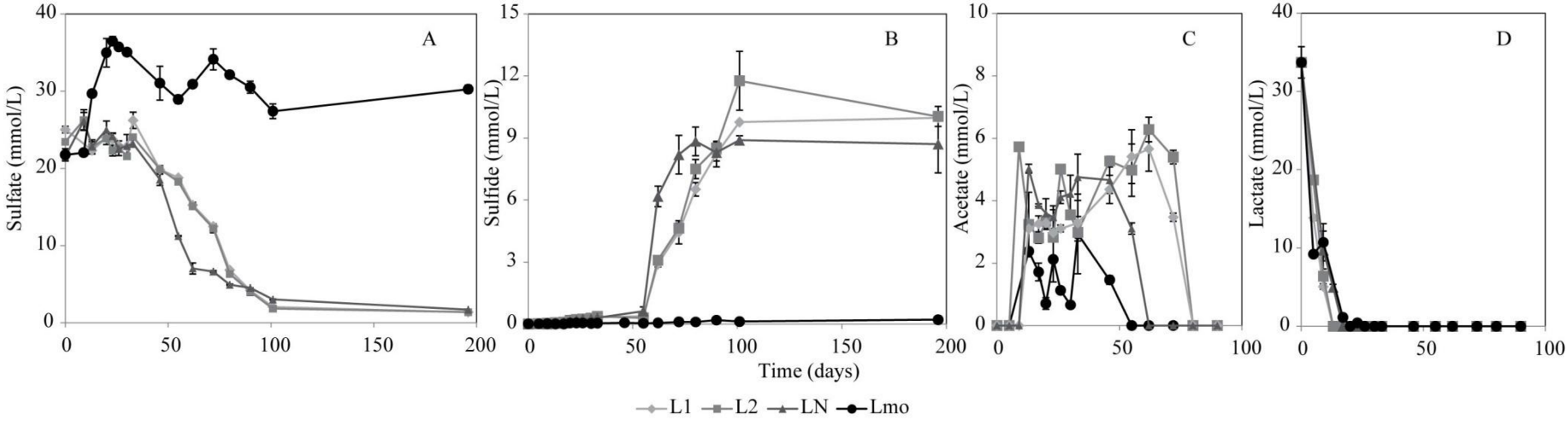

Figure 3.5: Profiles of sulfate (A), sulfide (B), acetate (C), and lactate (D) in batch reactors amended with lactate. Data are shown as average values of triplicate measurements and error bars represent standard deviation.

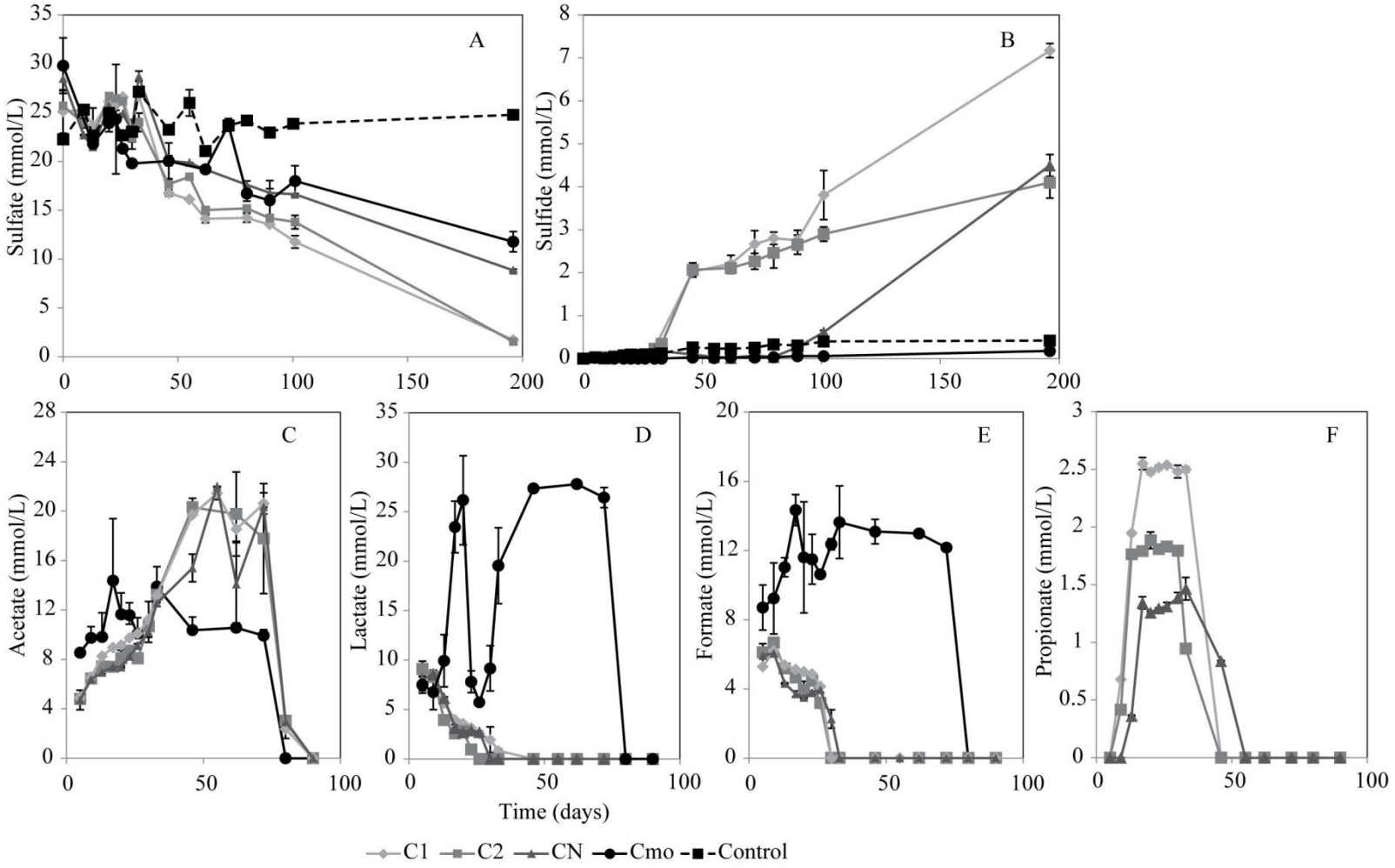

Figure 3.6: Profiles of sulfate (A), sulfide (B), acetate (C), lactate (D), formate (E), and propionate (F) in batch reactors amended with complex carbon mixture. Data are shown as average values of triplicate measurements and error bars represent standard deviation.

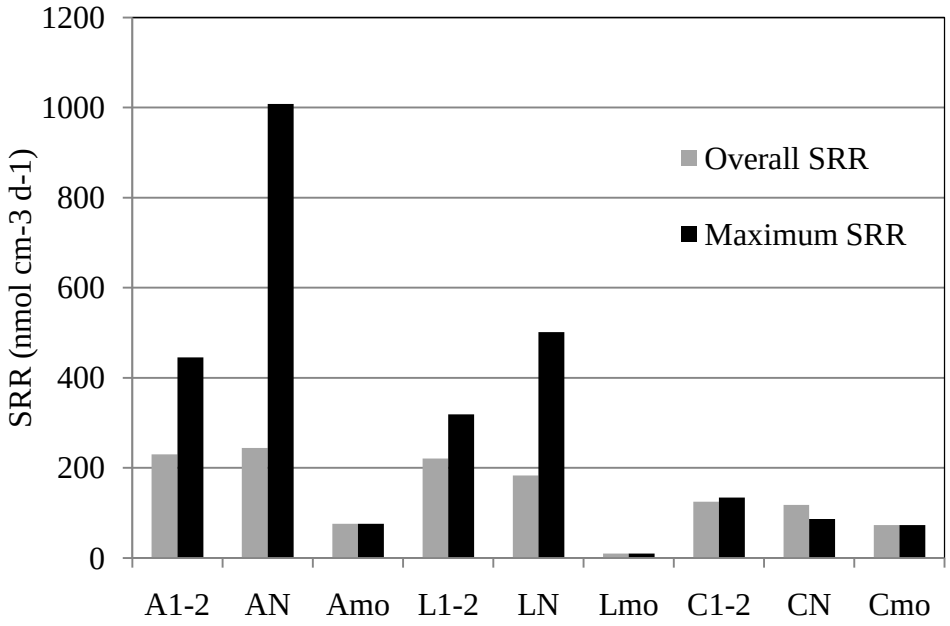

Figure 3.7: Sulfate reduction rates in batch reactors with different carbon materials. Values for A1-2, L1-2, and C1-2 are shown as average values between the two. Statistical t-test confirmed no significant differences between SRR of duplicate reactors.

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CHAPTER 4

CORRELATION OF BACTERIAL COMMUNITY STRUCTURES SUPPORTED BY VARIOUS ORGANIC MATERIALS WITH SULFATE REDUCTION IN METAL-RICH LANDFILL SEEPAGE

4.1 INTRODUCTION

Mine drainage, which is often acidic (acid rock drainage or ARD), is the most common pollution problem related to mining in many parts of world. For example, the Canadian metal ore mining industry, with British Columbia being prominent among the provinces containing mines, released 1,600 tonnes of polluted effluent in 2004 according to the National Pollutant Release Inventory (Government of Canada, 2006). During and after mining, rocks with sulfide minerals are exposed to air and water causing their oxidization. This generates seepage, which can contain high concentrations of sulfate and dissolved metals (INAP, 2009). Also, legacy technologies, such as roasting, generated large quantities of dust that contained, among other metals, arsenic, which, having no economic value, was landfilled. Now seepage from these landfills poses a threat to aquatic life in receiving environments and treatment to remove the toxic compounds is required (Government of Canada, 1996). The most widely used approach for treating mine effluents is to use chemical reagents for neutralization and precipitation; the high density sludge process that uses lime being the current industry standard (INAP, 2009). However, this process may not be economically feasible at remote and inaccessible sites or where long-term treatment is necessary (Johnson and Hallberg, 2005a). As well, safe storage of the metal-laden sludge is a major challenge. Alternatively, passive bioremediation systems, such as constructed sub-surface flow wetlands and permeable reactive barriers, are less expensive since they rely on natural processes such as sulfate reduction by sulfate-reducing bacteria (SRB) that raise the pH and

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generate sulfide, which causes metals to precipitate (Gusek, 1995; Gusek et al., 2007; Eccles, 1999; Reisinger et al., 1999; Younger et al., 2002; PIRAMID Consortium, 2003; Smyth et al., 2004). Although bioremediation can remove metals down to very low concentrations, it is not always reliable or sustainable. In particular, diagnosing causes of treatment failure is difficult and passive treatment systems often operate only for very short periods of time before failing. This is because the mechanisms responsible for metal removal have not been well characterized. Bacteria are deemed to play an important role in treatment but their presence and activity are rarely measured. Knowledge about the dynamics and diversity of microbial consortia and the rates of organic material degradation are areas that need to be addressed so as to assess the longevity of these systems (Johnson and Hallberg, 2003; Neculita et al., 2007). These knowledge gaps impede successful and sustainable implementation of passive treatment systems and their acceptance by regulatory bodies.

Passive treatment systems designed for removal of metals other than Fe and Al are also referred to as anaerobic bioreactors (ABRs) since they are excavated areas that are filled with natural or waste organic-rich materials through which the metal-contaminated water flows. The organic material supplies nutrients for anaerobic microbes that facilitate reduction and precipitation of metals. To minimize costs, waste organics close to the mine site are used, such as woodchips and sawdust (Johnson and Hallberg, 2005b; Doshi, 2006), manure (Zaluski et al., 2003; EPA, 2006), agricultural byproducts such as hay and alfalfa (Bechard et al., 1994), composted food waste or natural vegetation (McIntire et al., 1990; Stark et al., 1990), or industrial wastes such as pulp mill waste (Duncan et al., 2004; Hulshof et al., 2006). The consensus of most of the laboratory- and pilot-scale studies to date is that a mixture of different materials is more effective than one single type with respect to sulfate-reduction, metal removal rates and pH increase (Waybrant et al., 1998; Neculita et al., 2007; Brown, 2007). However, the long-term *in situ* performance of different organic materials has not been addressed (Neculita et al., 2007). In addition, it is still not known why certain complex organic materials or mixtures are better at supporting sulfate reduction. This makes choice of an appropriate organic matrix for a particular treatment system still an arbitrary one based mainly on what is locally available.

Depending on the characteristics of the organic compounds used, different microbes will proliferate that degrade high molecular weight polymers, such as hemi-cellulose and cellulose, into monomers. Because SRB utilize only certain simple carbon sources that are generally not present in complex organic wastes, they rely on these other microbes, including hydrolytic, acidogenic and acetogenic bacteria, to supply low molecular weight electron donors. Therefore,

often, the bottle neck and rate-limiting step of sulfate-reduction in ABRs for mine drainage treatment is the production rate of low molecular weight electron donors from hydrolysis of complex organics (Waybrant et al., 1998; Castro et al., 1999; Gibert et al., 2003). However, no information is available on how bacterial communities supported by different types of organic material differ, and how this correlates with the SRB diversity and the potential for sulfate reduction, which is the aim of the current study.

In this study, organic materials typical of those used in mine passive treatment systems were suspended in the plume of metal-rich (As, Zn, Cd) effluent flowing through a constructed ABR in Trail, British Columbia, Canada. These included pulp mill wastes (biosolids), silage, vegetable and wood compost, partially decomposed *Typha latifolia* plant litter (cattails), and molasses mixed with hay. The Trail ABR was constructed with pulp mill biosolids, but no sulfate is reduced in the current system. Therefore, the goal of this study was find out if the presence and activity of sulfate-reducing bacteria correlate with properties and decomposition of the organic substrate. After five months *in situ*, bacterial communities in sealed mesh bags were assessed using SSU rRNA and *dsrA* genes as molecular markers. Quantitative PCR (qPCR) was also designed to determine the abundance of SRB. Additionally, easily degradable material fraction (EDM), dissolved and particulate organic carbon (DOC/POC), particulate nitrogen (PN), and carbon to nitrogen ratio (C/N) parameters of the organic carbon materials were measured throughout the sampling period to explore any relationships between organic carbon and the bacterial community. The potential sulfate-reduction rate (SRR) of each material was assessed in batch laboratory tests.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Set-Up

A constructed wetland treatment system (Fig. 4.1) was built near Trail, British Columbia, Canada by Nature Works Remediation Corporation (http://nature-works.net/) to treat leachate containing high amounts of zinc, cadmium, and arsenic coming from a historic landfill in the proximity of the Teck zinc and lead smelter. The first step in a series of wetlands and ponds is a vertical, sub-surface flow bioreactor filled with a pulp mill biosolids mix (60 % kraft pulp mill biosolids, 35 % sand, 5 % cow manure) and limestone. Concentrations of dissolved metals and

sulfate in the influent and effluent of the first ABR during the study period are presented in Figure 4.2. The following materials were each sealed in a separate screen mesh bag (5x7 cm, ca. 1 mm mesh size): thirteen grams of alfalfa silage (Poundmaker Agriventures, Lanikan, SK); 7 g of a mixture of fresh and partially decomposed cattails (taken from the *Typha latifolia* pond); 7 g of vegetable and woody debris compost (University of British Columbia, Vancouver, BC); 8 g of a dried molasses of sugar beet pulp (sweet 45, Westway Feed Products, Tomball, TX) and hay (mixed crop of alfalfa and orchid grass 65:35) mixture (2:1 w/w); 12 g of kraft pulp mill biosolids (Celgar Pulp and Paper Mill, Castlegar, British Columbia). All materials were obtained fresh from source, homogenized and cut to ca. 0.5 cm^3 pieces. Two duplicate bags were made for each material. All 10 bags were submerged into a piezometer located approximately in the middle of the 1st anaerobic bioreactor that is a part of the Nature Works treatment system in Trail, BC on May 3, 2006 (Fig. 4.1). The first set was removed on August 19, 2006 and the second set was taken out on October 23, 2006. All bags were immediately placed on ice and kept frozen until chemical and molecular analysis. In addition, a sample from the anaerobic bioreactor was taken with a PVC corer (0.5 m in length, 10 cm in diameter) from ca. the mid-layer of the bioreactor and kept frozen until DNA extraction.

4.2.2 Chemical Analysis of Organic Materials

The following parameters were measured before and after the *in situ* exposure. Dissolved organic carbon (DOC) was measured using TOC-Vcph analyzer (Shimadzu, Columbia, MD). Approximately 4 g of wet material from each mesh bag was thawed and placed in a 50 mL tube. Thirty mL of deionized water was added and tubes were shaken at 250 rpm for 2 h, centrifuged at 8000 rpm for 10 min, and the supernatant was syringe-filtered (0.2 μm) and analyzed. The pellet was dried at 60°C overnight and used for particulate organic carbon/particulate nitrogen (POC/PN) and easily degradable material (EDM) analysis. POC and PN were measured using the method by Verardo et al. (Verardo et al., 1990). EDM was analyzed by modified gravimetric forage fibre analysis as described in Prasad et al. (Prasad et al., 1999).

4.2.3 DNA Extraction

Genomic DNA was extracted from thawed materials in the mesh bags using the MoBio® PowerSoil DNA extraction kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions with the following modifications: alternative protocol for maximum yields was used; the spin column was rinsed twice with 300 μL of solution C4; and finally DNA was eluted in 100 μL of 10 mM Tris. Total nucleic acid concentration and purity were measured spectrophotometrically with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm.

4.2.4 PCR and Quantitative PCR (qPCR)

DNA was extracted from the organics contained in the mesh bags at time 0, and in the materials after 109 and 174 days of exposure in the ABR water. The DNA was subjected to PCR for SSU rRNA gene fragments targeting different SRB groups and qPCR for targeting the *dsr* gene.

Based on the multiple alignments of *dsr*A genes from both cultured SRB and environmental sequences from GenBank, conserved regions of the gene were selected as primers for SRB quantification. The forward primer DSR1F' (5'-ACSCACTGGAAGCACGGC-3') was modified from previously published primer DSR1F (Wagner et al., 1998). A degenerate reverse primer DSR210R (5'-CGGTGGMRCCRTGCATRTT-3') was designed to match the majority of *dsr* sequences currently available and yield a target product of ca. 200 bp (Schmidtova et al., 2009). The primers were tested by amplification of several pure SRB strains: *Desulfobacterium autotrophicum* (DSM 3382), *Desulfobacter curvatus* (DSM 3379), *Desulfosarcina variabilis* (DSM 2060), and *Desulfovibrio desulfuricans* subsp. *desulfuricans* (DSM 1926). Total eubacterial primers used were 27F (Lane, 1991) and degenerate 519R (5'-GNTTTACCGCGGCKGCTG-3').

QPCR of SRB was performed on the ABI PRISM® 7000 (Applied Biosystems) real-time thermocycler. The reaction mixture (12 μl) contained iTaqTM SYBR® Green Supermix with ROX (Biorad), each primer at a final concentration 300 nM, nanopure water and template DNA. MicroAmp 96-well reaction plates (Applied Biosystems) were used. The amplification conditions were as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 94°C followed by 1 min at 60°C. Each genomic DNA sample representing different organics at

different sampling times was amplified in triplicate. Further, to confirm the uniformity of amplification runs, several samples again in triplicates were reamplified, which resulted in 6 technical replicates. The external standard curve for *dsr* quantification was constructed with a total extracted genomic DNA of *Desulfobacterium autotrophicum* (DSM 3382). The detection limit was 100 *dsr* copies per reaction, the efficiency ($E=10^{(-1/slope)}$); where 2 indicates an exact doubling per cycle) was 1.84, and $R^2=0.96$. Concentrations of the samples were extrapolated from the standard curve using ABI Prism 7000 SDS Software (Version 1.0, Applied Biosystems). QPCR of total eubacteria was performed on the Miniopticon system (Biorad, Hercules, CA). The reaction mixture (25 µl) contained iTaqTM SYBR® Green Supermix (Biorad), each primer at a final concentration 300 nM, nanopure water and template DNA. The amplification conditions were as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 94°C followed by 1 min at 60°C. The external standard curve was constructed as described by Zaikova et al. (2009). The gene copy number was diluted from 100 to 10^8 copies.

4.2.5 Clone Library Construction

Clone libraries of SSU rRNA and *dsr* genes were constructed from 4 samples: mesh bags carrying silage, compost, and molasses and hay taken from the reactor after 174 d, and pulp mill biosolids taken directly from the bioreactor. PCR amplification of SSU rRNA genes was carried out on an iCycler® (Biorad) using universal bacterial primers 27f and 1492f (Lane, 1991). Taq DNA polymerase (Invitrogen) was used and the following reaction conditions were applied: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 40 s, 55°C for 1.5 min, 72°C for 2 min; 1 cycle at 72°C for 10 min. PCR amplification of *dsr* genes was carried out using primers DSR1F and DSR4R (Wagner et al., 1998). The same conditions applied except for the melting temperature, which was 60°C in this case. Products were further purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were ligated into the pCR®2.1-TOPO® vector as described in the protocol of TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). Ligation reaction mixtures were transformed into One Shot® TOP10 competent E. coli cells (Invitrogen). Transformants were selected by blue and white screening. 288 white colonies were randomly selected from each sample for the SSU rRNA library and 196 white colonies were randomly selected from each sample for the *dsr* library, and stored in a glycerol stock solution in 96-well culture plates at -80°C. Plasmid inserts

from several colonies stored in glycerol stock were checked by direct PCR using standard M13F and M13R primers and confirmed with agarose electrophoresis.

4.2.6 Sequencing and Phylogenetic Analysis

Clones were purified with QIAquick® PCR purification kit and sequenced bidirectionally by the Michael Smith Genome Sciences Centre (www.bcgsc.ca) (Vancouver, Canada) using M13F and M13R primers. Assembled and trimmed sequences of SSU rRNA inserts (Sequencher; Gene Codes, Ann Arbor, MI) were imported and aligned with the ARB phylogeny computer program (Ludwig et al., 2004). All sequences were checked for chimeras with Ribosomal Database Project II chimera check program (Cole et al., 2003). Sequences with higher than 97 % similarity were combined into single OTUs using DNAdist from the PHYLIP package version 3.68 (Felsenstein, 2005) and DOTUR (Schloss and Handelsman, 2005). The closest phylogenetic neighbors were found using BLAST search for the NCBI database (Altschul et al., 1990). The phylogenetic trees and the bootstrap analysis (100 replicates) were constructed with the PhyML software package (Guindon and Gascuel, 2003) by using the maximum likelihood method. Good's coverage was calculated by using the following formula: $C = (1 - (n_1/N)) \times 100$, where n_1 is the number of clones that occurred only once in the clone library and N is the total number of clones analyzed (Mullins et al., 1995). Chao1 was calculated using DOTUR. Trimmed *dsr*A sequences were compared to the NCBI database and closely related phylogenetic neighbors were found using BLAST searches. The final phylogenetic tree was constructed with selected closest relatives and additional cultured SRB species using methods described above. Comparison of libraries and cluster diagram was constructed with UniFrac (Lozupone et al., 2007).

4.2.7 Sulfate Reduction Rate

Laboratory-scale batch reactions with seepage water and the five organic materials were set up to determine their potential sulfate reduction rate (SRR). Duplicate 150 mL glass bottles containing: 10 g of silage or 15 g of pulp mill biosolids or 5 g of molasses + 2.5 g of hay or 6 g of fresh and partially decomposed cattails, plus a control bottle without any material were set up. 140 mL of N_2-purged water taken from the wetland piezometer containing sulfate, 5 mL of mixed laboratory SRB inocula enriched in Postgate B medium (Postgate, 1984) from sulfate-rich

sediment (Lac DuBois (Brown, 2007)), and 1 mL of sodium thioglycolic acid was added and pH was adjusted to pH 7.5-8. The bottles were kept in the dark at room temperature and sulfate was measured using the barium chloride precipitation method (Clesceri et al., 1998) at times 0, 4, 8, 12, 15, 22 days. The SRR was determined from the slope of sulfate changes over time and the values are reported as average of duplicate reactors.

4.3 RESULTS

4.3.1 Characteristics of the System

The constructed wetland near Trail, B.C. (Fig. 4.1) had been treating landfill seepage containing mainly As, Zn and Cd for 5 years at the time of this study. At the end of April 2006, dissolved As entered the treatment system at 27 mg L^{-1} (Fig. 4.2). The hydraulic retention time of each ABR is approximately 2 weeks. One month later the dissolved As concentration in the second ABR effluent was 0.24 mg L^{-1}, indicating that 99.1 % As was retained in the ABRs: 55 % As is estimated to be removed in the first ABR. Similarly, Zn was effectively removed in the two ABRs over the same period (93.6 %). Cadmium concentrations also decreased, however dissolved Fe and Mn increased due to the reducing environment of the ABRs. No sulfate was reduced (Fig. 4.2) as the concentration remained unchanged through the treatment system or appeared to increase. Thus, it seems that there are features of this system that are not ideal for SRB. Therefore, the focus of this study was to determine if the type of organic material used in these passive treatment reactors influences the amount and nature of SRB that proliferate under the same *in situ* conditions. Temperature of the porewater inside the piezometer was around 6.5°C, 17°C and 8°C in April, July and October, respectively. Typically, some dissolved oxygen was present in the spring months (e.g., 1.5 mg L^{-1} in April) and decreased to less than 0.3 mg L^{-1} in the summer and fall months accompanied by negative redox potential of -172 to -180 mV. Thus, the environment within the ABR was circum neutral in pH, mild temperature, reducing with low oxygen concentrations, which are the environmental conditions usually deemed suitable for growth of SRB, especially with sulfate concentrations in the water greater than 500 mg L^{-1}.

4.3.2 Chemical Characteristics of the Organic Materials

Organic materials vary in terms of their biodegradability depending on their content of dissolved organic compounds, labile and more recalcitrant solid portions as well as presence of inhibiting substances. Several tests were performed to compare the organic materials and to monitor their utilization over time (Table 4.1). As expected, the molasses and hay mixture contained the highest amount of dissolved organic carbon (DOC) initially as well as the most easily degradable material (EDM). The molasses used in this experiment contained a minimum of 45 % of soluble sucrose, as defined by the manufacturer, which greatly contributed to the DOC and EDM. Significant DOC levels were found also in compost and silage. The lowest initial EDM of 20.8 %, indicating the highest amount of complex cellulosic and lignin compounds was found in the compost. The C/N ratios in the starting material ranged from 25.4 (cattails and pulp biosolids) to 52.5 (silage). After 174 days of exposure *in situ*, the molasses/hay mixture DOC decreased by 99 % (corresponding to 4.1 mg day^{-1}) and the EDM by 50 % (1.4 mg day^{-1}). The smallest change in DOC occurred for pulp mill biosolids (decrease of 0.07 mg day^{-1}) and in EDM in cattails (decrease of 0.3 mg day^{-1}). The amount of particulate organic carbon (POC) and nitrogen (PN) remained relatively unchanged with the exception of molasses, where the PN dropped from 1.6 % to 0.7 %. The C/N of both plant-derived materials (cattails and silage) decreased by ca. 15 %. The remaining materials' C/N ratios increased by 11 % (pulp biosolids), 48 % (compost) and 131 % (molasses and hay).

4.3.3 Quantification of Bacterial Species

Initially, before the mesh bags were added to the ABR, we were unable to detect any SRB, via qPCR of the *dsr* gene, in any of the organic materials. However, we found that other bacteria were present at the start of the experiment in amounts from $2.5x10^6$ copies g^{-1} dry weight in the compost to $5.5x10^8$ copies g^{-1} dry weight in silage (Fig. 4.3). Significant numbers of SRB (3.2-$7.1x10^6$ copies g^{-1} dry weight) were found in all four materials after 109 days of incubation in the ABR, and this amount further increased by more than 10-fold during the remaining exposure time. At the end of the incubation period, silage contained the most SRB ($1.1x10^9$ *dsr* copies g^{-1} dry weight). The highest amount of total bacteria ($9.3x10^9$ copies g^{-1} dry weight) after the incubation period was found also in silage. Assuming that there is only one *dsr* copy per sulfate-

reducing bacterium (Kondo et al., 2004) the fraction of SRB in the total bacteria population can be estimated. In August 19, less than 1 % of the total bacteria population comprised SRB. Whereas, by October 23, SRB as a fraction of the total community had increased to 11.7 ± 1.7 % in silage, followed by compost (8.2 ± 2.0 %), and molasses and hay mix (7.1 ± 1.6 %). The pulp mill biosolids samples, in contrast, did not contain any measurable amount of SRB (data not shown) at any time. In addition to the mesh bags, a sample taken directly from the ABR containing the substrate (mainly pulp mill biosolids) was also analyzed. In this we found an average value for total bacteria and SRB of 6.06×10^8 copies of SSU rDNA g^{-1} dry weight and 6.46×10^6 *dsr* copies g^{-1} dry weight, respectively.

4.3.4 Bacterial Diversity Evaluated from a Phylogenetic Analysis of SSU rRNA Genes

Materials with highest amount of SRB after 174 d *in situ* (silage, compost, and molasses with hay) as well as the core sample taken directly from the ABR (ABR pulp mill biosolids sample) were analyzed for bacterial diversity. This was done so as to indentify the major groups of bacteria involved in degrading these organic materials. A total of 816 clones were chosen and sequenced for SSU rRNA analysis. To avoid microdiversity, clones that were ≥97 % identical were grouped into operational taxonomic units (OTUs), yielding 366 OTUs. Good's coverage estimating the fraction of the total bacterial community targeted suggests that the ABR pulp mill biosolids contained the most diverse bacterial community of which ca. 51 % was covered by the clone library. The remaining samples' coverage values were between 64 and 68 % (Table 4.2).

Over 50 % of all SSU rRNA clones belonged to *Bacteroidetes* (52 %). Second most abundant class was *Clostridia* (25 %), followed by Candidate division TG3, *δ-Proteobacteria*, and *Spirochaetes* (Fig. 4.4). Overall, the majority of the clones were closely related (>97 %) to other environmental sequences, often recovered from anaerobic reactors, aquifers, or soils. Figure 4.5 shows a phylogenetic tree constructed with representative OTUs and their close phylogenetic neighbors. All OTUs and their closest relative, together with the frequency in each library are listed in Table D.1. The calculated distances between clone libraries of different materials are outlined in cluster diagram (Fig. 4.6). The bacterial diversity associated with different materials is described below.

Silage

Bacteroidetes, specifically members of *Cytophaga* and related sp., were dominant with 130 out of 211 clones (61.6 %). Moreover, 56 % of these sequences were represented by single OTU TR27_R_4, whose nearest relative is an environmental sequence GW-32 obtained from a household biogas digester (Fig. 4.5, Table D.1). Further, eight clones were similar to a sequence from the sediment of the anoxic lake Kinneret (Schwarz et al., 2007). Few clones were also similar (93 %) to a sequence from a biodegraded oil reservoir (Grabowski et al., 2005) and ferric-iron reducing enrichment culture (Lin et al., 2007). The second most abundant bacterial group was *Firmicutes* (21.3 %). Eight clones were closely related (97 % similar) to a *Ruminococcus* clone from uranium contaminated soil (Brodie et al., 2006). Remaining clones clustered together with sequences from biogas digesters, wastewater, coal seam groundwater, and soil. One clone was 99 % identical to cultured acetogen *Acetobacterium woodii*. *δ-Proteobacteria* were represented in the silage sample with 13 clones, which was the highest amount among all samples. The majority of these fell into the *Desulfovibrionales* order. Four clones were 99.9 % and 99.5 % identical to *Desulfomicrobium baculatum* and *Desulfomicrobium norvegicus*, respectively. Three clones were affiliated with sequences from PCB-dechlorination environments. *Spirochaetes* were represented by 11 clones (5.2 %). One clone was 95.5 % similar to sugar-fermenting *Spirochaeta stenostrepta* and two clones 93.3 % similar to *Spirochaeta caldaria*. Five sequences were affiliated with a clone from a SRB fluidized-bed reactor treating ARD and fed by ethanol (Kaksonen et al., 2004). Another three clones were similar to sequence from paper pulp column. The remaining clones belonged to *β-Proteobacteria*, *Planctomycetes*, Candidate divisions, and *Verrucomicrobia*.

Compost

The composition of main phyla of this library was similar to silage. *Bacteroidetes* and *Firmicutes* also dominated the clone library comprising 55 % and 20 %, respectively. Again, majority of *Bacteroidetes* (53 %) were represented with clone TR27_R_4. Four clones were associated with a clone from a biotrickling filter removing sulfide. Two *Bacteroidetes* clones were 93.5 % identical to a sequence derived from a methanogenic consortium degrading long-chain fatty acids (Shigematsu et al., 2006). However, several bacterial classes were found uniquely in this sample, such as clones affiliated with *Acidobacteria*, *Rhodobacter*, and *Acidithiobacillus*. Also specific to this library, two *Clostridia*-related clones were >99 % similar to a clone from a rice straw composting sample. Highest amount of sequences from candidate division TG3 was found in this

library with 19 clones (10.6 %). All clones were 92.4-96.5 % similar to a sequence retrieved from termite gut (Hongoh et al., 2006). The remaining clones belonged to α- and δ-*Proteobacteria* and *Fibrobacteres*.

Molasses and Hay

Similarly, the phylogeny of this sample resembled the libraries from the silage and compost samples. Again, OTU TR27_R_4 was highly represented with 42.3 % of all sequences in the library. Two γ-*Proteobacterial* sequences were found uniquely in this sample: one *Rickettsiella* OTU related to a sequence from tundra soil and one *Aeromonas* OTU related to a sequence from geothermal hot spring (Fig. 4.5, Table D.1). Five sequences belonged to *Nitrospira* sp. and were closely related to a sequence from a sulfur-oxidizing membrane reactor (Vannini et al., 2008). Two phyla were found only in this sample: *Cyanobacteria* and *Chlorobi*. The closest relatives of these sequences were found in an acid-impacted lake (Percent et al., 2008) and contaminated sediment (Abulencia et al., 2006). Together with the compost SSU rRNA library, this library also contained a significant amount of candidate division TG3 sequences (7.7 %).

ABR Pulp Mill Biosolids

The clone library of this sample differed significantly from the others (Fig. 4.6). *Clostridia* dominated the library with 40 % of the clones and exhibited the highest diversity among all the libraries, with sequences from *Bacillales* (6 sequences), *Mollicutes* (7 sequences), and *Clostridiales* (70 sequences) present. Ten *Clostridia* rel. sequences were similar to sequences from methanogenic reactors. *Cytophaga* et rel. sequences that dominated the material in the mesh bags, were less present in the ABR biosolids. Instead, a different cluster of *Bacteroidales* was found with sequences similar to bacterium clone WU75 obtained from anaerobic digester sludge. Two high G-C Gram-positive bacteria (*Actinobacteria*), seven *Verrucomicrobia* and four *Lentisphaerae* clones were obtained only from this library. The highest amounts of α-*Proteobacteria*, mostly *Rhizobiales*, were found in this library. On the contrary, no δ-*Proteobacterial* sequences were retrieved. One OTU (TR8_R_785) of γ-*Proteobacteria* was closely related (98 %) to a sequence from chromium-contaminated soil (Desai et al., 2009). Other sequences were similar to environmental clones recovered from ice, soil, and oil-contaminated sites.

4.3.5 Phylogenetic Analysis of SRB

In addition to the SSU rRNA phylogenetic analysis, clone libraries of the *dsr* gene, specific for sulfate-reducing bacteria, were also constructed for the same four materials in order to gain higher resolution of SRB species. A total of 78, 68, 74, and 121 clones were sequenced from silage, compost, molasses and hay, and ABR pulp mill biosolids, respectively. These sequences were grouped to 39 OTUs based on ≥ 97 % sequence similarity. The distribution and phylogenetic relatives of sequenced clones are shown in Figure 4.7 and Table D.2. The majority of SRB in the samples are affiliated with *Desulfovibrio* and *Desulfomicrobium* sp. However, the sequences from ABR biosolids clustered separately from the remaining libraries. Over 92 % of all sequences in the biosolids library, represented by clones TR8A_213 and TR8A_214, were closely related (98 %) to a single SRB *Desulfovibrio* sp. related sequence (NTUA-1A-DSR3) from an upflow fixed-bed reactor fed with lactate (Remoundaki et al., 2008). This sequence NTUA-1A-DSR3 appeared solely at the bottom of the reactor, where conditions were highly reducing and lactate was present in high amounts. The cultured species most closely related (97.5-97.7 %) to TR_8A_213 and TR_8A_214 is *Desulfovibrio desulfuricans*. The OTU represented by the largest number of clones in both the compost and molasses libraries (TR29_99) is related to a clone NTUA-5A-DSR22 recovered from the same SRB fixed-bed reactor, but its closest cultured relative is *Desulfovibrio aminophilus* (Fig. 4.7). In many cases, same OTU was found in all libraries except for biosolids (Table D.2). For example, OTU TR27_22, which belongs to *Desulfonema/Desulfococcus/Desulfosarcina* cluster, represented 24 % of clones in silage, 14.7 % clones in compost, and 20.3 % in molasses and hay. Of the remaining groups, *Desulfomicrobium* sp. were mostly found in silage library and spore-forming *Desulfutomaculum* sp. were found in silage and molasses, and in lesser amounts in compost.

4.3.6 Sulfate Reduction Rates

Potential sulfate reduction rates attainable in the organic materials were determined during a 22-day batch reactor study (Fig. 4.8). The reactors with silage as a carbon source reduced 550 ± 3 nmol SO_4^{2-} mL^{-1} d^{-1}. Approximately four times less sulfate was reduced in reactors with molasses (142 ± 18 nmol SO_4^{2-} mL^{-1} d^{-1}) and compost (133 ± 59 nmol SO_4^{2-} mL^{-1} d^{-1}). Finally, reactors with cattails and pulp mill biosolids reduced the least sulfate with

56 ± 16 nmol SO_4^{2-} mL^{-1} d^{-1} and 28 ± 21 nmol SO_4^{2-} mL^{-1} d^{-1}, respectively. These results correlate with the field-scale SRB quantification using QPCR, suggesting that this molecular technique is a suitable proxy to estimate the sulfate reducing potential of in situ material.

4.4 DISCUSSION

In this study, we combined quantitative and qualitative molecular techniques as well as chemical characteristics to assess five different materials and their potential effectiveness for supporting sulfate reduction in an anaerobic wetland system treating metal-rich water. The treatment system near Trail, B.C. uses a passive biological system to decrease high levels of metals from landfill seepage. The anaerobic bioreactor, which is first in the series of cells (Fig. 4.1), was designed to sustain an active community of SRB within organic-rich pulp mill biosolids media that would reduce sulfate and precipitate metals as sulfides. While some arsenic, zinc, and cadmium are retained within the reactor, no sulfate, iron, or manganese are removed (Fig. 4.2). This means that sulfate reduction is likely not significant and other processes, such as metal reduction, adhesion, or adsorption onto the solid media, are responsible for removed metals. With the limited knowledge about these systems, we cannot determine the factors that prevent the establishment of an active SRB population and therefore predict the long-term efficiency of this ABR.

One of the crucial parameters in such bioreactors is the choice of organic material (Neculita et al., 2007). Since complex organic mixtures are used, SRB are dependent on other microbes to provide them with low molecular weight electron donors. These, such as cellulolytic organisms, often are the rate-controlling step in the treatment kinetics. In this study, we evaluated five different organic materials that were readily available in or around the treatment system to assess their suitability for such systems, as well as to obtain understanding on the bacteria involved in the degradation processes and how these correlate with the SRB and metal removal. Ultimately, in future work based on this study, obtaining sequences for new functional genes can lead to proteomic methods for tracking activity of metabolic pathways in these natural treatment systems.

4.4.1 Correlation Between Organic Carbon Properties and SRB

The samples of different materials were analyzed for carbon parameters at the beginning, and after ca. 3, and 5 months of *in situ* exposure in the Trail, B.C. bioreactor. In the case of molasses, hay was added to the mesh bag to ensure sufficient surface area for support of bacterial biofilms. By assessing the initial carbon characteristics, it would appear that the molasses and hay mixture is likely to achieve the highest sulfate reduction rates since it contained the most amount of readily available carbon, represented by high DOC and EDM levels. We found that the materials with high initial DOC (silage, compost, and molasses) did have the highest absolute amount of SRB as well as the highest SRB to total bacteria ratio after 174 d of field experiment (Fig. 4.3). However, the soluble organic carbon fraction was washed rapidly away in the continuous system and likely did not substantially affect the bacterial colonization. Although, it is probable that micro-niches within the material inside the mesh bags, which were not directly exposed to the flow, retained some of the DOC. If that were the case then DOC associated with the original material would be available for establishment of bacterial communities. Zagury et al. (2006) found that the materials with the lowest initial TOC were least effective in removing metals from ARD during an 80 d batch operation. In this study, however, no significant trends can be seen.

Several authors claim that low C/N ratio (~10) improves sulfate reduction and metal precipitation (Bechard et al., 1994; Prasad et al., 1999). In contrast to these studies, the highest initial (day 0) C/N ratio (52.5) was found in silage that contained the largest final SRB population based on *dsr* gene quantification. In fact, we found strong positive correlation (r^2 = 0.89) between the initial C/N ratio and SRB fraction of the bacterial community (Fig. 4.9). This finding, although with less statistical confidence, was further extended to the correlation of C/N ratio with actual sulfate reduction rate measured during a batch experiment (Fig. 4.10). Zagury et al. (2006) also found that the materials with C/N close to the theoretical value (~6.6) did not promote sulfate reduction. On the other hand, maple wood chips with C/N of 567 performed the best. It is important to note that different C/N ratios were reported for the same material in different studies. The C/N ratio of 70, as opposed to 25.4 in this study, was reported for cattails in Florida wetlands (Corstanje et al., 2006). Also, higher C/N ratio of pulp mill biosolids (up to 100) was measured in Ontario paper mill (Price and Voroney, 2007). Therefore, the C/N ratio largely depends on the nature, location, and decomposition state of each material and is not uniform for a particular material. For example, in the Trail ABR, there are high concentrations of nitrate and ammonium in the influent water, which means that nitrogen as a nutrient source for

the bacteria is not limiting. However, in other environments where the organic material is the only source of nitrogen, a lower C/N ratio close to the theoretical value would promote microbial growth. Overall, the C/N ratio was the only parameter that could be correlated with sulfate reduction and should be considered during the design of a treatment system and choice of the organic substrate. This would likely be particularly important at the early stages of reactor operation, because, as we can see from microbial analysis using molecular markers, SRB population initially increases after fresh material is added to the treatment system, but is most likely suppressed as the availability of suitable electron donor decreases.

4.4.2 Variations in Bacterial Population Among Different Materials

Pulp mill biosolids did not contained measurable amounts of dsr gene at any point during the study. As well, the lowest sulfate reduction rate was determined for this material during a bench-scale batch experiment. Since we were studying materials that are most suitable for supporting high numbers of SRB, the pulp mill biosolids and cattails were excluded from further phylogenetic analysis. In addition to the remaining three mesh bag materials that were placed into the bioreactor for 174 d, a sample of the biosolids taken directly from the ABR that has been in operation for ca. 4 y, was also analyzed. We found that the composition of SSU rRNA genes in the ABR biosolids differs significantly from the others (Figs. 4.3, 4.5). Besides the difference in the organic material, the bacterial structure of this sample has also been forming for much longer when compared to 174 days in the mesh bag samples. Interestingly, the older ABR biosolids community was more diverse than those in the mesh bag samples. Although, the coverage of the ABR biosolids library increased to 86 % on the genus level (≤ 10 % distance between SSU rRNA sequences) and was comparable with the coverage of the other three libraries (86-89.6 %) (data not shown). As opposed to a high total bacterial diversity in the ABR biosolids SSU rRNA library, the *dsr* library revealed very low diversity of SRB with over 79 % of clones being ≥ 97 % identical and forming a single OTU TR8A_214. These results indicate that while there is great competition between species degrading the complex carbon molecules, only few SRB species are capable to survive in these conditions.

Overall, most sequences fell into two bacterial classes: Gram-negative *Bacteroidetes* and Gram-positive fermentative *Clostridia*. Similar distribution was also observed in an anaerobic sludge digester (Chouari et al., 2005) and the dominance of *Clostridia* was also found by

employing two different molecular methods on a bench-scale permeable reactive barrier columns filled with a mixture of wood shavings, cattle manure, alfalfa pellets, and wetland sediment (Hong et al., 2007). The dominance of these groups can be attributed to their ability to degrade a variety of carbon macromolecules, which complex carbon materials mostly contain. Whereas *Clostridia* sp., widely distributed in most soils and sediments, dominated the ABR biosolids SSU rRNA library, *Bacteroidetes* sp. were most abundant is the libraries from mesh bag materials. These species are mostly found in soils with high carbon mineralization rates, which could explain why they were found in high frequency in freshly added materials (Fierer et al., 2007). The ABR biosolids SSU rRNA library also differed from the other libraries in the composition of less abundant groups. Specifically, higher amounts of α- and γ-*Proteobacteria*, *Lentisphaerae*, *Verrumicrobia*, and *Actinobacteria* and no δ-*Proteobacteria* (Fig. 4.4) were found in this library, indicating that these may be organisms that use the degradation byproducts produced by the hydrolytic microbes that were more active in the earlier stages of decomposition. The uniformity of the composition of most frequently occurring sequences in silage, compost, and molasses with hay samples reveals that the complex carbon degrading bacteria are the same, regardless of the source of organic carbon.

In the *dsr* library, all 121 clones from the ABR biosolids library belonged to the genus *Desulfovibrio* sp., and this cluster was closely related to *Desulfovibrio desulfuricans* (DQ092635) (Fig. 4.7). Although we were able to obtain *dsr* gene sequences from this sample, no SRB were detected in the SSU rRNA library suggesting that SRB represent only small fraction of the total bacteria in the sample. Diversity of *dsr* clones in silage, compost, and molasses samples was higher than that from the ABR biosolids. Although many clones from compost and molasses also belonged to the *Desulfovibrio* genus, they clustered differently from the ABR TR8A_214: The most predominant clone, TR29_99 (37 % in compost and 42 % in molasses with hay) was related to other uncultured bacteria from sludge and fixed-bed reactors (EU552479 and EF645675). The only prevalent *dsr* clone that was present in all three libraries from the mesh bag experiment (compost, silage, molasses), TR27_22, was distantly related to a sequence from near-surface sediment of uranium-contaminated mine pit lake (Suzuki et al., 2005). Silage also contained many sequences from *Desulfomicrobium* sp. (Fig. 4.7). To author's knowledge, there is no published study examining the diversity of *dsr* gene in a constructed wetland treating ARD, therefore no comparisons with other studies can be made. Phylogenetic analysis of bacteria from batch sulfate reducing reactors amended with pure carbon compounds

and molasses detected species *Desulfotomaculum* sp. as the most common SRB (Geets et al., 2005). These spore-forming acetate-oxidizing species were also detected in our study. *Desulfovibrio* sp. were also found in fluidized-bed reactor fed with lactate and ethanol treating metal-contaminated wastewater (Kaksonen et al., 2004). The results in the present work indicate that, although all the materials were placed in the same aqueous environment, they are quite dissimilar in the SRB species that they support. This is in contrast with the SSU rRNA gene library composition, and suggests that the materials might not have significant effect on bacteria degrading complex molecules, but the composition of complex carbon materials affects the SRB population.

The results indicate that the amount of SRB (as well as the sulfate reduction potential) increases with increased phylogenetic *dsr* diversity. Mixed community has advantage over single species by improved response to changing factors, such as availability of electron donors, increased stress levels due to higher metal loading, changes in temperature, etc. (White and Gadd, 1996). Therefore, for increased SRB activity in passive treatment systems, it is advisable to choose a material that supports high diversity of SRB species.

4.4.3 Ecological Significance of Dominant Species

The purpose of this study was to identify dominant bacterial species in a metal-contaminated seepage treatment system with different organic substrates, and thus bring more understanding into the geochemical processes, especially related to carbon degradation that occur in these systems. Although we understand the limitations of relating phylogenetic analysis to metabolic functions, the robustness of the database provides excellent tool to study phylogenetic variations and some functional processes can be deduced. In MME treatment systems where sulfate concentrations in the influent are high, SRB are considered as the most functionally important organisms since the generation of sulfide and alkalinity are essential for effective treatment. However, the most prevalent sequences, understandably, in these systems are associated with complex carbon degradation. Based on the species and close relatives metabolic information we can supplement the carbon degradation pathway presented in Chapter 1 (Fig. 1.4) with species found during this study (Fig. 4.11).

The majority of sequences that were recovered from our study are likely involved in hydrolysis of complex molecules such as cellulose and chitin and further fermentation to produce

low-molecular weight organic molecules such as short-chain fatty acids or alcohols. The most frequently occurring clones TR27_R_4 is closely related to uncultured sequence from the biogas digester (acc. EU407215) and unfortunately nothing is known about its metabolic function. The closest cultured representative is *Cytophaga* sp. AN-B14 strain isolated from deep-sea oxic-anoxic interface of Bannock basin (Daffonchio et al., 2006). The isolate was able to ferment a variety of sugars and biopolymers as sole source of carbon and energy. The highest frequency of this clone was obtained from the molasses and hay sample, which consists mainly of sucrose and other sugars. This supports the hypothesis that bacteria represented by this clone utilize the same hydrolytic and fermentative processes for growth. A genomic study of *Cytophaga hutchinsonii* determined that this bacterium is able to digest crystalline cellulose (Xie et al., 2007). Nineteen sequences from compost and 18 sequences from molasses and hay were shown to be associated with TNR-I-16 clone of termite group TG3 retrieved from rice paddy soil (Hongoh et al., 2006). The bacteria are possibly degrading dead plant material, although their exact ecological role is not known.

A number of sequences have also shown to be associated with metal-contaminated environments. Six sequences were similar to *Bacteroidetes* clone G1DMC-151 obtained from chromium-contaminated landfill sediment (Desai et al., 2009). Also, 26 sequences were related to iron-reducing enrichment cultures from the mining environment, thus we expect that iron reduction also occurs in the ABR. Sequences from uranium- and gold-contaminated sites were also similar to sequences from this study. These bacteria, which are repeatedly found in environments with high concentrations of metals, must have developed metal-resistant mechanisms that enable them to thrive in such conditions, which are toxic to the majority of bacteria.

Interestingly, close relatives of many sequences come from oil-contaminated environments (Fig. 4.5). For example, several sequences from dominant groups *Bacteroidetes* and *Clostridia* were closely related to uncultured bacteria from oil reservoir in western Canada that are probable hydrocarbon degraders (Grabowski et al., 2005). Eight OTUs from *Clostridia* sp. were similar to sequences from tar-oil contaminated sediment that contains bacterial community degrading toluene (Winderl et al., 2008). Also, many sequences, including highly represented sequence TR27_R_4, are similar to sequences from biogas digester. Other sequences are also found in various reactors treating wastewater, paper mill waste, PCB, toluene contamination, etc. This also supports the hypothesis that the majority of microbial activity in

treatment systems containing complex carbon source is restricted to processes involved in breakdown of complex organic molecules.

It is interesting to note that many sequences, especially from ABR biosolids library were closely related to sequences from methanogenic reactors (Fig. 4.5, Table D.1). Even though we did not focus on Archaea and thus the majority of methanogenic organisms, it is reasonable to assume that methanogens are present and possibly compete with SRB for electron donors. Although, phylogenetic analysis of SRB suggests that most of them do not utilize acetate, which is the primary electron donor for methanogens.

Despite the dominance of the hydrolytic, acidogenic and acetogentic bacteria, it does appear that the nature of the material selects for different SRB species. In terms of *dsr* gene distribution, we find that all sequences from ABR biosolids library belong to *Desulfovibrio* sp., which are known as incomplete oxidizers that cannot utilize acetate. On the other hand, silage, compost, and molasses and hay libraries contained both members of incomplete and complete oxidizers, enabling larger amount of electron donors to be used up by SRB. We argue that increased operation time of the system (4 y for biosolids vs. 174 d for the other samples) depleted the system of most available carbon source for SRB and thus the diversity rapidly decreased.

4.5 CONCLUSION

In this study we compared five different organic materials for their ability to support sulfate-reducing bacteria when immersed under anaerobic conditions in seepage water containing arsenic, zinc and cadmium. We found that pulp mill biosolids that were taken from the same source as the organics used in the actual Trail anaerobic bioreactor treating this effluent, did not support any SRB and demonstrated very low SRRs in the laboratory. This observation was corroborated in the phylogenetic analysis of the ABR biosolids in which we found no sequences related to *δ-Proteobacteria*, the phylum containing most of the known SRB. Although the *dsr* library did reveal that some SRB are present in the biosolids, they do not have any measurable effect on the SRR. This confirms the observation that no reduction of sulfate occurs between the influent and effluent in the treatment system. Nevertheless, some arsenic and zinc are removed from the effluent in the ABR, but we conclude that sulfate reduction is not a major mechanism in metal removal. In our SSU rRNA clone library, we did find some sequences related to other

environmental sequences from iron-reducing enrichments. Iron reduction under highly reducing conditions is known to remove As through co-precipitation of Fe(II) minerals (Lloyd et al., 2004). Therefore, future work should be directed at establishing what biological processes, if any, are responsible for successful metal removal in this treatment system.

In contrast, the other organic materials tested all supported measurable amounts of SRB, ranging from 2 to 12 % of the total bacterial community. The amount of SRB as measured by qPCR correlated with the potential SRRs for each material indicating that quantification of SRB using qPCR can be used as an estimate of SRR in cases where it is impossible to measure SRR *in situ*. The order of preference of SRB for the organic materials tested is silage > compost mix > molasses/hay > cattails > biosolids. Based on the uniform bacterial phylogeny of the most abundant sequences of the three most successful organics (silage, compost mix and molasses/hay), we conclude that similar species may be involved in biodegradation of these complex materials. Although, the nature of the degradation products might vary accounting for the diversity in SRB species seen in each material. While many sequences in compost mix and molasses/hay *dsr* libraries clustered with *Desulfovibrio* sp., silage library was represented by sequences from *Desulfomicrobium* sp. and *Desulfonema-Desulfococcus-Desulfosarcina* cluster.

We have shown that the choice of organic material does influence the numbers of SRB and their diversity. We were able to find positive correlation between C/N ratio of the raw organic materials and the amount and activity of SRB. However, there are other factors, particularly related to the complex molecules degradation, which affect the SRB. To determine the bottlenecks that exist to sulfate reduction as these materials decompose, we recommend monitoring the species identified in this study, using qPCR, over time to see if there are any correlations between abundance of any particular groups and SRB activity. Also, this study pioneered in investigating the *dsr* gene diversity in passive MME treatment systems and also only few other studies focused on the overall SSU rRNA gene diversity. The collection of such data from other systems is highly encouraged, as with the increased datasets, new possibilities involving multivariate statistics can be explored, and, ultimately, trends and relationships can be developed between the microbial composition and system parameters that can directly enhance the passive treatment system performance.

4.6 TABLES AND FIGURES

Table 4.1: Carbon characteristics of organic substrates before and after treatment.

	DOC (mg g^{-1})[a]			POC (% w/w)			PN (% w/w)			EDM (% w/w)			C/N (molar)		
Substrate / Time (days)	0	109	174	0	109	174	0	109	174	0	109	174	0	109	174
Pulp mill biosolids	19.3	10.3	7.9	41.8	50.1	45.4	1.9	2.0	1.9	36.8	28.8	25.5	25.4	29.3	28.4
Silage	86.4	23.4	6.5	42.7	37.1	45.1	0.9	2.0	1.2	45.8	40.7	29.9	52.5	21.6	44.3
Cattails	16.3	12.7	3.0	24.3	32.4	23.3	1.1	2.1	1.3	37.6	32.0	31.8	25.4	18.2	21.5
Vegetable compost	111.1	7.8	2.2	45.0	44.6	45.6	1.3	1.0	0.9	20.8	15.8	13.6	41.2	50.9	60.9
Molasses and hay	725.6	16.8	4.6	42.0	44.7	45.4	1.6	1.3	0.7	47.7	26.1	24.0	31.1	38.8	71.9

[a] measured on leachates as described in Materials and Methods

Table 4.2: Parameters of SSU rRNA clone libraries.

	Silage	Compost	Molasses + hay	ABR biosolids
Number of clones	211	179	246	180
Number of OTUs	104	86	97	121
Good's coverage (%)	64	64	68	51
Chao1[a]	243 (172-379)	244 (163-409)	361 (226-634)	276 (205-406)

[a] values in brackets represent 95% confidence interval

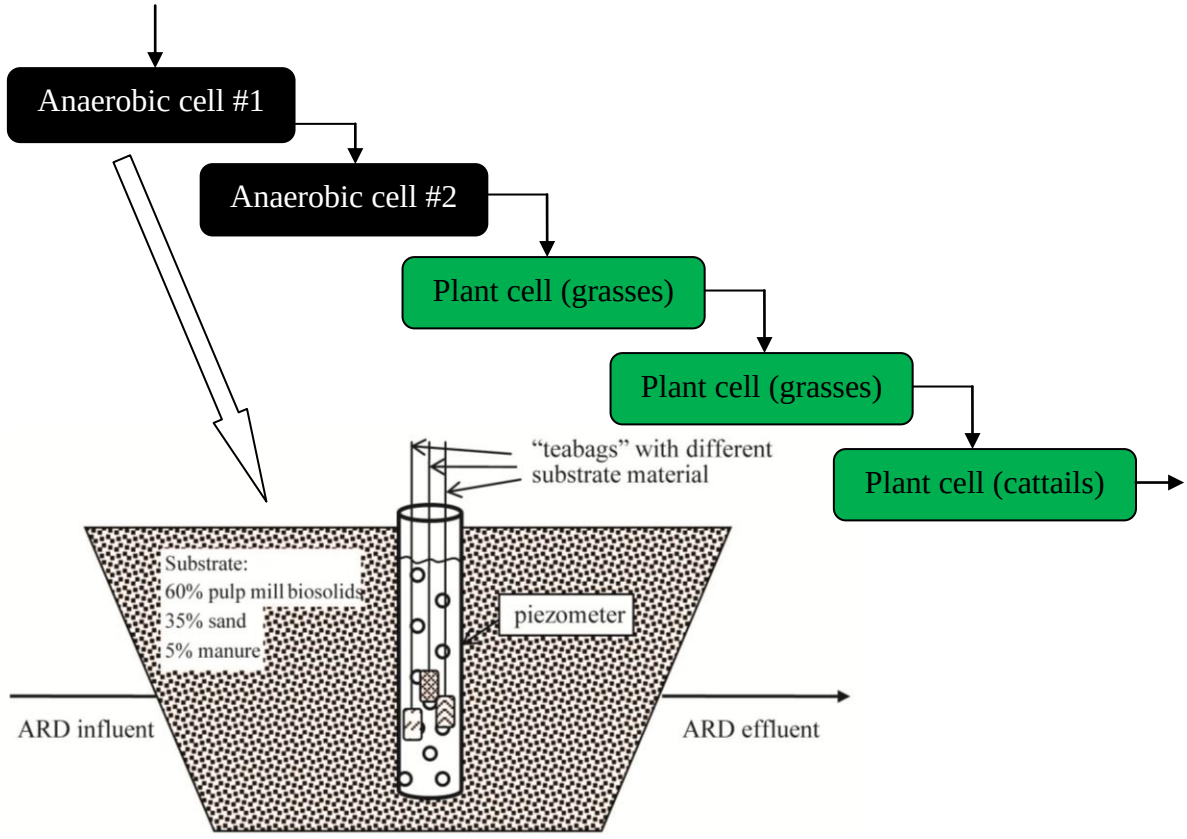


Figure 4.1: Schematic diagram of passive treatment system in Trail, B.C.

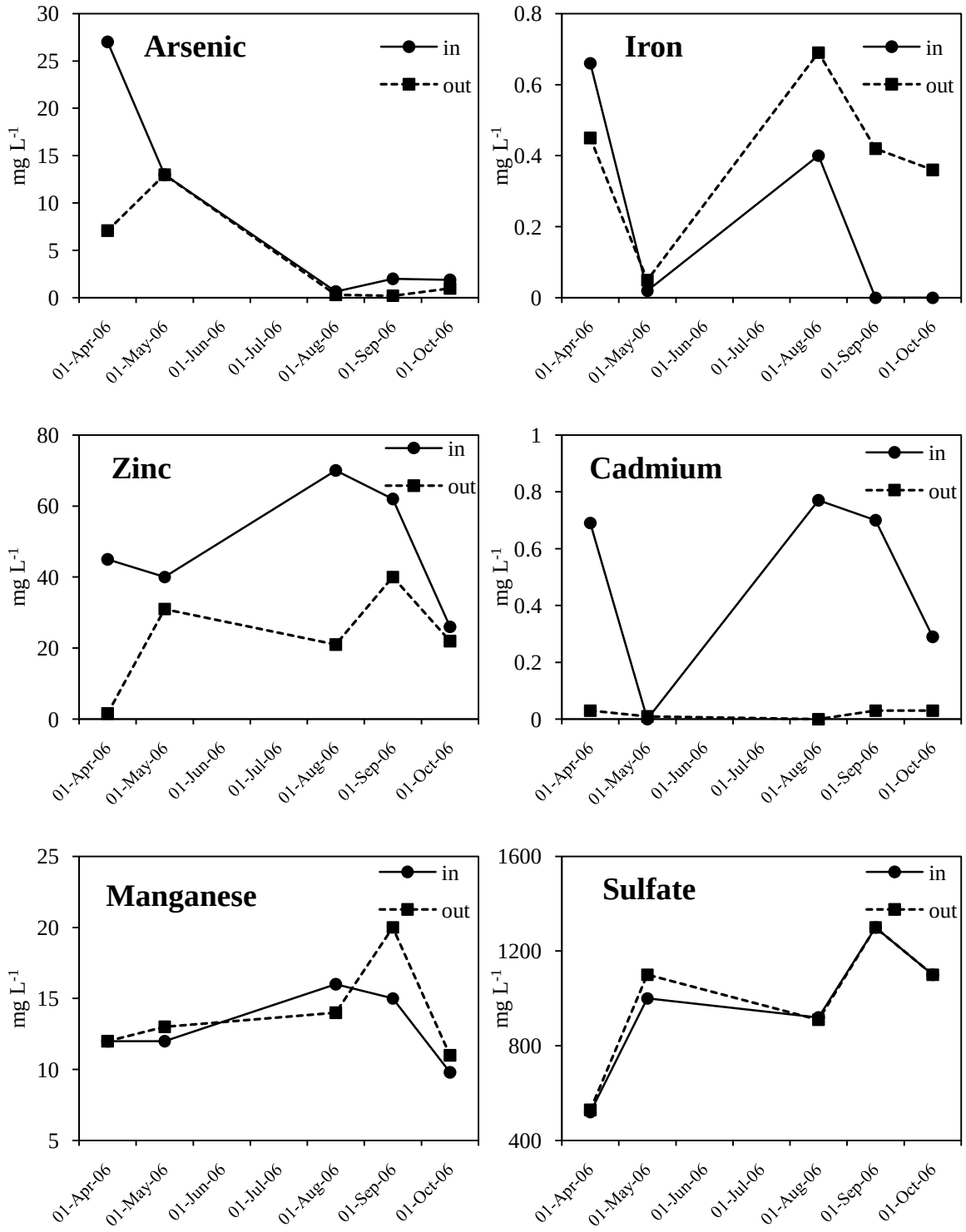


Figure 4.2: Removal of dissolved metals and sulfate from anaerobic bioreactor #1 during the course of the study.

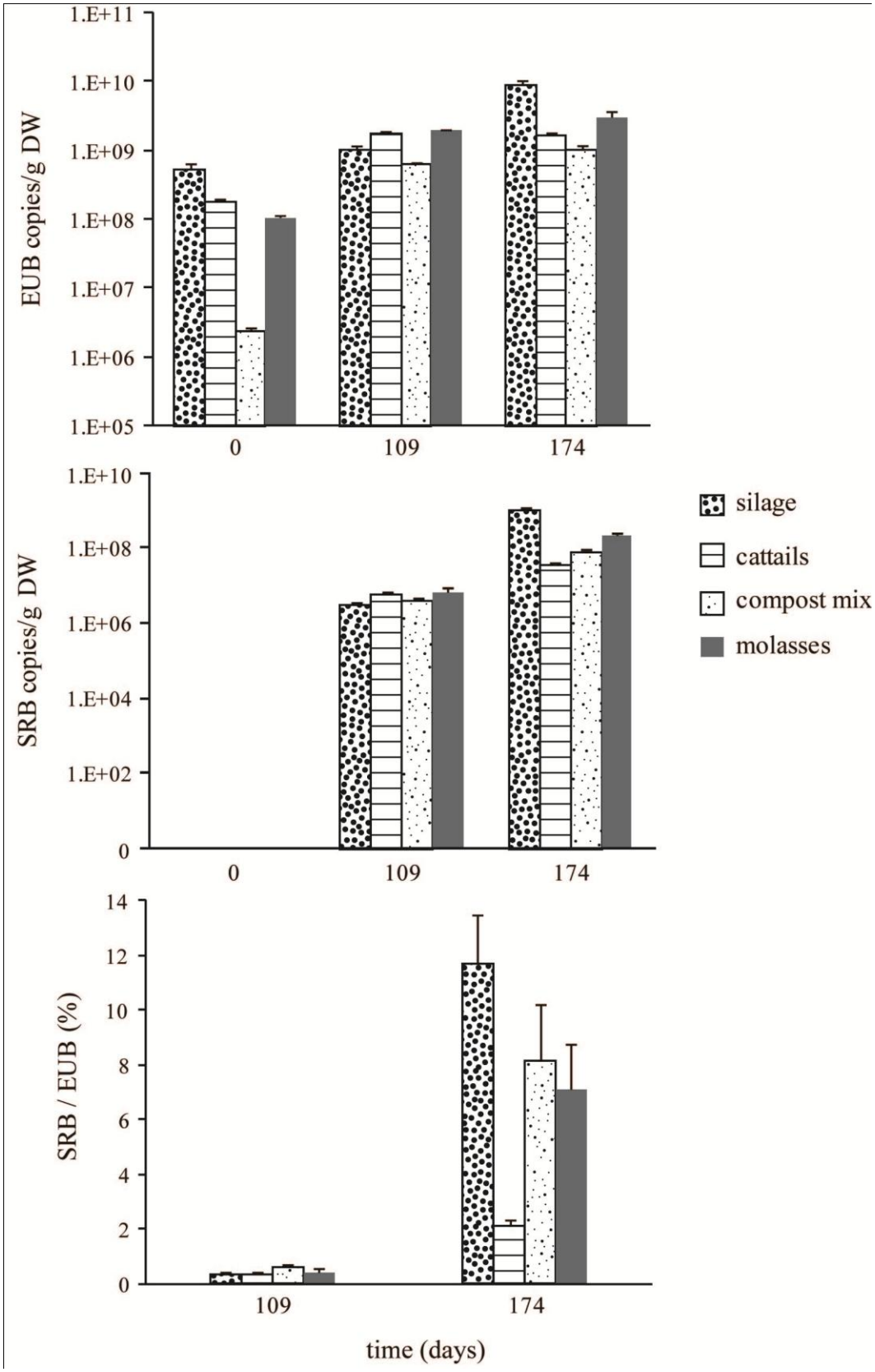


Figure 4.3: Changes in total bacteria and SRB during the length of the experiment as determined by qPCR. Error bars represent standard deviation of 3 or more analytical replicates.

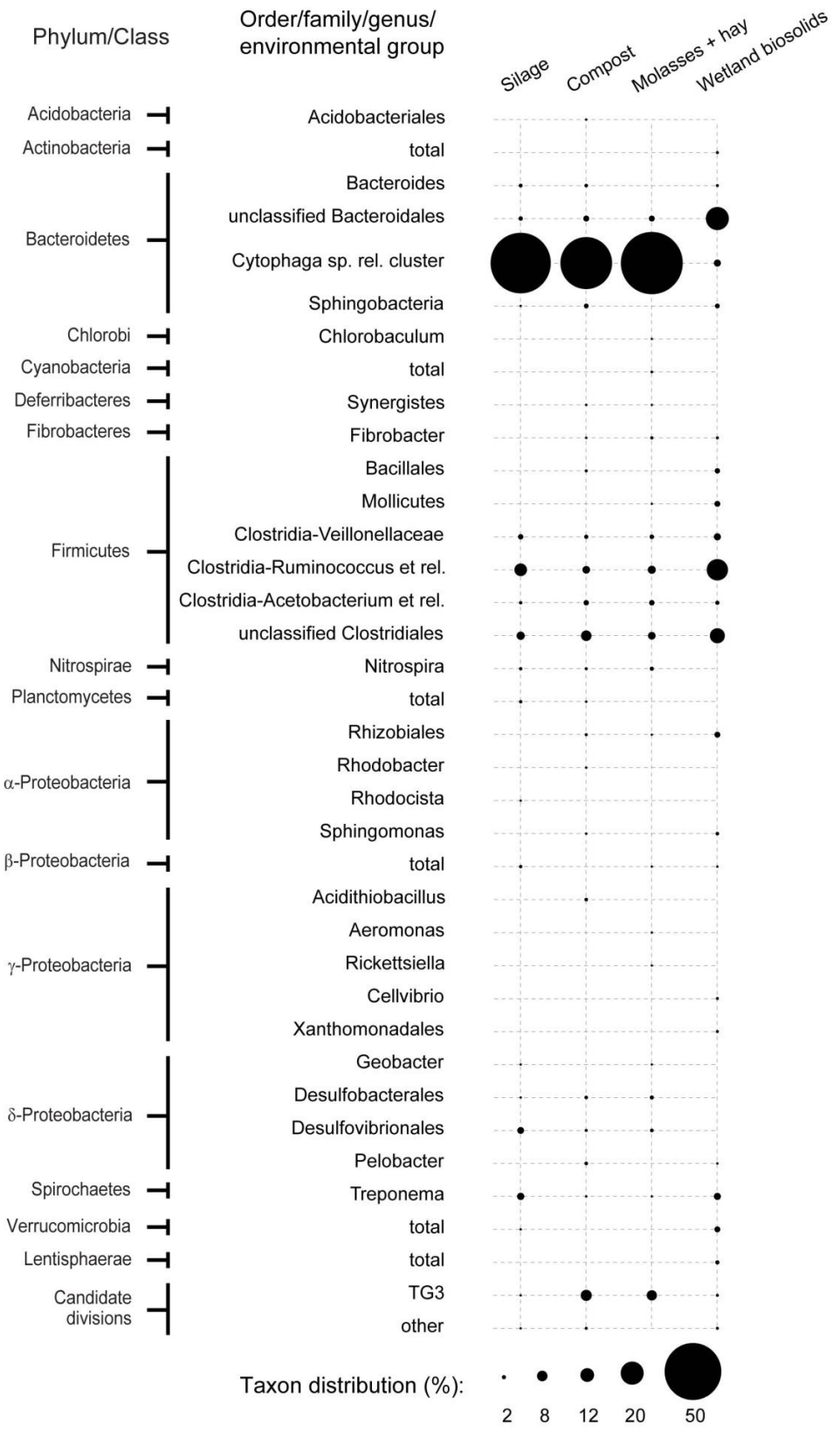
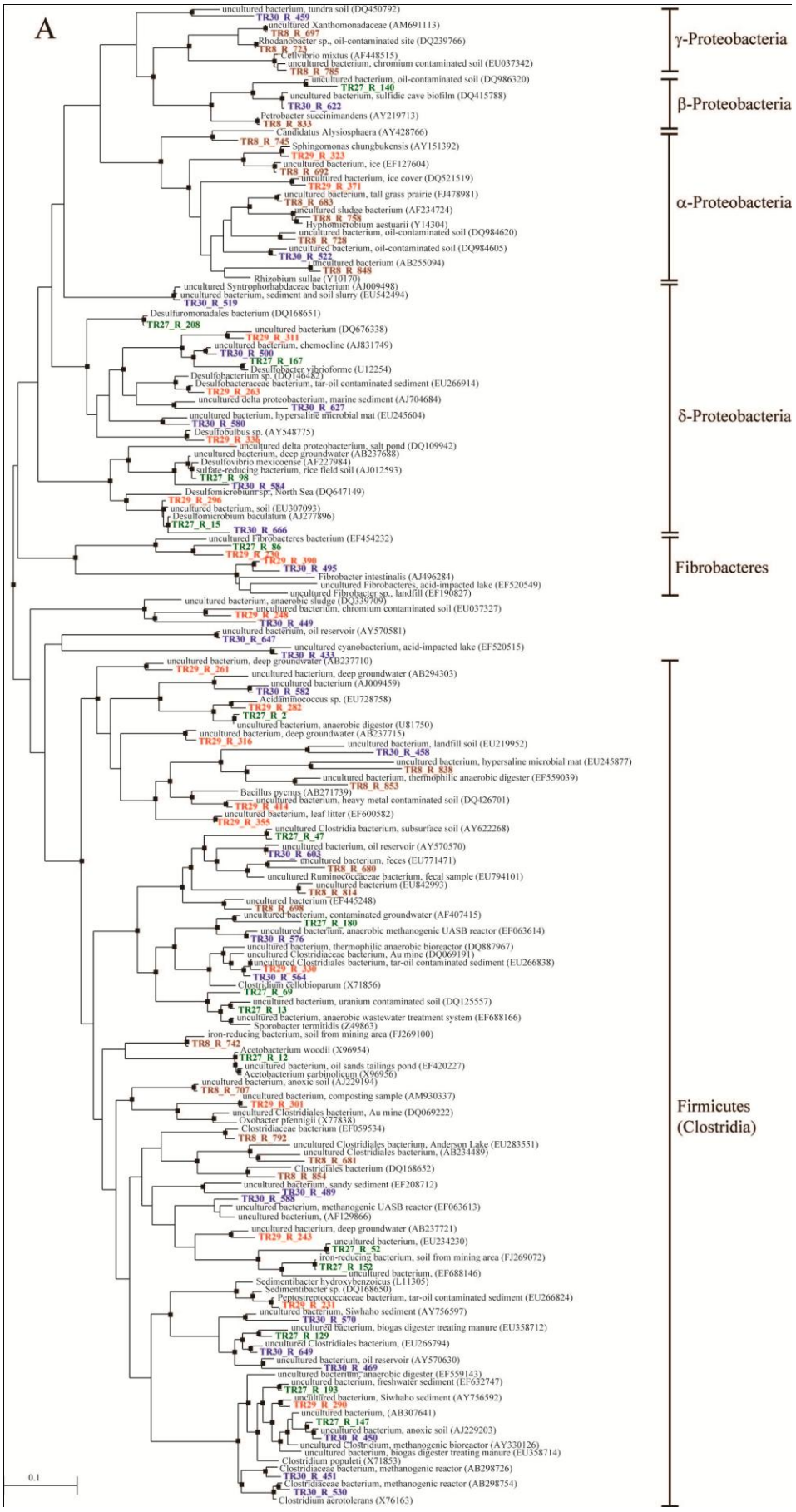


Figure 4.4: Dot representation of bacterial diversity in different carbon materials based on phylogenetic proximity to relevant reference groups and environmental sequences. The circumference of closed circles determines the percentage of clones falling within certain group.


A
γ-Proteobacteria
β-Proteobacteria
α-Proteobacteria
δ-Proteobacteria
Fibrobacteres
Firmicutes
(Clostridia)
0.1

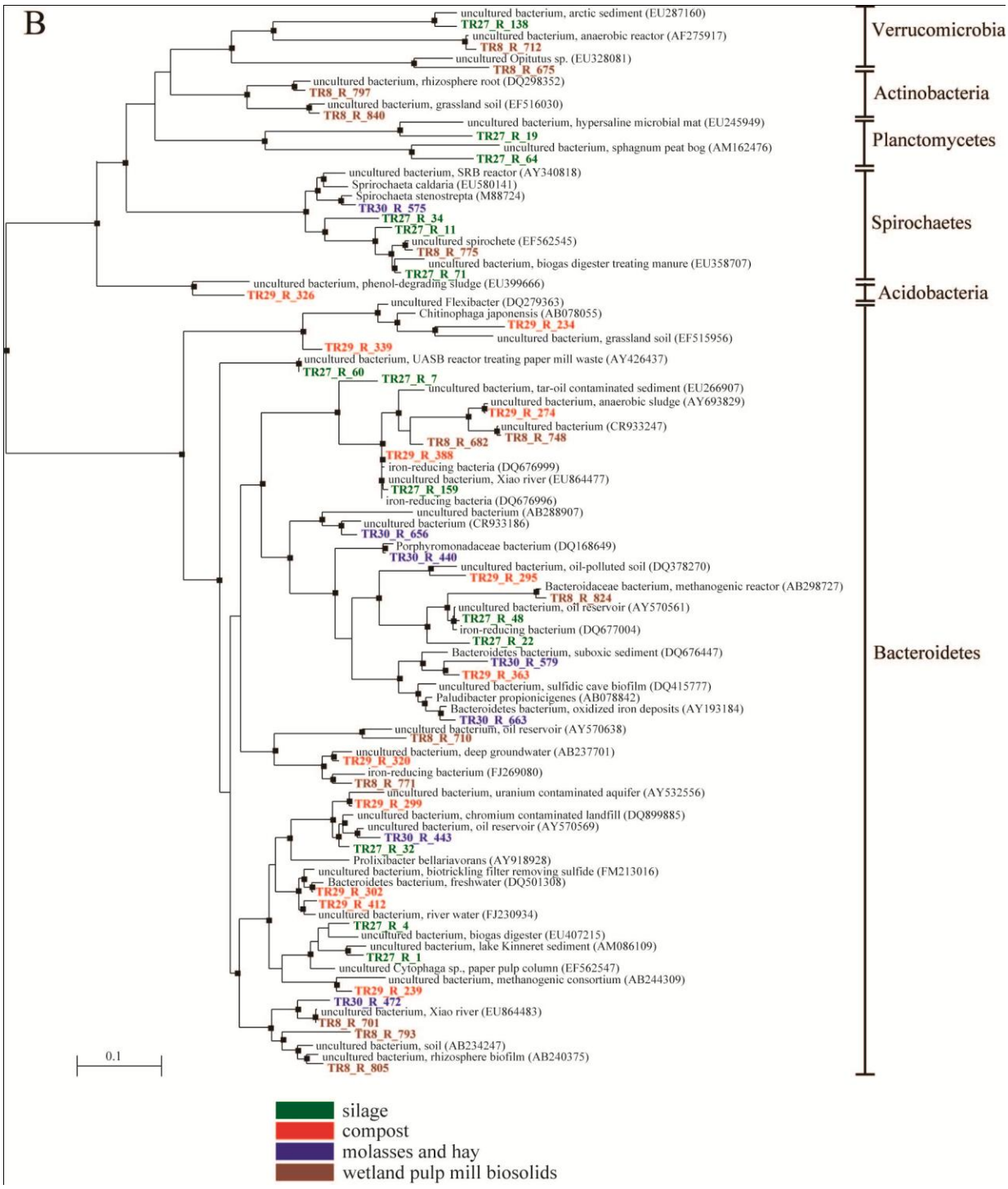


Figure 4.5: Unrooted maximum likelihood phylogenetic tree showing SSU rRNA gene sequences from different carbon materials and related sequences. Clones from this study are colored as per legend. Bootstrap values are shown with a closed square for >50 % (for 100 iterations). Values below 50 % are not shown.

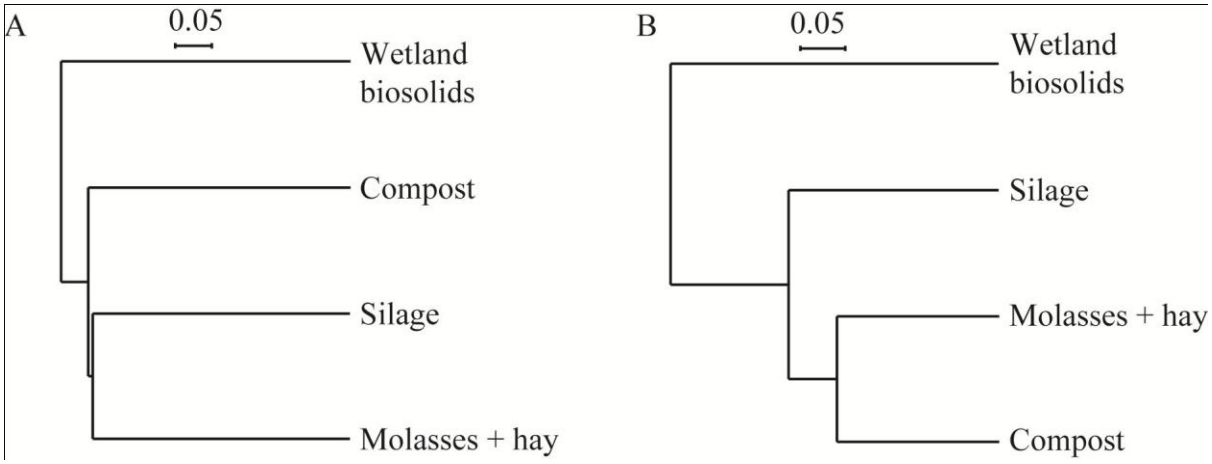


Figure 4.6: Similarity of SSU rRNA (A) and *dsr* clone libraries (B) as determined by Unifrac. The scale bar represents 5 % difference.

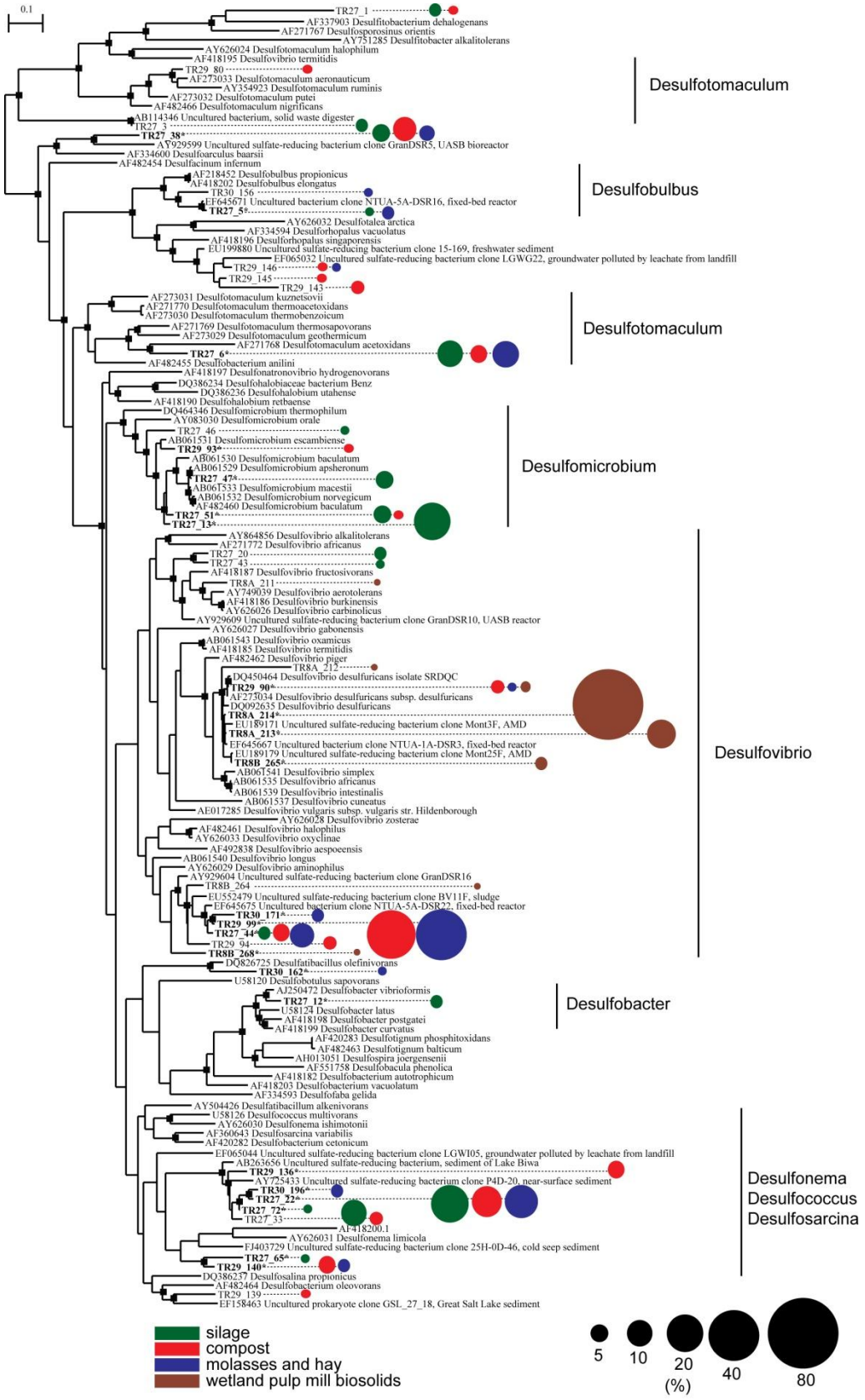


Figure 4.7: Unrooted maximum likelihood phylogenetic tree showing *dsr* gene sequences from different carbon materials and related sequences. The dots represent frequency of particular sequence in a library and are colored as per legend. Clones that are targeted by qPCR primers are shown bold and with asterisk (*). Bootstrap values are shown with a closed square for >50 % (for 100 iterations).

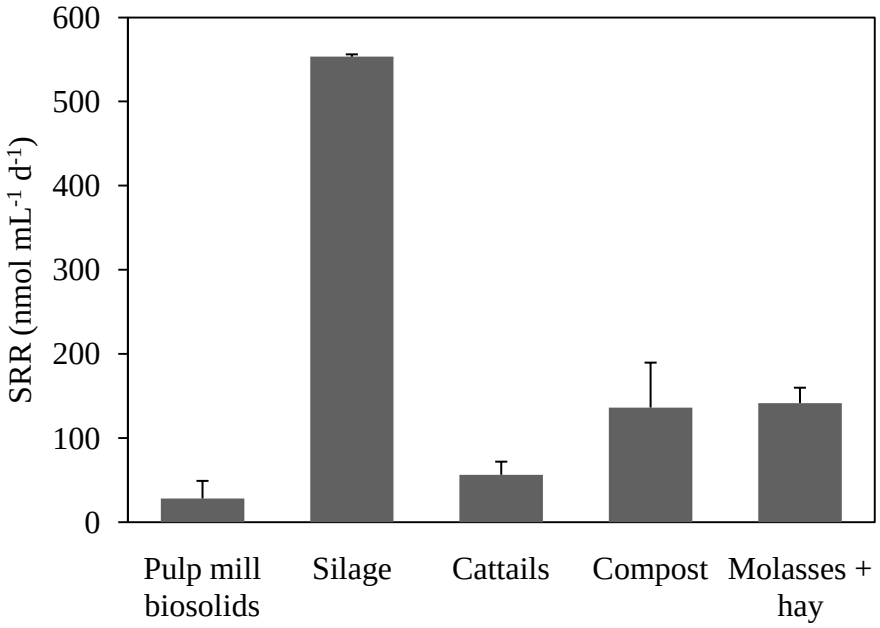


Figure 4.8: Sulfate reduction rate in batch reactors amended with complex carbon sources.

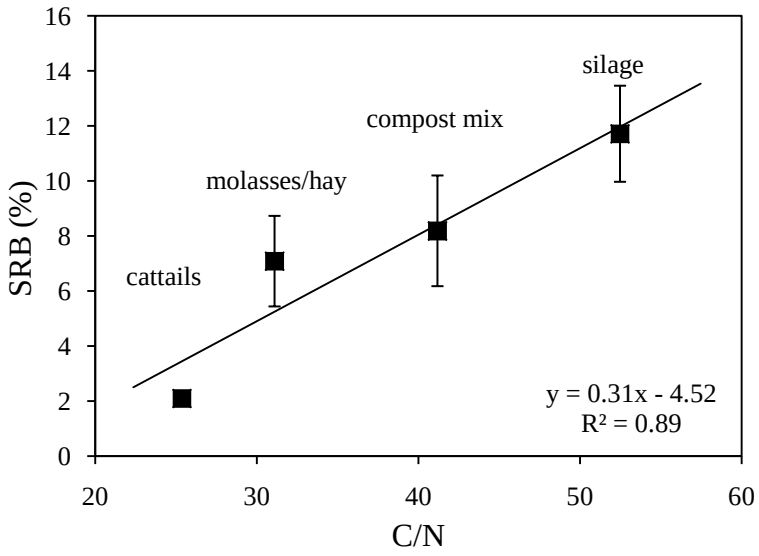


Figure 4.9: Relationship between SRB amount and C/N ratio of initial carbon material samples. The error bars represent standard deviations from multiple measurements.

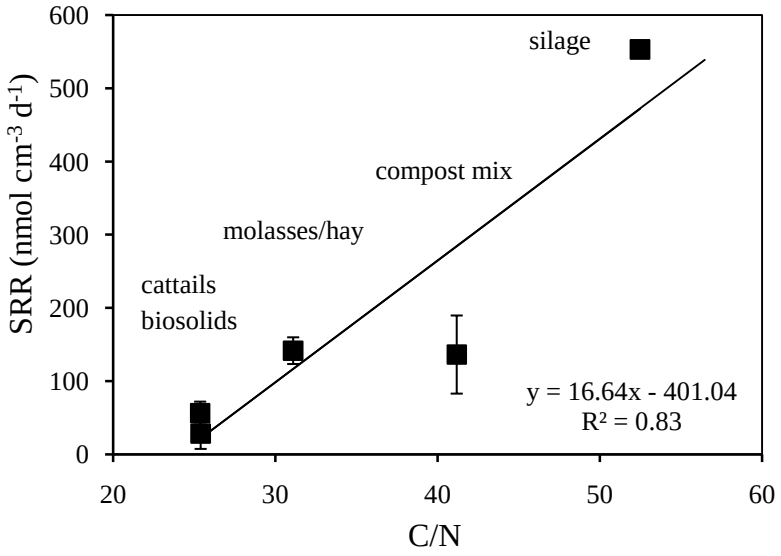


Figure 4.10: Relationship between sulfate reduction rate as determined from the batch reactor experiment and C/N ratio of initial carbon material. The error bars represent standard deviations from multiple measurements.

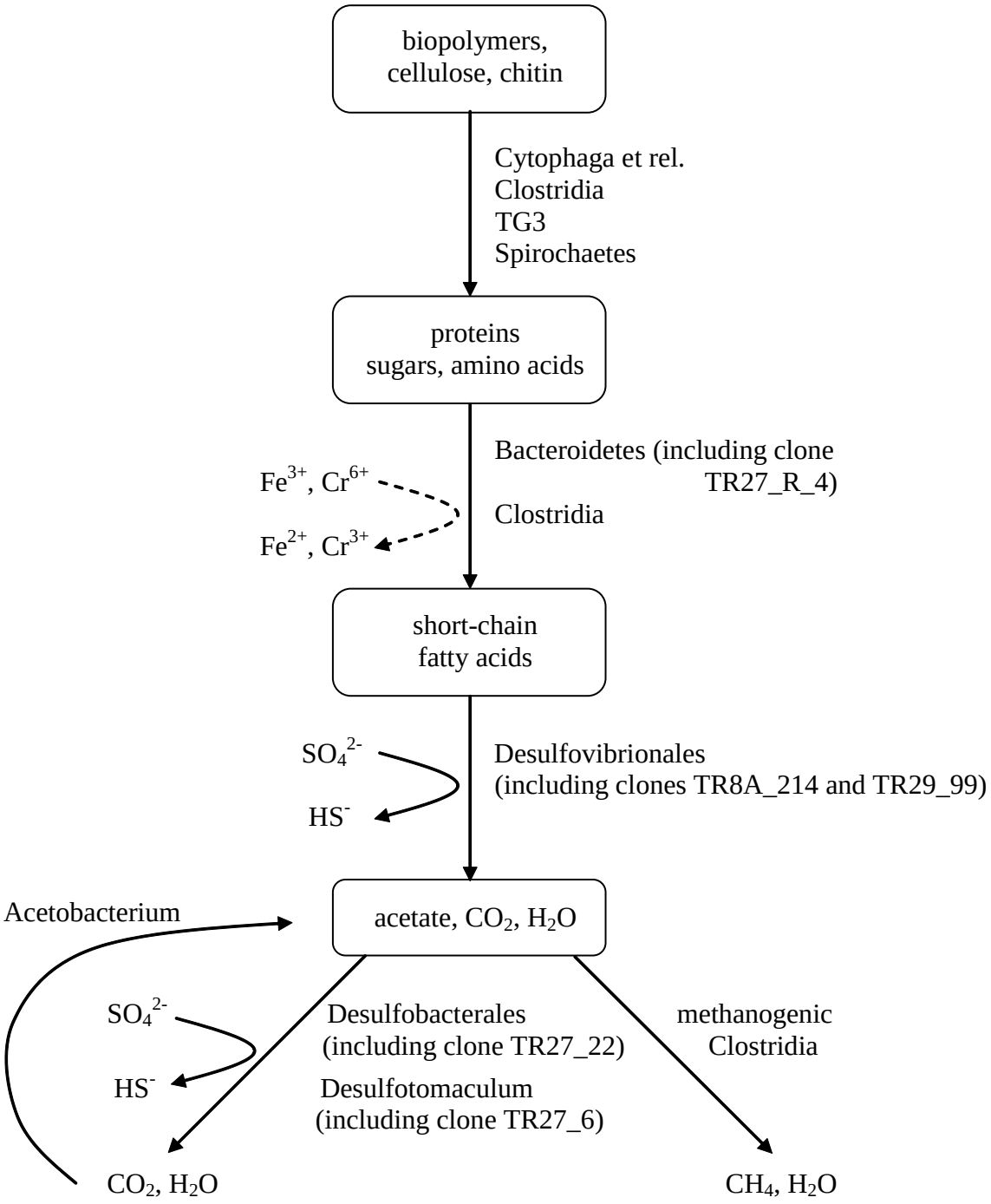


Figure 4.11: Diagram of potential microbial processes occurring in the Trail anaerobic bioreactor.

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CHAPTER 5

CONCLUSIONS

5.1 SUMMARY AND SIGNIFICANCE

In this concluding chapter, outcomes and significance of this thesis work are summarized and possibilities for future research directions are suggested.

Waste from the mining industry has the potential to harm ecosystems if dissolved metals and sulfate infiltrate into natural environments surrounding the mine site. Because of this, regulating authorities have strict guidelines for existing mines on the quality of mine effluent so as to prevent discharge of any toxic compounds to the environment. However, abandoned mines and outdated metal extraction technologies have left a legacy of affected areas where seeps containing toxic metals and sometimes acid are discharging into the aquatic environment. The ease of construction and low maintenance of passive bioremediation systems for these MME seeps led to their widespread implementation in areas where traditional active treatment is not preferred. The evaluation of treatment efficiency is typically based on short-term monitoring of influent and effluent water quality and in some cases the treatment systems failed to continue operating effectively in the long-term. Therefore, there is a need for a more accurate evaluation of their long-term treatment potential. The one area that has not been sufficiently addressed is the microbiology within these systems (Neculita et al., 2007). Thus, the research presented in this thesis offers much needed insight into the microbial communities with the emphasis on the rate-limiting processes of complex carbon degradation as well as the environmental factors that influence the presence and activity of SRB, the group of bacteria important for the long-term functioning of passive treatment systems. The biological data obtained from this approach can be used both to provide insight into processes that should be considered during the design of the system, as well as biomonitoring tools to rapidly and reliably assess the state of a current treatment system.

The first field site used in this study for the implementation of molecular tools and to characterize the microbiology of a natural stratified, high sulfate environment, was the anoxic

Nitinat Lake. Although this fjord is natural and is not affected by MME, it can serve as an analog to mine pit lakes due to its permanent stratification with no oxygen below 11- 40 m (Pawlowicz et al., 2006). In addition, the expansion of anoxic and oxygen minimum zones throughout the world's water bodies, largely due to the increase eutrophication and climate change, has increased interest in the biochemical processes within these systems, as they can potentially have an impact on global elemental cycling (Helly and Levin, 2004; Stramma et al., 2008). While other known anoxic bodies have been studied extensively (Vetriani et al., 2003; Lin et al., 2006; Stevens and Ulloa, 2008), this work is the first to address Nitinat Lake's microbial diversity. The hypothesis leading into this work was that significant sulfate reduction would be found in an anoxic, sulfate- and sulfide-rich aquatic system. However, surprisingly, SRB could not be detected in the deep waters with a phylogenetic analysis of SSU rRNA genes (Schmidtova et al., 2009). Instead, complete dominance by an uncharacterized bacterial group belonging to *ε-Proteobacteria*, which was named NITEP5, was found throughout the anoxic layer in two stations during both spring and summer samplings. Similar sequences have also been found in other anoxic aquatic environments (Madrid et al., 2001; Koizumi et al., 2004; Campbell et al., 2006), but they have only been representative of a small % of the total sequence collection unlike in Nitinat Lake where they comprised up to 97 % of the library. Although it is likely that this group is involved in anaerobic sulfide oxidation, the SSU rRNA gene sequence is not sufficient to determine its exact metabolic function. Therefore, I suggest that this organism is further explored by other techniques such as isolation and culturing studies in the laboratory, or by whole genome sequencing. Besides novel information on species distribution, several conclusions can be drawn with respect to application of sulfate reduction for treatment of MME using mine pit lakes. Since it was found that SRB do not represent a significant part of the anoxic water bacterial population, it cannot be assumed in the remediation of mine pit lakes that high sulfate concentration and anaerobicity are necessary and sufficient conditions for stimulating the growth of SRB.

The outcome from the research presented in Chapter 2 regarding the SRB activity suggested that sulfate reduction is restricted to the sediment layer. In Chapter 3, this hypothesis was confirmed by both quantitative and qualitative measurements. The *in situ* sulfate reduction rate of 250 ± 60 nmol cm^{-3} d^{-1} was determined with a radiotracer. The phylogenetic analysis of all bacteria, as well as solely SRB also showed that SRB constitute significant part of the bacterial community in the top 20 cm of the sediment. An important observation was that the

majority of SRB belonged to *Desulfobacteraceae* family, whose cultured representatives are members of the complete-oxidizing division, therefore they are able to utilize acetate and hydrogen as terminal electron donors (Postgate, 1984). This was also confirmed by the laboratory carbon amendment study where amendment with acetate resulted in the highest sulfate reduction rate when compared to lactate and complex carbon mixture amendments. The approach of monitoring short chain fatty acids as potential electron donors during the experiment revealed more information on the metabolic pathways that have been missing in similar studies (Fauville et al., 2004; Logan et al., 2005).

The second field site was an existing constructed passive treatment system for landfilled mine waste seepage which was particularly rich in arsenic, zinc, and cadmium. The system has been mostly successful in removing metals from the influent since 2002. Therefore, it was of interest to find out which microbial processes take place in the system and how can the efficiency be maintained or improved. In addition, the anaerobic bioreactor, which is the first processing step in a multilevel system, was used for an *in situ* experiment to assess the potential of different carbon materials to enhance the treatment systems. Many low cost materials and mixtures of materials have been tested as a substrate for treatment systems based on sulfate reduction (e.g., (Annachhatre and Suktrakoolvait, 2001; Cocos et al., 2002; Martins et al., 2009). Several parameters, such as C/N ratio, DOC, TOC, lignin and cellulose content, have been proposed to determine the reactivity and suitability of each material. However, no clear trends could be drawn; the results were variable, depending on the length of the study, reactor set-up, and the origin of the materials (Neculita et al., 2007). In my study, I took a different approach to study five different agricultural and industrial waste materials. In addition to analyzing general carbon characteristics, metabolic processes were estimated using molecular tools and phylogenetic analyses. The materials were suspended in the aqueous phase within the anaerobic bioreactor and left in that environment for five months from early spring to fall, when the most microbial activity was assumed to take place. At the end of the experiment, the materials, which were sealed in individual mesh bags, were taken out and analyzed. Understandably, there are limitations to this approach. Because all materials were placed next to each other into the same environment, the initial soluble portion of the materials was instantly released and likely did not influence the microbial colonization. However, the results from the carbon and microbial analyses suggested that microniches formed within the individual materials where some of the carbon was preserved (Chapter 4). A very different bacterial community was found in the materials after the *in situ* exposure compared to the sample from the actual bioreactor. Whereas

the SRB library from the introduced materials showed relatively high diversity with many species representing different SRB groups, the *dsr* library for biosolids taken directly from the bioreactor was uniform with 80 % sequences forming a single OTU that belongs to incomplete-oxidizing *Desulfovibrio*. In fact, very few SRB sequences were retrieved from this sample, suggesting that sulfate reduction is not a significant process. Accordingly, the measurements of the effluent from the bioreactor contained similar amounts of sulfate when compared to the influent. Therefore, even though this system was designed to remove metals using biological sulfate reduction, the process does not occur substantially and other processes must be responsible for metal removal. When comparing the introduced materials, silage contained largest number of SRB at the end of the experiment. The superiority in facilitating sulfate reduction of this material was also confirmed by a short-term sulfate reduction rate batch reactor study. A positive trend in SRR was observed between the amount and activity of SRB and the initial C/N ratio in the materials. Although the SRB were found in all but pulp mill biosolids, they were not the dominant species. All phylogenetic libraries from sampled materials contained high amounts of sequences from *Bacteroidetes* and *Firmicutes* phyla. These phyla are expected to contribute to the degradation of complex carbon molecules, the rate-limiting step in the breakdown process.

Understanding the complexity of microbial processes and how they correlate with their local environment is the key to the success in passive bioremediation of ARD. To the author's knowledge, this work pioneered application of new molecular tools to reveal how organic materials and the microbes that they support determine the sulfate reduction potential of natural and constructed systems. Improved methods were developed, such as qPCR, for species quantification that can enumerate SRB more rapidly and accurately than previously used culture-based approaches. Certain correlations could be made between the molecular data and the actual system operation and performance. Unquestionably, this is the first step that can lead to the application of molecular methods for this complex biological system's evaluation. The application of phylogenetic tools is powerful and simple, and should be a starting point for every attempt to characterize a biological system. Still, there are limitations that need to be overcome. The amount of data to characterize the microbial community with statistical confidence is significant. Every week, hundreds of new sequences are submitted to databases. Therefore, it becomes increasingly difficult to analyze the substantial datasets and a demand for improved bioinformatics tools is inevitable. Also, the phylogeny provides only indirect information on the functions of the system and thus on the performance. Addition of methods that result in

information directly associated with design parameters, such as metabolic rates, using protein or mRNA expression levels, or stable isotope probing, should follow.

This work characterized two different environments, both with strong sulfate reducing potential. It showed that the bacterial communities varied greatly and different processes driving the geochemistry were identified. From a scientific point of view, a unique bacterial group was discovered in the Nitinat Lake anoxic water column. Also other unique clusters of uncharacterized microbes that are involved in hydrolytic processes were found, as well as SRB adapted to specific organics materials. In addition, 1779 SSU rRNA gene and 450 *dsr* sequences were analyzed and will be deposited to the publicly available database.

5.2 FORMAL RECOMMENDATIONS FOR THE DESIGN IMPROVEMENTS OF MME PASSIVE TREATMENT SYSTEMS

The work presented here has emphasized the need for understanding the microbial activity in MME treatment systems. Although many interactions and relationships remained unexplored, the results can still provide guidelines and suggestions on improving the passive systems design and operation:

1. Mine pit lakes

   It was shown in Chapter 2 and 3 that sulfate reduction in stratified basin is restricted to the sediment intervals. In Nitinat Lake, the overall fjord SRR is 0.5 nmol cm^{-3} d^{-1}, which results in 1.38 Mmol of produced sulfide per day (of which around 50 % is in a dissolved form). Considering a MME containing 17 mmol L^{-1} of dissolved metals, we can calculate that a maximum of 450 L s^{-1} of MME could enter the fjord. Diffusion limitations of sulfide produced only in the upper sediment, as well as bacterial sulfide removal, will further decrease the metal removal capabilities. Therefore, addition of more permeable organic materials to increase the volume of the SRB reactive zone, or structural modifications to increase the contact area between water and the reactive zone, are some recommended design modifications. If MME is designed to enter the lake at the bottom and even permeate through a reactive barrier, diffusion limitations will be overcome promoting higher overall volumetric SRRs within the lake. Identification of likely SRB species in the sediments can help decide what organic amendment to add. For example,

addition of acetate showed to be effective to boost sulfate reduction rate and can be added to provide sufficient electron donors for establishment of active SRB population.

2. Passive anaerobic bioreactors and subsurface wetlands

   The results of Chapter 4 showed that sulfate reduction was likely not the mechanism responsible for metal removal in the Trail anaerobic bioreactor, at least not after 4 years of operation. This is in agreement with many other systems also in operation for several years using a variety of substrate materials. Therefore, other processes, such as metal adsorption onto solid surfaces, chemical precipitation, direct microbial metal uptake or metal precipitation mediated by bacteria other than SRB must be responsible for reducing the dissolved metal concentrations. More work needs to be done to identify the microbial processes, if any, in this system that do promote metal sequestration if we are to understand how to improve the treatment performance with nutrient addition. Multiple processes other than sulfate reduction for metal removal are sufficient if decreasing the sulfate concentration is not a prerequisite. However, in British Columbia, water quality regulations for aquatic life recommend sulfate concentrations less than 100 mg L^{-1}. Therefore, it is important to identify organic substrates that support the highest SRB activity to use in passive treatment systems for MME containing sulfate concentrations greater than 100 mg L^{-1}. Our study showed that a greater diversity of SRB species corresponded with increased total numbers of SRB and a high SRR. Also, the relationship was found between C/N ratio of the raw material and SRB activity. Thus, organic material capable of supporting many different SRB species should be used in passive treatment systems.

5.3 FUTURE WORK RECOMMENDATIONS

The following areas of research are recommended stemming from the presented work:

- NITEP5 bacterial group found in the Nitinat Lake water column:

  More research is needed to determine the metabolic capabilities and the role this bacteria play in the overall geochemical cycling. It is recommended that the species are isolated and an attempt to culture be made in a laboratory setting. The uptake of different sulfur species can be then examined. Another approach is to sequence the whole genome so

that the sequence can be surveyed for functional genes and the metabolic activity can be deduced.

- Determination of processes responsible for metal removal in passive systems

  Due to the time-related and analytical constraints, only limited number of samples was subjected to phylogenetic analysis. It is clear that the bacterial community changes overtime, as could be seen when clone libraries of 5-month old samples were compared to the sample that was in the system for ca. 4 years. Long-term evolution of microbial population since the system's start-up, and their correlation to the metal removal processes, needs be explored. New technologies that can process giga-base pairs at one time and lower the price of sequencing will enable us to gather more information on spatial and temporal variations in microbial diversity and thus dominant geochemical processes.

- Molecular and analytical techniques:

  The use of molecular techniques has become a standard approach in the field of microbial ecology, and now these tools are becoming increasingly popular in applied science, such as in the monitoring of engineered biological processes. The phylogenetic analysis is a powerful technique for overall microbial diversity evaluation. However, it does not provide information on specific biochemical processes and their rates. Therefore, in addition to microbial diversity, I suggest to use complementary techniques, such as activity and substrate uptake tests using radiotracers, or rapidly developing metagenomic approaches. For example, using larger fosmids as opposed to clones enables to identify bigger portion of the genome, where genes related to specific metabolic processes can be found. Alternatively, microarrays with different functional genes or metabolic pathways can rapidly determine the presence of particular processes. After these processes are identified, other approaches such as mRNA expression levels or protein quantification can determine the kinetic rates that are of interest.

- Mathematical modeling:

  Ultimately, the goal should be to develop a mathematic model that would predict the kinetic rates and the long-term process efficiency of these passive bioremediation systems. Attempts have been made, with the latest one from Hemsi et al. (Hemsi et al., 2005), however, the understanding of the complex processes is not sufficient yet to create a model that would be accurate. Therefore, the combination of molecular

techniques with kinetic rate tests should expand the knowledge of the systems in order to design an accurate model.

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APPENDICES

APPENDIX A

CONSIDERATIONS OF QPCR

Suitability of qPCR for dsr quantification

This technique was chosen due to its high sensitivity and high specificity of dsr gene, occurring solely in species that perform dissimilatory sulfate reduction. A number of pure SRB cultures, as well as a nixed laboratory cultures were tested and yielded positive results and linear calibration curves. The method was also tested for false-positive amplification using negative controls of both pure non-SRB species, and environmental DNA samples that did not contain SRB (such as aerobic communities). Although certain error in absolute quantification is inevitable stemming from variable PCR efficiencies, non-specific amplifications, non-specific fluorescent dye binding, it is especially reliable in between-sample comparisons. In addition, the designed primers do not detect all cultured and non-cultured sequences available in the database, so it is likely that the absolute cell number is underestimated.

SYBR Green vs. probe-based assays

The quantification of dsr gene optimized in this work uses SYBR Green technology. Although the addition of a specific probe tends to result in higher specificity of qPCR reaction, we chose to use SYBR Green due to the high variability of the gene of interest. Therefore, designing primers and a probe that would target high number of dsr sequences was not possible.

Amplification efficiency

The assumption was made that the reaction efficiency of environmental genomic DNA sample was the same as the one extracted from the pure SRB species and used as a standard. This was confirmed by evaluating the increase of fluorescent signal between two cycles in environmental samples and standards. The efficiency of all reaction was between 80-100 %.

Inhibitors

It has been shown that environmental samples contain substances that potentially inhibit PCR reactions (e.g., humic acids). The effect of inhibitors was tested by serial dilution of the environmental DNA samples and subsequent qPCR amplification. The variations in quantification results between different dilutions were less than the analytical error and therefore the inhibition effect was excluded.

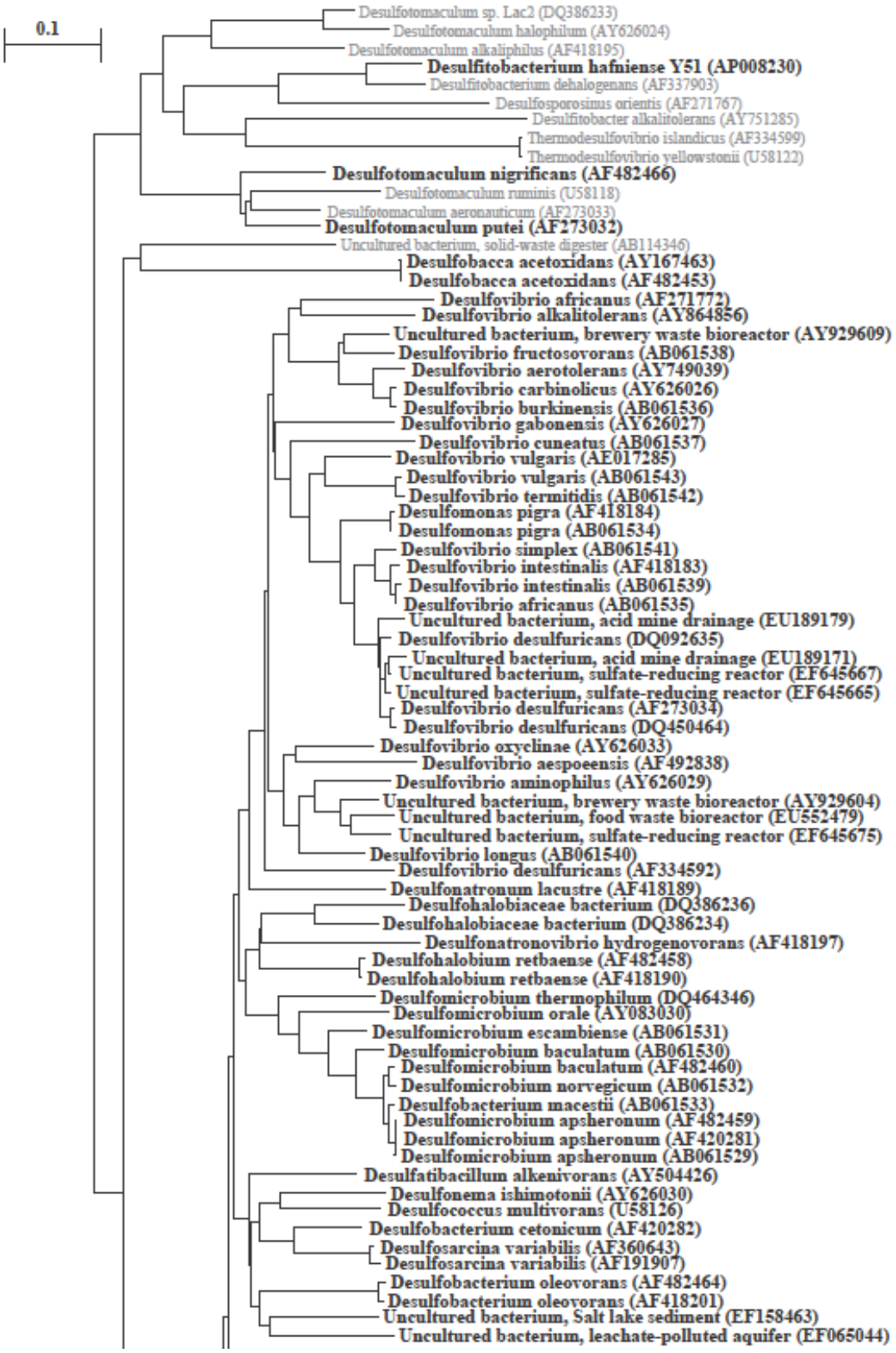


Figure A.1: Neighbor-joining phylogenetic tree of *dsr* sequences used for qPCR primer design. Sequences that match the primers (with up to 1 mismatch) are in bold.

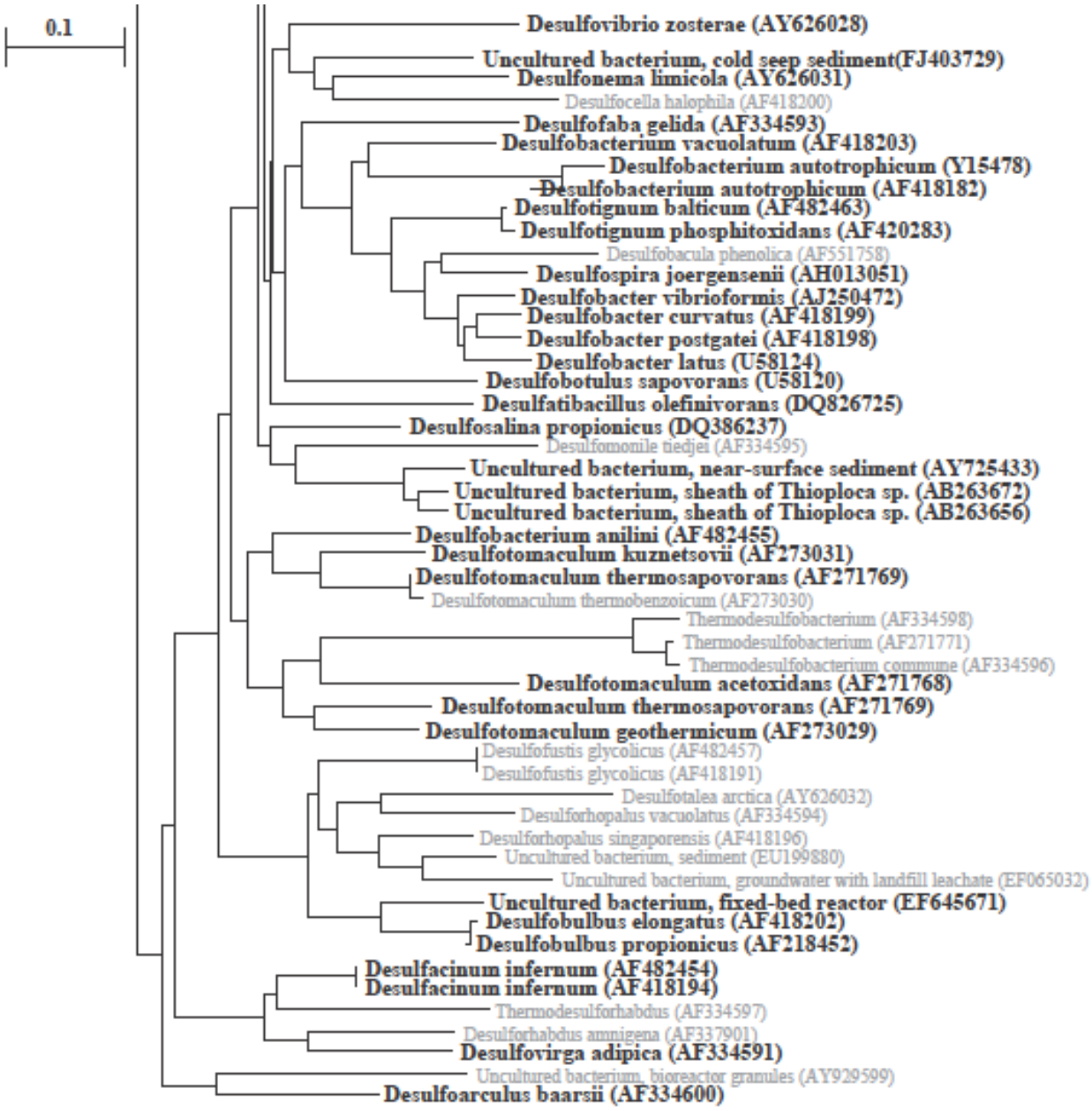
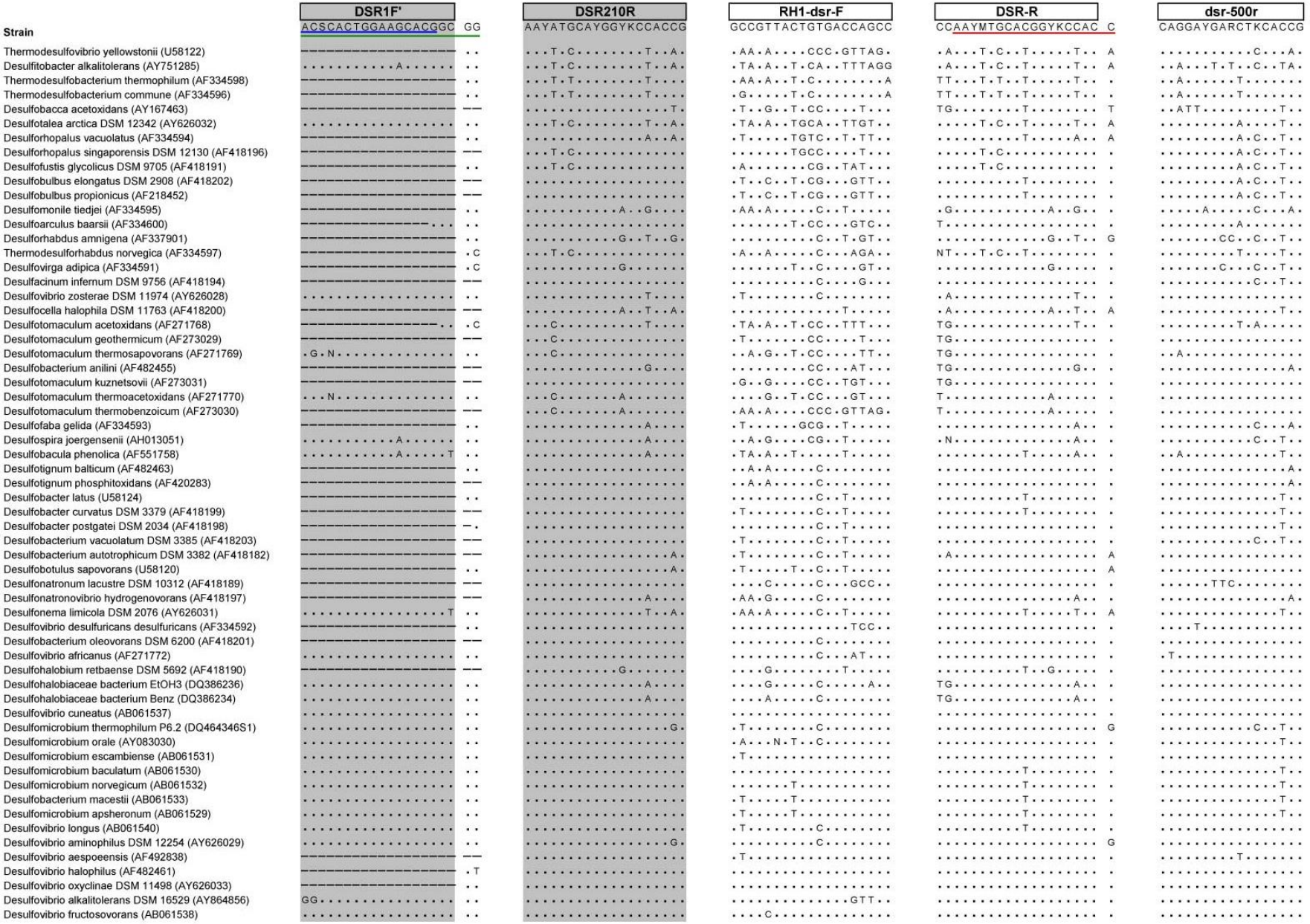
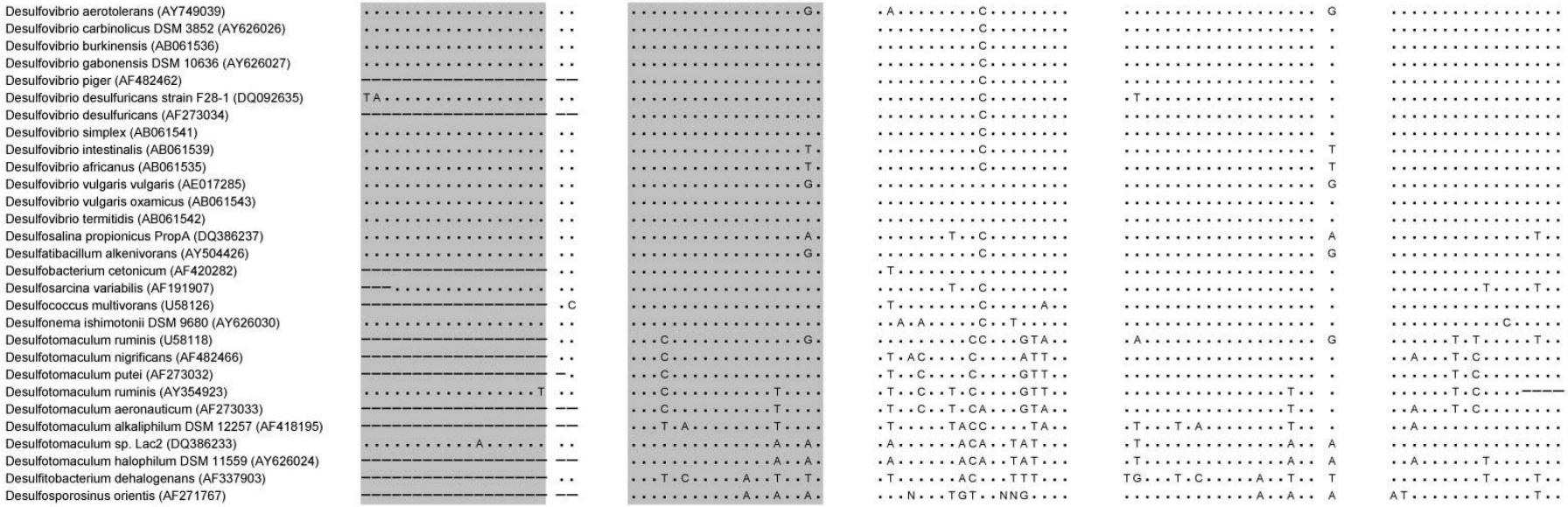


Figure A.1 continued

Table A.1: Comparison of qPCR primers used in this study (shaded) to other published qPCR primers. Dots represent matching nucleotides.





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APPENDIX B

SUPPLEMENTARY DATA FOR CHAPTER 2

Table B.1: Abundance and nearest phylogenetic neighbors of all OTUs obtained from Nitinat Lake SSU rRNA clone libraries. OTUs with asterisk (*) belong to the NITEP5 group of epsilon Proteobacteria and match 100 % to specific qPCR primers developed in this study.

OTU / Accession no.		No. of clones in library								Nearest phylogenetic neighbor		
		S02 April		S02 August		S05 April		S05 August		Closest match	Accession no.	Similarity (%)
		26	50	37.5	50	22	50	13	28			
Ø-Proteobacteria												
Nit2A0620_19	EU265946							1		Uncultured bacterium, marine bacterioplankton	EF016465	99
Nit2A0626_86	EU266011	1								Uncultured Sulfitobacter sp. clone F457	AY794211	91
Nit5Au0613_684	FJ628301							3		Uncultured Sulfitobacter sp. clone F457	AY794211	92
Nit2Au0637_99	EU570849			8	2					Uncultured bacterium, Arctic sediment	EU050749	97
Nit2Au0637_398	FJ628225			1						Uncultured bacterium, Arctic sediment	EU050749	91
Nit2Au0637_100	EU570831			2						Rickettsia limoniae strain	AF322442	89
Nit2Au0637_403	FJ628227			1						Rickettsia limoniae strain	AF322442	89
Nit2Au0637_102	EU570832			1						Uncultured bacterium, Arctic sediment	EU287008	93
Nit2Au0637_340	FJ628205			1						Uncultured bacterium, Arctic sediment	EU287008	93
Nit2Au0637_405	FJ628229			1						Uncultured bacterium, Arctic sediment	EU287008	93
Nit2Au0637_107	EU570836			1		1		1		Uncultured clone Arctic96A-20, Arctic ocean	AF353208	99
Nit5Au0613_177	EU570899							1		Magnetic bacterium	Y13209	98
Nit2Au0637_374	FJ628217			1						Uncultured bacterium	AB294318	82
Nit5Au0613_722	FJ628324							1		Candidatus Pelagibacter ubique	CP000084	97
Nit5Au0613_753	FJ628338							1		Uncultured bacterium, cold seep sediment	AB188773	98
Ø-Proteobacteria												
Nit2A0650_47	EU265973		1							Delftia sp.	EF426439	99
Nit5Au0613_721	FJ628323							1		Nitrosospira sp. III7	AY123809	95
δ-Proteobacteria												
Nit2A0620_3	EU265930	1				1		5		Uncultured bacterium, submarine hot spring	AB294972	87
Nit5Au0613_741	FJ628335							1		Uncultured bacterium, submarine hot spring	AB294972	87
Nit2A0626_74	EU265999	1		2	3			7		Uncultured bacterium, submarine hot spring	AB294952	89
Nit5Au0613_711	FJ628318							1		Uncultured bacterium, submarine hot spring	AB294952	89
Nit5Au0613_680	FJ628300							1		Uncultured bacterium, submarine hot spring	AB294950	95
Nit2A0626_193	FJ628173	2								Uncultured bacterium, hydrothermal plume	AB112451	95
Nit2Au0650_423	FJ628238				1					Uncultured bacterium, hydrothermal plume	AB112451	93
Nit5A0622_575	FJ628285					1				Uncultured bacterium, hydrothermal plume	AB112451	94
Nit2Au0637_354	FJ628207			1						Uncultured bacterium, hydrothermal plume	AB112458	95
Nit5Au0613_695	FJ628308							1		Uncultured bacterium, hydrothermal plume	AB112458	87
Nit5Au0613_739	FJ628333							1		Uncultured bacterium, hydrothermal field	DQ270612	82
Nit5Au0613_674	FJ628296							1		Uncultured bacterium, seafloor lavas	EU491836	89
Nit5Au0613_697	FJ628309							1		Uncultured bacterium, seafloor lavas	EU491308	89
Nit5Au0613_710	FJ628317							1		Uncultured bacterium, seafloor lavas	EU491308	89
Nit2A0626_73	EU265998	1								Uncultured bacterium, arctic surface sediment	EU287364	88
Nit5Au0613_685	FJ628302							1		Uncultured bacterium, arctic surface sediment	EU287345	91
Nit5Au0613_176	EU570898							1		Uncultured bacterium, marine sediment	EU700149	90
Nit5Au0613_725	FJ628326							1		Uncultured bacterium, marine sediment	DQ289906	94
Nit5A0622_503	FJ628257					1		1		Uncultured bacterium, Yellow Sea sediment	EU652574	90
Nit5Au0613_672	FJ628295							1		Uncultured bacterium, Yellow Sea sediment	EU652574	91
Nit5Au0613_746	FJ628337							1		Uncultured bacterium, Yellow Sea sediment	EU652561	99
Nit2A0620_9	EU265936			1						Uncultured bacterium, mangrove soil	DQ811846	92
Nit2A0620_20	EU265947					1				Uncultured bacterium, mangrove soil	DQ811846	90
Nit5A0622_535	FJ628270					1				Uncultured bacterium, mangrove soil	DQ811846	88
Nit2A0620_26	EU265953				1			11		Uncultured bacterium, soil	DQ451463	87
Nit2A0620_31	EU265958				3					Uncultured bacterium, soil	EU134767	91
Nit2A0626_82	EU266007	2								Uncultured bacterium, soil	EU134767	96
Nit2A0626_224	FJ628188	1								Uncultured bacterium, soil	DQ083107	87
Nit5A0650_160	EU570885						1			Uncultured bacterium, sulfide-oxidizing mat	EF687339	98
Nit2A0620_16	EU265943							3		Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	91
Nit2A0650_36	EU265963		1	1	1					Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	98
Nit2A0626_64	EU265989	1		11	10			2		Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	97
Nit2A0626_78	EU266003	1						1		Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	97
Nit2A0626_84	EU266009	1								Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	85
Nit2Au0637_97	EU570847			1			1			Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	98
Nit2Au0650_132	EU570861	2		8	9	1		1		Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	97
Nit2A0626_201	FJ628179	1								Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	92
Nit2A0626_219	FJ628184	1		1						Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	94
Nit2A0626_233	FJ628191	1								Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	97
Nit2A0626_237	FJ628192	1		5	5	2		5		Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	97
Nit2Au0650_461	FJ628248				2					Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	92
Nit2Au0637_369	FJ628216			2						Thiotrophic endosymbiont of Bathymodiolus sp.	AJ745718	95
Nit2A0626_67	EU265992	1		11	1	2	2	2		Thiotrophic endosymbiont of Bathymodiolus sp.	AM236328	98
Nit2Au0637_357	FJ628209			2						Thiotrophic endosymbiont of Idas sp.	AM402957	91
Nit2Au0650_459	FJ628247				1					Thiotrophic endosymbiont of Idas sp.	AM402957	93
Nit2Au0637_106	EU570835			1		2		2		Bathymodiolus sp. methanotrophic gill symbiont	AB036710	98
Nit2Au0637_414	FJ628234			1						Bathymodiolus sp. methanotrophic gill symbiont	AB036710	94
Nit5Au0613_713	FJ628319							1		Uncultured bacterium, upwelling near African shelf	FM246508	90
Nit5Au0613_728	FJ628328							1		Uncultured bacterium, Cariaco basin	AF285612	90

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		26	50	37.5	50	22	50	13	28			
Nit2A0626_77	EU266002	1			2	1		3		Uncultured gamma proteobacterium	AY094499	95
Nit2Au0650_450	FJ628246				1					Uncultured gamma proteobacterium	AY094499	92
Nit5Au0613_676	FJ628298							1		Uncultured gamma proteobacterium	AY094499	94
Nit2Au0650_122	EU570852				1					Uncultured gamma proteobacterium	AJ704661	94
Nit2Au0650_126	EU570856	1			1					Uncultured gamma proteobacterium	AJ704661	98
Nit5Au0613_178	EU570900							1		Uncultured gamma proteobacterium	AJ704661	92
Nit5Au0613_756	FJ628340							1		Uncultured gamma proteobacterium	AJ704661	90
Nit2Au0637_347	FJ628206			2						Uncultured gamma proteobacterium	AF382102	97
Nit5Au0613_745	FJ628336							2		Uncultured gamma proteobacterium	AF382102	99
Nit2Au0637_377	FJ628218			1						Uncultured gamma proteobacterium	AF382100	93
Nit2Au0637_388	FJ628222			1						Uncultured gamma proteobacterium	AF382104	91
Nit5Au0613_706	FJ628313							1		Marine gamma proteobacterium	AY386341	99
Nit2A0626_212	FJ628183	1								Enterobacter sp. J11	EU099377	95
Nit2Au0637_384	FJ628221			1						Uncultured Marinomonas sp.	DQ421510	86
Nit5Au0613_720	FJ628322							1		Colwellia sp.BSs20120	EU330346	97
δ-Proteobacteria												
Nit2A0626_53	EU265979	1						1	1	Uncultured delta proteobacterium, Barents Sea	AJ704694	97
Nit2A0626_95	EU266020	1								Uncultured delta proteobacterium, Barents Sea	AJ704694	93
Nit2A0626_220	FJ628185	2								Uncultured delta proteobacterium, Barents Sea	AJ704694	96
Nit2Au0637_367	FJ628214			1						Uncultured delta proteobacterium, Barents Sea	AJ704694	97
Nit2A0626_60	EU265986	1						1		Uncultured bacterium, marine sediment	AJ535238	95
Nit2A0626_70	EU265995	1								Uncultured bacterium, marine sediment	AJ535238	90
Nit2A0626_232	FJ628190	1								Uncultured bacterium, marine sediment	AJ535238	89
Nit2Au0637_381	FJ628219			1						Uncultured bacterium, marine sediment	AJ535238	96
Nit5A0622_549	FJ628276					1				Uncultured bacterium, marine sediment	AJ535238	94
Nit2A0626_66	EU265991	1								Uncultured bacterium, marine sediment	AM882645	89
Nit2A0626_76	EU266001	1								Uncultured bacterium, marine sediment	AM882645	91
Nit5A0622_152	EU570879					4		1		Uncultured bacterium, marine sediment	DQ521817	93
Nit2A0626_210	FJ628182	2								Uncultured bacterium, methane seep	EU622296	94
Nit2A0626_223	FJ628187	1				1				Uncultured Desulfobulbaceae bacterium	AB294967	92
Nit2A0626_243	FJ628194	1								Uncultured Desulfobulbaceae bacterium	AB294967	92
Nit2A0626_230	FJ628189	1								Uncultured delta proteobacterium, Barents Sea	AJ704685	94
Nit2A0650_326	FJ628201		1							Uncultured bacterium, Lake Kauhako	AY344393	96
Nit2Au0637_104	EU570833			1						Uncultured delta proteobacterium	AY922197	90
Nit5Au0613_709	FJ628316							1		Uncultured delta proteobacterium	AY922197	90
Nit5A0622_136	EU570864					4				Uncultured bacterium, marine sediment	AB177128	92
Nit5A0622_143	EU570871					3				Uncultured bacterium, sulfidic biofilm	EU101251	81
Nit5A0622_520	FJ628265					1				Desulfobacterium phenolicum	AJ237606	94
Nit5A0622_540	FJ628272					1				Uncultured delta proteobacterium	AY771966	88
Nit5A0622_541	FJ628273					1		1		sulfate-reducing bacterium	AJ006853	98
Nit5Au0613_687	FJ628304							3		sulfate-reducing bacterium	AJ006853	96
Nit5A0650_161	EU570886						1	1		Uncultured bacterium, hypersaline mat	DQ397431	90
Nit5A0650_164	EU570889						1			Uncultured bacterium, namibian upwelling	EU290686	92
Nit5Au0613_174	EU570896							1		Uncultured bacterium, marine sediment	AJ704693	93
Nit5Au0613_184	EU570906							1		Uncultured bacterium, marine sediment	AJ704693	88
Nit5Au0613_186	EU570907							1		Uncultured bacterium, freshwater calcareous mat	EF580965	86
Nit5Au0628_766	FJ628345								1	Uncultured bacterium, lake sediment	AM086118	95
e-Proteobacteria												
* Nit2A0620_5	EU265932	19	153	10	21		131		155	Uncultured Arcobacter sp.	AB250570	95
* Nit2A0650_45	EU265971		4						1	Uncultured Arcobacter sp.	AB250570	92
* Nit2A0650_48	EU265974		2		1					Uncultured Arcobacter sp.	AB250570	92
* Nit2A0626_56	EU265982	1			1					Uncultured Arcobacter sp.	AB250570	96
* Nit2A0626_63	EU265988	1	1							Uncultured Arcobacter sp.	AB250570	96
* Nit2Au0650_124	EU570854				1					Uncultured Arcobacter sp.	AB250570	84
* Nit2Au0650_129	EU570859				1					Uncultured Arcobacter sp.	AB250570	91
* Nit5A0650_154	EU570880						1			Uncultured Arcobacter sp.	AB250570	96
* Nit5A0650_157	EU570882						1			Uncultured Arcobacter sp.	AB250570	94
* Nit5A0650_165	EU570890						2			Uncultured Arcobacter sp.	AB250570	96
* Nit5A0650_166	EU570891						1			Uncultured Arcobacter sp.	AB250570	95
* Nit5Au0628_190	EU570911								1	Uncultured Arcobacter sp.	AB250570	91
* Nit2A0626_250	FJ628196	1							1	Uncultured Arcobacter sp.	AB250570	92
* Nit2A0650_268	FJ628197		1				1			Uncultured Arcobacter sp.	AB250570	95
* Nit2A0650_294	FJ628198		1							Uncultured Arcobacter sp.	AB250570	91
* Nit2A0650_311	FJ628200		1						2	Uncultured Arcobacter sp.	AB250570	94
* Nit2Au0637_358	FJ628210			1					3	Uncultured Arcobacter sp.	AB250570	91
* Nit2Au0650_430	FJ628239				1					Uncultured Arcobacter sp.	AB250570	93
* Nit2Au0650_439	FJ628242				1					Uncultured Arcobacter sp.	AB250570	92
* Nit5A0650_598	FJ628291						1			Uncultured Arcobacter sp.	AB250570	89
* Nit5A0650_627	FJ628292						1			Uncultured Arcobacter sp.	AB250570	86
* Nit5A0650_645	FJ628293						1			Uncultured Arcobacter sp.	AB250570	89

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		26	50	37.5	50	22	50	13	28			
* Nit5A0650_665	FJ628294						1			Uncultured Arcobacter sp.	AB250570	95
* Nit5Au0628_769	FJ628346								1	Uncultured Arcobacter sp.	AB250570	96
* Nit5Au0628_776	FJ628347								1	Uncultured Arcobacter sp.	AB250570	96
* Nit5Au0628_805	FJ628348								1	Uncultured Arcobacter sp.	AB250570	90
* Nit5Au0628_822	FJ628349								1	Uncultured Arcobacter sp.	AB250570	93
* Nit5Au0628_826	FJ628350								1	Uncultured Arcobacter sp.	AB250570	94
* Nit5Au0628_832	FJ628351								1	Uncultured Arcobacter sp.	AB250570	92
* Nit5Au0628_833	FJ628352								1	Uncultured Arcobacter sp.	AB250570	91
* Nit5Au0628_844	FJ628353								1	Uncultured Arcobacter sp.	AB250570	92
* Nit2A0650_40	EU265967		1							Uncultured Arcobacter sp.	AY697901	95
* Nit2A0620_13	EU265940			2	3			1		Uncultured bacterium, marine sediment	DQ521787	98
* Nit2Au0637_418	FJ628236			1						Uncultured bacterium, marine sediment	DQ521787	94
* Nit2Au0650_464	FJ628250				1					Uncultured bacterium, marine sediment	DQ521787	94
Nit2A0650_44	EU265970		1							Endosymbiont of Alviniconcha sp.	AB235231	92
Nit2A0626_54	EU265980	2			1			1		Endosymbiont of Alviniconcha sp.	AB235231	97
Nit2A0626_58	EU265984	2		3				4		Endosymbiont of Alviniconcha sp.	AB235231	96
Nit2A0626_59	EU265985	9	1	18	43			1		Endosymbiont of Alviniconcha sp.	AB235231	98
Nit2A0626_68	EU265993	1		12			1			Endosymbiont of Alviniconcha sp.	AB235231	95
Nit2Au0637_116	EU570844			1						Endosymbiont of Alviniconcha sp.	AB235231	97
Nit2Au0650_128	EU570858				1					Endosymbiont of Alviniconcha sp.	AB235231	96
Nit2Au0650_133	EU570862				3					Endosymbiont of Alviniconcha sp.	AB235231	96
Nit2Au0650_134	EU570863				1					Endosymbiont of Alviniconcha sp.	AB235231	87
Nit2A0626_199	FJ628178	1								Endosymbiont of Alviniconcha sp.	AB235231	92
Nit2A0626_247	FJ628195	1								Endosymbiont of Alviniconcha sp.	AB235231	94
Nit2Au0637_338	FJ628203			1						Endosymbiont of Alviniconcha sp.	AB235231	93
Nit2Au0637_339	FJ628204			2						Endosymbiont of Alviniconcha sp.	AB235231	93
Nit2Au0637_407	FJ628230			1						Endosymbiont of Alviniconcha sp.	AB235231	96
Nit2Au0637_410	FJ628231			1						Endosymbiont of Alviniconcha sp.	AB235231	94
Nit2Au0637_411	FJ628232			1						Endosymbiont of Alviniconcha sp.	AB235231	91
Nit2Au0650_432	FJ628241				1					Endosymbiont of Alviniconcha sp.	AB235231	94
Nit2Au0650_463	FJ628249				1					Endosymbiont of Alviniconcha sp.	AB235231	95
Nit2Au0637_110	EU570839			1						Bacterial endosymbiont of Idas sp.	AM402958	89
* Nit2A0650_33	EU265960		1							Uncultured bacterium, marine sediment	EU652637	93
* Nit2A0626_72	EU265997	1								Uncultured bacterium, marine sediment	DQ521818	92
* Nit2A0626_196	FJ628175	1								Uncultured bacterium, marine sediment	DQ521818	97
* Nit2Au0637_360	FJ628212			1						Uncultured bacterium, marine sediment	DQ521818	97
Nit2A0626_90	EU266015	1								Uncultured marine bacterium	DQ071101	96
Nit2Au0637_417	FJ628235			1						Uncultured marine bacterium	DQ071101	88
Nit2A0626_92	EU266017	1								Uncultured epsilon proteobacterium	AY211671	91
Nit2Au0637_108	EU570837			1						Uncultured epsilon proteobacterium	AY211671	94
Nit2Au0637_117	EU570845			1						Uncultured epsilon proteobacterium	AY211671	96
Nit2A0626_221	FJ628186	1								Uncultured epsilon proteobacterium	AY211671	93
Nit2Au0637_337	FJ628202			1						Uncultured epsilon proteobacterium	AY211671	93
Nit2Au0637_397	FJ628224			1						Uncultured epsilon proteobacterium	AY211671	91
Nit2Au0650_486	FJ628251				1					Uncultured epsilon proteobacterium	AY211658	90
Nit2Au0637_98	EU570848			2	4					Uncultured epsilon proteobacterium	AY211658	90
Nit2Au0637_105	EU570834			1				1		Unclultured epsilon bacterium, cold seep sediment	AB189370	86
* Nit2Au0650_125	EU570855				3					Uncultured epsilon proteobacterium	AY922183	87
Nit5A0650_158	EU570883						1			Uncultured marine bacterium	AY548997	95
Nit2Au0637_359	FJ628211			2	2					Uncultured bacterium, marine sediment	AY211670	95
Nit2Au0637_412	FJ628233			1	1					Uncultured bacterium, marine sediment	AY211670	93
Nit2Au0637_368	FJ628215			1						Uncultured bacterium, marine sediment	EU652652	90
Nit2Au0637_382	FJ628220			1						Uncultured bacterium, methane seep sediment	FJ264760	94
* Nit5A0650_155	EU570881						1			Uncultured bacterium, marine sediment	AY627570	92
Nit2Au0650_445	FJ628244				2					Uncultured bacterium, marine sediment	EU652648	94
Nit2Au0650_448	FJ628245				1					Uncultured Campylobacterales bacterium	DQ234141	88
* Nit2Au0650_131	EU570860			1	2					Uncultured bacterium, iron-oxidizing mat	EF687192	93
* Nit2Au0637_402	FJ628226			1						Uncultured bacterium, iron-oxidizing mat	EF687491	90
Nit2A0626_55	EU265981	1		9	8					Uncultured bacterium, hydrothermal vent	AF420352	97
Nit2Au0650_443	FJ628243				1					Uncultured bacterium, hydrothermal vent	AF420352	94
Nit2Au0650_487	FJ628252				1					Uncultured bacterium, hydrothermal vent	AF420352	92
Nit2A0626_94	EU266019				2					Uncultured bacterium, hydrothermal vent	AY197379	95
Nit2Au0650_431	FJ628240				1					Uncultured bacterium, hydrothermal vent	AY197379	88
Nit2A0626_198	FJ628177	1								Uncultured bacterium, hydrothermal vent	AY197410	98
Nit5Au0628_845	FJ628354								1	Uncultured bacterium, hydrothermal vent	EF218996	90
Chlorobia												
Nit2A0626_50	EU265976	1		1						Chlorobium phaeovibrioides DSM 265	CP000607	92
Nit5Au0613_686	FJ628303							1		Chlorobium phaeovibrioides DSM 265	CP000607	86
Nit5A0622_523	FJ628266					1				Chlorobium phaeovibrioides DSM 265	CP000607	94
Nit5A0622_552	FJ628278					2				Chlorobium phaeovibrioides DSM 265	CP000607	97

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Nit5Au0613_675	FJ628297							1		Chlorobium phaeovibrioides DSM 265	CP000607	97
Nit2A0626_57	EU265983	18			2	27	1	7		Chlorobium phaeobacteroides BS1	AAIC01000044	99
Nit2A0626_62	EU265987	1								Chlorobium phaeobacteroides BS1	AAIC01000044	91
Nit2A0626_65	EU265990	1			1			4		Chlorobium phaeobacteroides BS1	AAIC01000044	86
Nit2A0626_75	EU266000	4								Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit2A0626_79	EU266004	1							1	Chlorobium phaeobacteroides BS1	AAIC01000044	90
Nit2A0626_80	EU266005	2				24			2	Chlorobium phaeobacteroides BS1	AAIC01000044	97
Nit2A0626_85	EU266010	1				1				Chlorobium phaeobacteroides BS1	AAIC01000044	94
Nit2A0626_87	EU266012	1						1		Chlorobium phaeobacteroides BS1	AAIC01000044	97
Nit2A0626_89	EU266014	1						1		Chlorobium phaeobacteroides BS1	AAIC01000044	90
Nit2A0626_93	EU266018	1								Chlorobium phaeobacteroides BS1	AAIC01000044	86
Nit5Au0613_689	FJ628306							1		Chlorobium phaeobacteroides BS1	AAIC01000044	85
Nit5Au0613_719	FJ628321							1		Chlorobium phaeobacteroides BS1	AAIC01000044	95
Nit5Au0613_726	FJ628327							1		Chlorobium phaeobacteroides BS1	AAIC01000044	95
Nit5A0622_140	EU570868					2	1	1		Chlorobium phaeobacteroides BS1	AAIC01000044	85
Nit5A0622_146	EU570873					1				Chlorobium phaeobacteroides BS1	AAIC01000044	92
Nit5A0622_147	EU570874					1				Chlorobium phaeobacteroides BS1	AAIC01000044	87
Nit5A0622_151	EU570878					1				Chlorobium phaeobacteroides BS1	AAIC01000044	92
Nit5Au0613_179	EU570901							1		Chlorobium phaeobacteroides BS1	AAIC01000044	84
Nit2A0626_206	FJ628180	1								Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit2A0626_207	FJ628181	1								Chlorobium phaeobacteroides BS1	AAIC01000044	99
Nit2A0626_239	FJ628193	1				1				Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit5A0622_499	FJ628255					1				Chlorobium phaeobacteroides BS1	AAIC01000044	92
Nit5A0622_505	FJ628258					1				Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit5A0622_515	FJ628263					1				Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit5A0622_525	FJ628267					2				Chlorobium phaeobacteroides BS1	AAIC01000044	97
Nit5Au0613_759	FJ628343							1		Chlorobium phaeobacteroides BS1	AAIC01000044	91
Nit5A0622_568	FJ628281					1				Chlorobium phaeobacteroides BS1	AAIC01000044	96
Nit5A0622_569	FJ628282					1				Chlorobium phaeobacteroides BS1	AAIC01000044	89
Nit5A0622_572	FJ628283					1				Chlorobium phaeobacteroides BS1	AAIC01000044	92
Nit2A0626_83	EU266008	1				2		1		Chlorobium sp. Mog4	EF149015	98
Nit5Au0613_187	EU570908							1	1	Chlorobium sp. Mog4	EF149015	91
Nit5Au0613_183	EU570905					1		2		Chlorobium sp. Mog4	EF149015	92
Nit5A0622_138	EU570866					21		2		Chlorobium sp. Mog4	EF149015	99
Nit5A0622_501	FJ628256					10		3		Chlorobium sp. Mog4	EF149015	99
Nit5A0622_139	EU570867					8	1	1		Chlorobium sp. Mog4	EF149015	99
Nit5A0622_495	FJ628254					4				Chlorobium sp. Mog4	EF149015	97
Nit5A0622_517	FJ628264					2				Chlorobium sp. Mog4	EF149015	98
Nit5A0622_538	FJ628271					1				Chlorobium sp. Mog4	EF149015	94
Nit5A0622_547	FJ628274					1				Chlorobium sp. Mog4	EF149015	92
Nit5A0622_548	FJ628275					1				Chlorobium sp. Mog4	EF149015	94
Nit5A0622_555	FJ628279					1				Chlorobium sp. Mog4	EF149015	91
Nit5A0622_557	FJ628280					1				Chlorobium sp. Mog4	EF149015	93
Nit5Au0613_690	FJ628307							1		Chlorobium sp. Mog4	EF149015	89
CFB group												
Nit2A0620_15	EU265942			1			1			Uncultured bacterium, sediment	DQ351745	87
Nit2A0626_51	EU265977	1								Uncultured bacterium, sulfide-oxidizing mat	EF687431	95
Nit2Au0637_114	EU570842			1						Uncultured bacterium, sulfide-oxidizing mat	EF687431	99
Nit5A0622_150	EU570877					1				Uncultured bacterium, sulfide-oxidizing mat	EF687431	99
Nit5A0650_167	EU570892						1			Uncultured bacterium, sulfide-oxidizing mat	EF687431	99
Nit2A0626_197	FJ628176	1					3			Uncultured bacterium, sulfide-oxidizing mat	EF687431	96
Nit5Au0613_758	FJ628342							1		Uncultured bacterium, sulfide-oxidizing mat	EF687431	95
Nit2A0626_52	EU265978	1								Flavobacteriaceae bacterium	AY353814	98
Nit2Au0637_115	EU570843			1						Uncultured Bacteroidetes bacterium	DQ070811	95
Nit2Au0637_109	EU570838			1				4		Uncultured bacterium, namibian upwelling	EF646129	96
Nit5A0622_141	EU570869					1				Uncultured bacterium, namibian upwelling	EF646129	99
Nit5Au0613_757	FJ628341							1		Uncultured bacterium, namibian upwelling	EF645959	96
Nit2Au0637_118	EU570846			2		1	1	7		Uncultured bacterium, hydrothermal mound	DQ832645	98
Nit5A0622_137	EU570865					1				Uncultured bacterium, sediment of Lake Kinneret	AM086105	91
Nit5A0650_162	EU570887						1			Uncultured bacterium, sediment of Lake Kinneret	AM086105	90
Nit5A0622_142	EU570870					1				Uncultured bacterium	AF382098	95
Nit5Au0613_171	EU570895							1		Uncultured bacterium	AF382098	91
Nit5Au0613_679	FJ628299							3		Uncultured bacterium	AF382098	99
Nit5A0622_149	EU570876					1				Uncultured bacterium	DQ015793	85
Nit2Au0637_356	FJ628208			1						Polaribacter glomeratus isolate S3-30	AY771729	93
Nit2Au0637_364	FJ628213			1						Uncultured bacterium, hydrothermal site	EF218998	90
Nit2Au0637_391	FJ628223			1						Uncultured bacterium, arctic surface sediment	EU287256	87
Nit2Au0637_419	FJ628237			1						Uncultured bacterium	EF573001	83
Nit5Au0613_688	FJ628305							1		Uncultured bacterium, seawater	DQ009088	99
Nit5Au0613_708	FJ628315							1		Uncultured bacterium	AF382106	89

OTU / Accession no.		No. of clones in library								Nearest phylogenetic neighbor		
		S02 April		S02 August		S05 April		S05 August		Closest match	Accession no.	Similarity (%)
		26	50	37.5	50	22	50	13	28			
Nit5Au0613_754	FJ628339							1		Uncultured bacterium	AF382106	91
Nit5Au0613_698	FJ628310							1		Uncultured bacterium, seawater	DQ295241	98
Nit5Au0613_718	FJ628320							1		Uncultured Flavobacteriaceae bacterium	AY794064	86
Nit5Au0613_729	FJ628329							1		Uncultured bacterium, seawater	DQ071103	91
Actinobacteria												
Nit5Au0613_175	EU570897							2		Marine metagenome 1096626833213	AACY020462030	89
Nit5Au0613_182	EU570904							1		Marine metagenome 1096626361887	AACY020288370	94
Nit5A0622_511	FJ628261					1				Marine metagenome 1096626361887	AACY020288370	95
Chloroflexi												
Nit5A0622_148	EU570875					1				Uncultured Chloroflexi bacterium	AJ441227	93
Nit5Au0613_699	FJ628311							1		Uncultured Chloroflexi bacterium	AJ441227	91
Nit5Au0613_707	FJ628314							1		Uncultured bacterium, seafloor lavas	EU491321	95
Nit5A0650_587	FJ628288						1			Uncultured Chloroflexi bacterium	AY592357	97
Nit5Au0613_723	FJ628325							1		Uncultured bacterium, seafloor lavas	EU491096	90
Nit5Au0613_736	FJ628331							1		Uncultured bacterium, hydrothermal sediment	AF419695	97
Nit5Au0613_737	FJ628332							1		Uncultured bacterium, hydrothermal sediment	AF419695	97
Verrumicrobia												
Nit2Au0650_492	FJ628253				1					Lentisphaera araneosa HTCC2155	ABCK01000003	98
Nit5Au0613_735	FJ628330							1		Bacterium Ellin514	ABOX01000003	87
Nit5Au0628_846	FJ628355								1	Uncultured bacterium	DQ513023	88
Nit2Au0637_404	FJ628228			1						Uncultured bacterium	DQ513023	96
Firmicutes												
Nit5Au0613_169	EU570894						1	1		Uncultured bacterium, methane seep sediment	EU142042	92
Nit5Au0628_188	EU570909								1	Uncultured bacterium, groundwater	AB237732	86
Planctomycetia												
Nit2A0626_195	FJ628174	1						3		Uncultured marine bacterium clone	DQ071077	84
Nit5A0622_506	FJ628259					1		1		Uncultured marine bacterium clone	DQ071077	84
Nit5A0622_532	FJ628269					1				Uncultured planctomycete 13FN clone	EF591889	95
Candidate division												
Nit2A0620_21	EU265948		1							Uncultured bacterium, sulfide-oxidizing mat	EF687393	91
Nit5A0622_574	FJ628284					1				Uncultured candidate division OD1	DQ676453	87
Nit5Au0613_761	FJ628344							1		Uncultured candidate division OD1	DQ676453	84
Nit5A0650_585	FJ628287						1			Uncultured bacterium, hypersaline mat	EU246208	82
Cyanobacteria												
Nit2A0650_301	FJ628199		1							Uncultured bacterium, hypersaline lake	EF031090	89
Nit5A0622_513	FJ628262					1				Thalassiosira pseudonana chloroplast	EF067921	86
Spirochetes												
Nit5A0650_584	FJ628286						1			Uncultured bacterium	AY592404	96
Nit5Au0613_703	FJ628312							1		Uncultured spirochete clone	AY605171	85
SAR406 cluster												
Nit5A0622_550	FJ628277					1		1		Uncultured bacterium	AY458631	91
Deinococci												
Nit5A0622_530	FJ628268					1				Deinococcus alpinitundrae strain	EF635408	96
unclasified												
Nit5Au0613_740	FJ628334							1		Uncultured bacterium, cold seep sediment	AB121106	94
Nit5A0622_508	FJ628260					1				Uncultured bacterium, cold seep sediment	AB121106	92
Nit5A0650_590	FJ628289						1			Uncultured bacterium, cold seep sediment	AB121107	94
Nit5A0650_159	EU570884						2			Uncultured bacterium	EF031090	89
Nit5A0650_591	FJ628290						1			Uncultured bacterium, deep-sea sediment	AJ567599	91

Note: OTUs marked with asterisk are targeted with NITEP5 qPCR primer pair

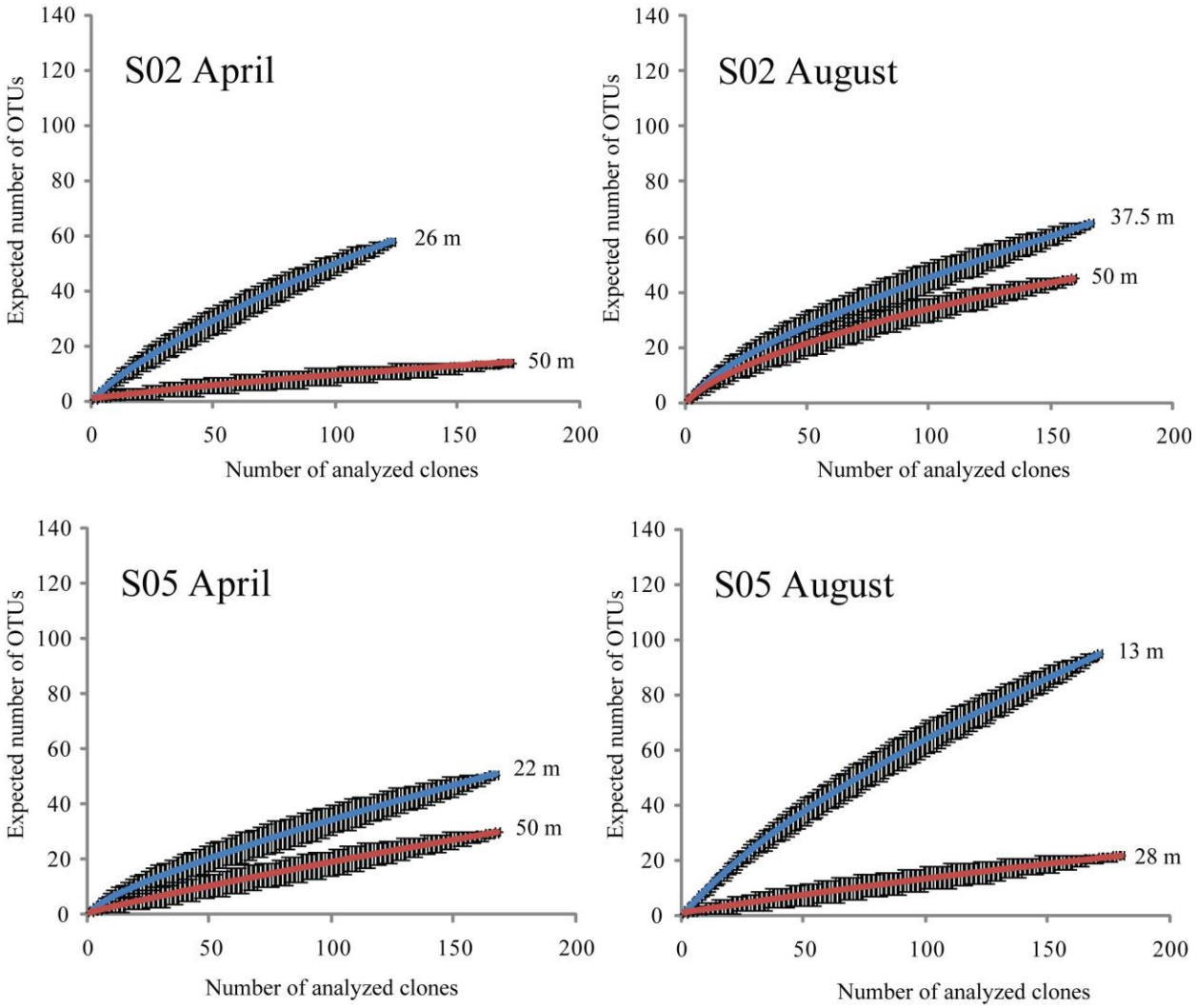

Figure B.1: Rarefaction curves constructed from Nitinat Lake SSU rRNA libraries. Black bars represent 95 % confidence intervals. The calculations were obtained from DOTUR.

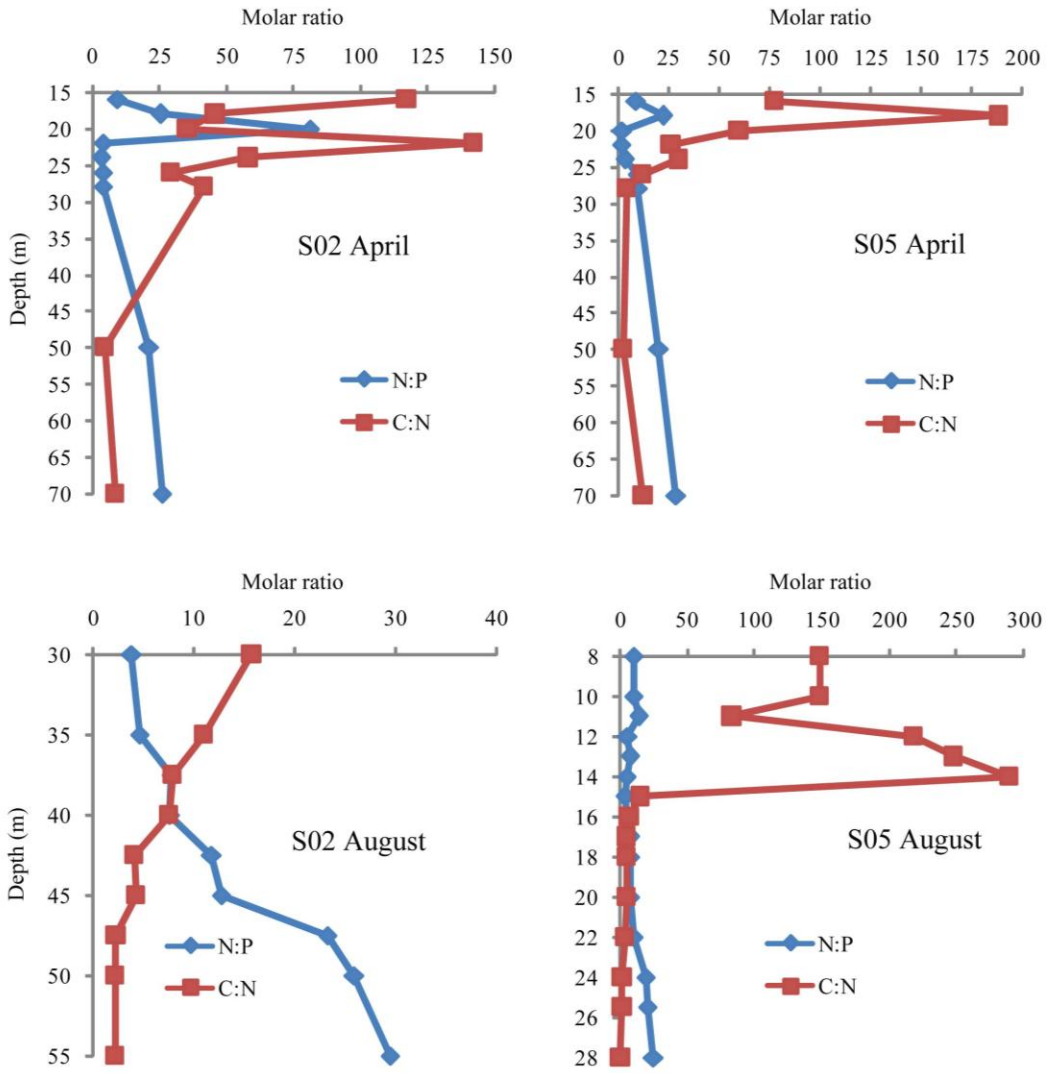

Figure B.2: Changes in molar ratios of carbon to nitrogen (C:N), and nitrogen to phosphorus (N:P) with depth during two seasons and at two stations of Nitinat Lake.

APPENDIX C

RFLP PATTERNS REPRESENTING NITINAT SSU rRNA CLONES

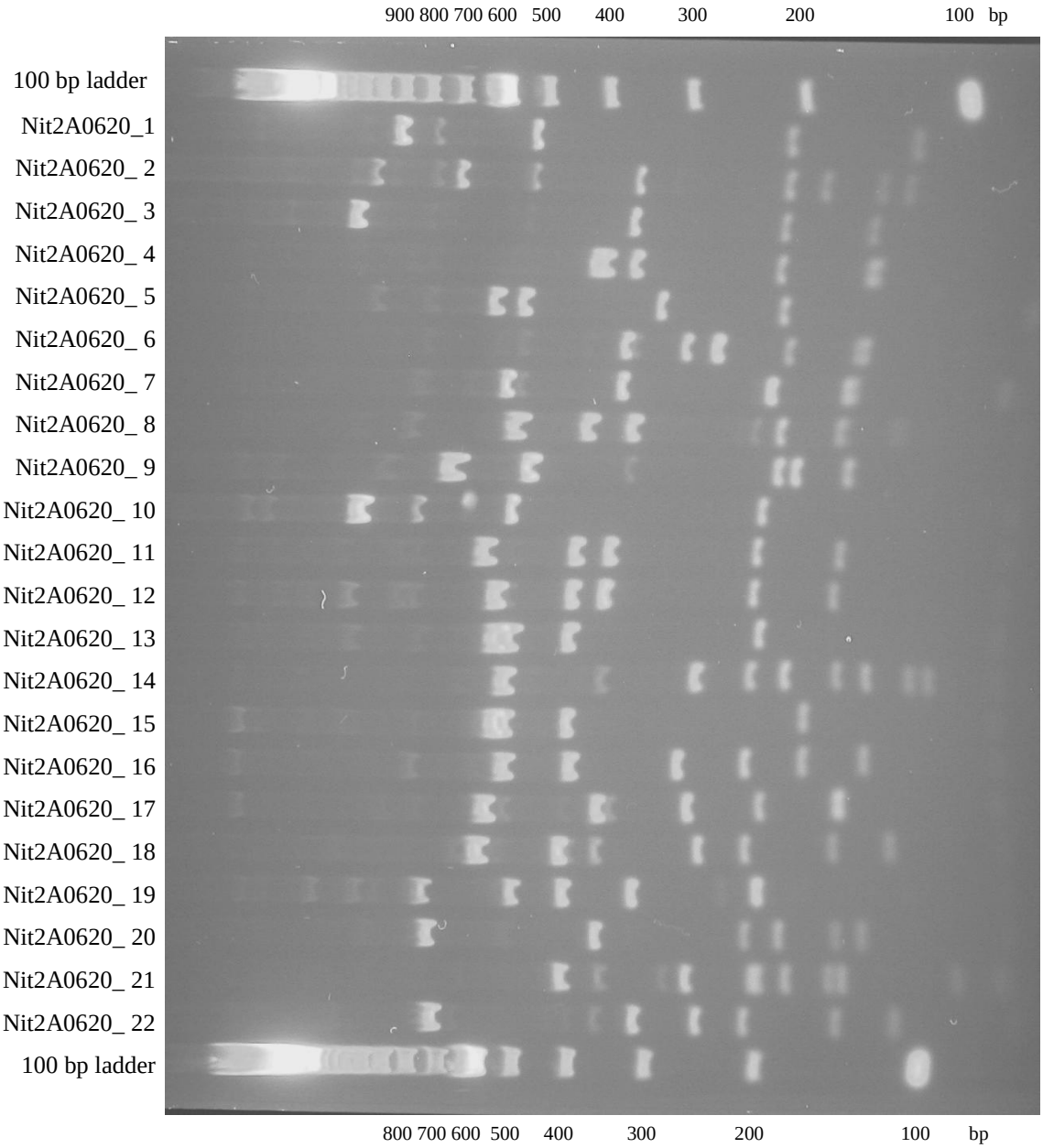

Figure C.1: RFLP patterns of SSU rRNA sequences from Nitinat Lake water column clone library after digestion with RSAI.

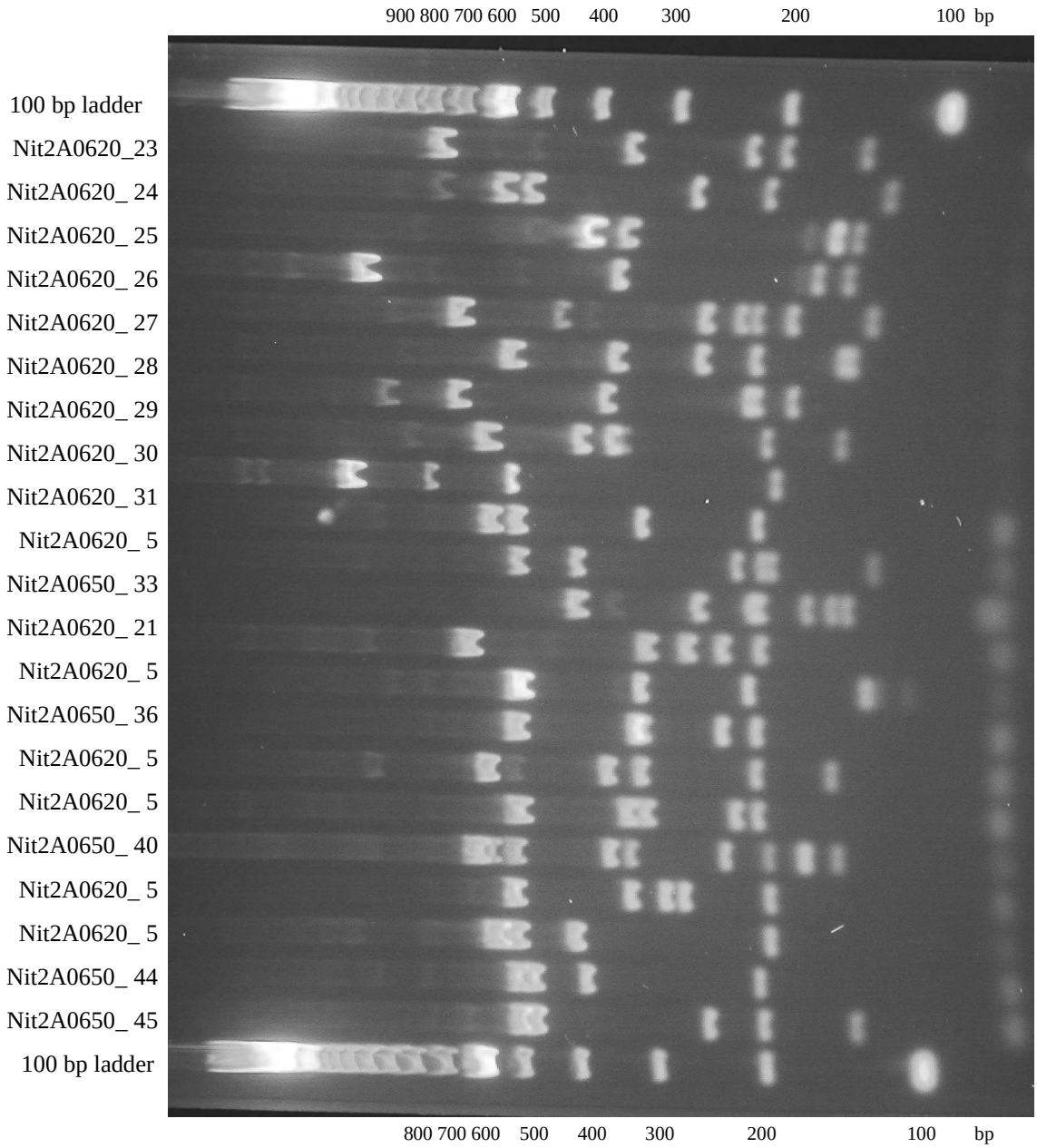

Figure C.1 continued

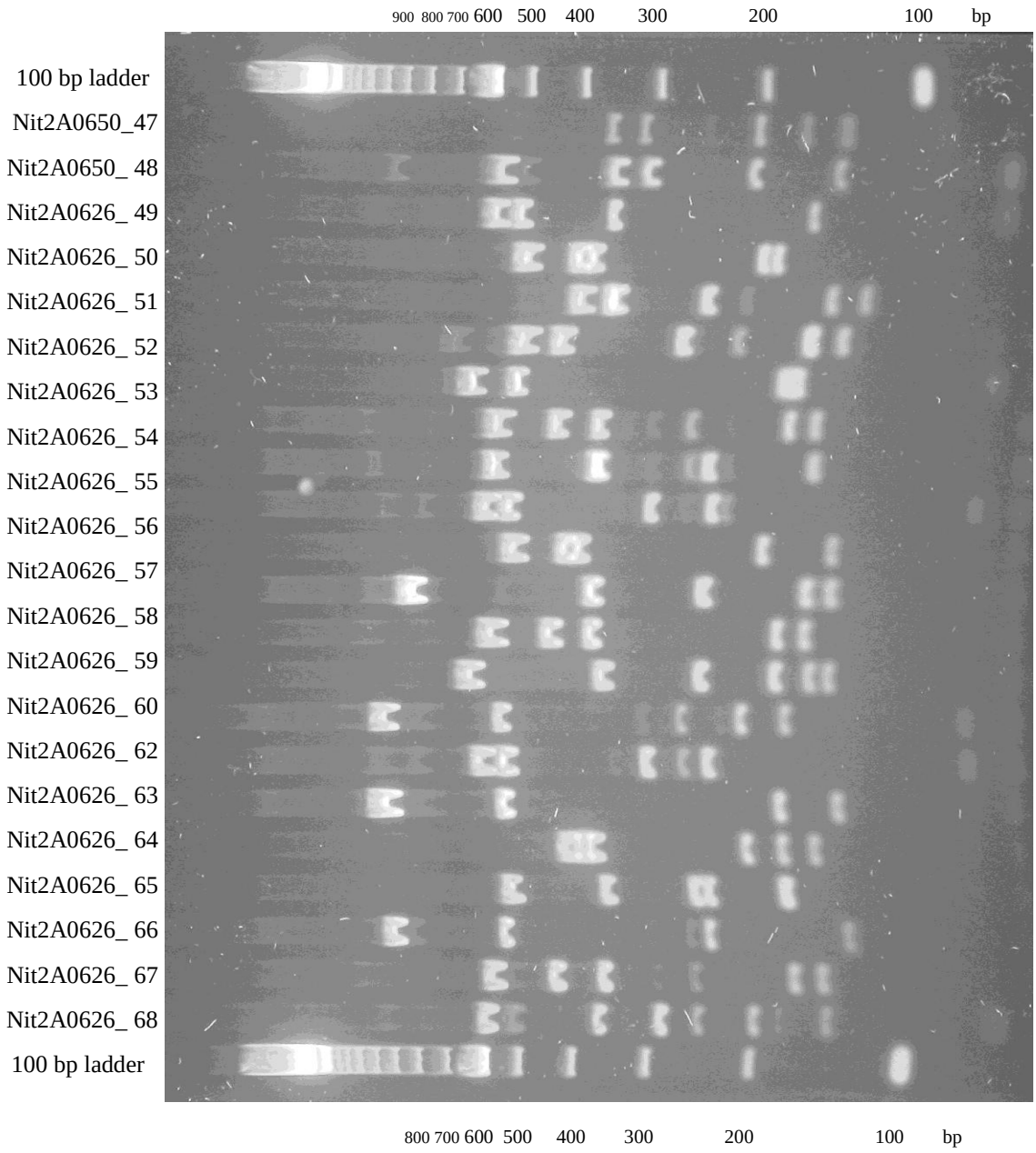

Figure C.1 continued

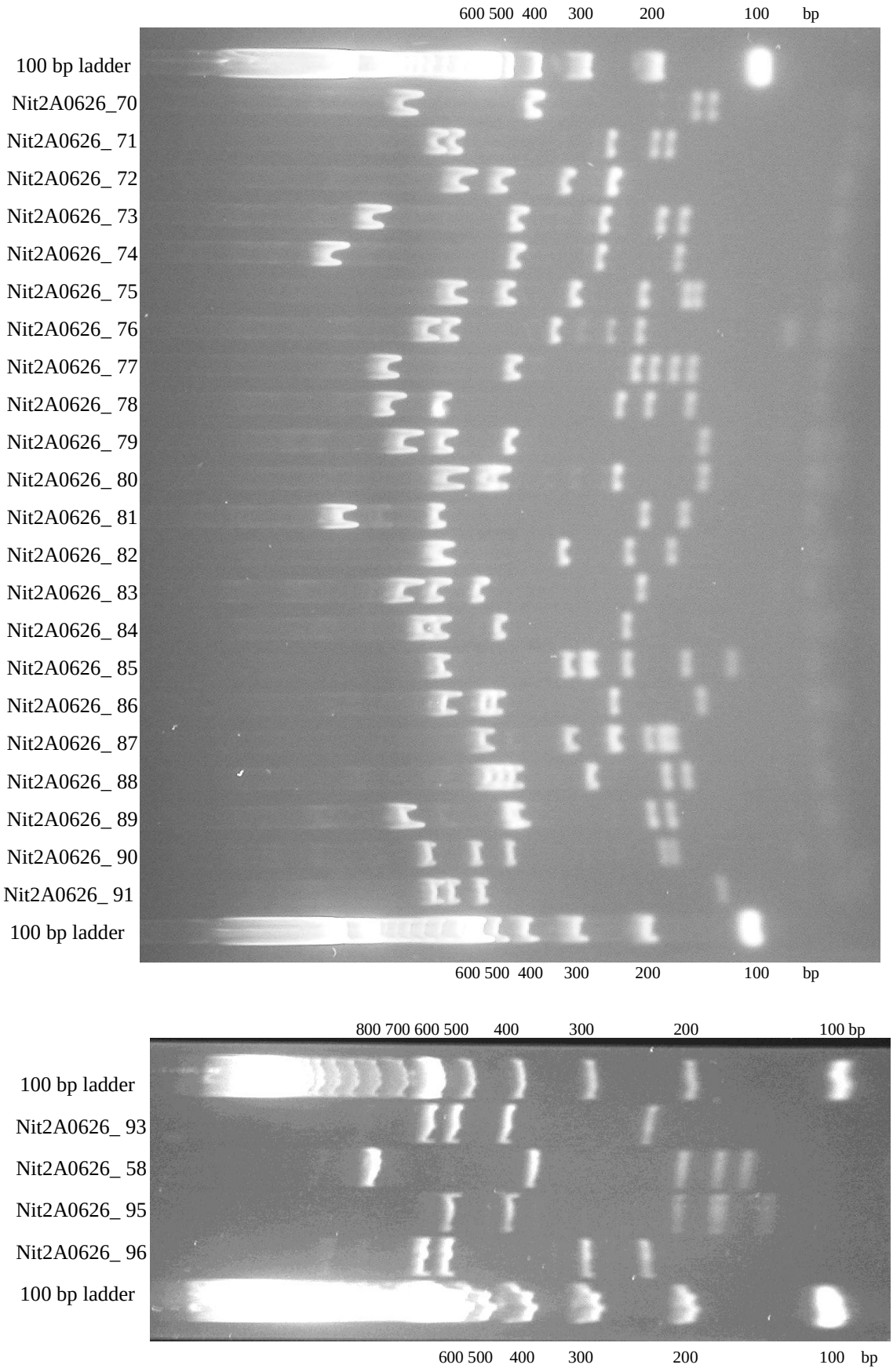

Figure C.1 continued

APPENDIX D

SUPPLEMENTARY DATA FOR CHAPTER 4

Table D.1: Abundance and nearest phylogenetic neighbors of all OTUs obtained from complex carbon material SSU rRNA clone libraries.

OTU	Frequency in library				Nearest neighbor			
	Silage	Compost	Molasses + hay	Wetland biosolids	Sequence	Source	Accession no.	Similarity %
Bacteroidetes								
TR27_R_3	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.24
TR27_R_4	73	53	104	4	Uncultured bacterium clone GW-32	household biogas digester	EU407215	96.49
TR27_R_23	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.15
TR27_R_24	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.89
TR27_R_58	3	1	3		Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.55
TR27_R_62	1	1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.38
TR27_R_66	1	2	1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.48
TR27_R_84	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.86
TR27_R_88	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	88.31
TR27_R_100	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.4
TR27_R_105	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	89.24
TR27_R_106	1	1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.07
TR27_R_116	1	3	2	1	Uncultured bacterium clone GW-32	household biogas digester	EU407215	96.24
TR27_R_118	3				Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.3
TR27_R_143	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	93.17
TR27_R_157	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	90.02
TR27_R_173	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.98
TR27_R_178	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.27
TR27_R_199	1	1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.05
TR27_R_205	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	90.63
TR27_R_210	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	91.63
TR29_R_235		3			Uncultured bacterium clone GW-32	household biogas digester	EU407215	93.65
TR29_R_249		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	93.4
TR29_R_283		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	87.17
TR29_R_337		1			Uncultured bacterium clone E20	river receiving wastewater	EU864448	86.95
TR29_R_338		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	91.21
TR29_R_374		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	94.2
TR29_R_395		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.81
TR29_R_398		1			Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.67
TR30_R_427			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	89.48
TR30_R_435			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.69
TR30_R_468			3		Uncultured bacterium clone GW-32	household biogas digester	EU407215	90.91
TR30_R_499			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	95.55
TR30_R_517			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	90.41
TR30_R_521			2		Uncultured bacterium clone GW-32	household biogas digester	EU407215	93.82
TR30_R_526			3		Uncultured bacterium clone GW-32	household biogas digester	EU407215	91.35
TR30_R_579			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	90.66
TR30_R_605			2		Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.7
TR30_R_610			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	92.24
TR30_R_636			1		Uncultured bacterium clone GW-32	household biogas digester	EU407215	96.23
TR27_R_1	5				Uncultured bacterium clone c5LKS10	lake profundal sediment	AM086109	96
TR27_R_102	2				Uncultured bacterium clone c5LKS10	lake profundal sediment	AM086109	95
TR27_R_206	1				Uncultured bacterium clone c5LKS10	lake profundal sediment	AM086109	90.72
TR27_R_218	1	1			Uncultured bacterium clone c5LKS10	lake profundal sediment	AM086109	90.16
TR27_R_79	1				Uncultured bacterium clone c5LKS10	lake profundal sediment	AM086109	95.38
TR27_R_7	2				Uncultured bacterium clone E43	river receiving wastewater	EU864477	91.67
TR27_R_120	2				Uncultured bacterium clone E43	river receiving wastewater	EU864477	91.74
TR30_R_472			1		Uncultured bacterium clone E39	river receiving wastewater	EU864483	94.71
TR8_R_701				1	Uncultured bacterium clone E39	river receiving wastewater	EU864483	99.86
TR27_R_21	2		1		Uncultured bacterium clone G1DMC-151	landfill sediments high Cr(VI) contamination	DQ899885	94.81
TR27_R_32	3				Uncultured bacterium clone G1DMC-151	landfill sediments high Cr(VI) contamination	DQ899885	98.35
TR27_R_22	2				Uncultured bacterium clone PL-26B10	low-temperature biodegraded oil reservoir	AY570561	93.24
TR27_R_68	1				Uncultured bacterium clone PL-26B10	low-temperature biodegraded oil reservoir	AY570561	92.84
TR30_R_419			1		Uncultured bacterium clone PL-38B1	low-temperature biodegraded oil reservoir	AY570569	87.99
TR30_R_443			2		Uncultured bacterium clone PL-38B1	low-temperature biodegraded oil reservoir	AY570569	96.1
TR30_R_573			2		Uncultured bacterium clone PL-38B1	low-temperature biodegraded oil reservoir	AY570569	93.15
TR8_R_710				1	Uncultured bacterium clone PL-7B5	low-temperature biodegraded oil reservoir	AY570638	93.6
TR8_R_816				1	Uncultured bacterium clone PL-7B5	low-temperature biodegraded oil reservoir	AY570638	93.48
TR27_R_20	1				Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	87.16
TR27_R_48	1	2		1	Iron-reducing enrichment clone Cl-A12	iron-reducing enrichment	DQ677004	99.39
TR27_R_159	1				Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	99.64
TR29_R_359		1			Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	99.73
TR29_R_388		1			Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	99.26
TR8_R_682				3	Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	96.58
TR8_R_699				2	Iron-reducing enrichment clone Cl-A4	iron-reducing enrichment	DQ676996	95.44
TR27_R_87	1				Uncultured Cytophaga sp. clone C6	paper pulp column	EF562547	89.87
TR29_R_322		1			Bacteroidetes bacterium clone CD_05	paper pulp column	EF562561	91.73
TR29_R_412		1			Bacteroidetes bacterium clone CD_05	paper pulp column	EF562561	97.18
TR27_R_60	1			1	Uncultured bacterium clone B1	bioreactor treating paper mill wastewater	AY426437	99.86
TR27_R_85	2				Uncultured bacterium clone RB353	rhizosphere biofilm	AB240349	92.28
TR27_R_175	1				Uncultured bacterium clone RB353	rhizosphere biofilm	AB240349	94.27
TR8_R_805				1	Uncultured bacterium clone RB442	rhizosphere biofilm	AB240375	95.97
TR27_R_136	1				Uncultured bacterium clone L15	biofilm on oxygen-transfer membrane	AY444993	90
TR27_R_183	1				Bacteroidetes bacterium clone FNE11-29	freshwater	DQ501308	97.44
TR27_R_216	1				Uncultured bacterium clone TTA	anaerobic hybrid reactor	AY661406	86.21
TR29_R_239		2			Uncultured bacterium clone LCFA-B02	methanogenic consortium	AB244309	93.5
TR29_R_252		2	1		Uncultured bacterium clone KD2-33	environmental sample	AY188300	91.95
TR29_R_274		1	1	1	Uncultured bacterium clone EUB50-2	anaerobic sludge	AY693829	98.79
TR8_R_762				1	Uncultured bacterium clone EUB50-2	anaerobic sludge	AY693829	98.79
TR29_R_295		1			Uncultured soil bacterium clone M56	oil-polluted soil	DQ378270	93.65
TR29_R_299		1			Uncultured bacterium clone 1013-1-CG48	uranium-contaminated aquifer	AY532556	96.37
TR29_R_320		2			Uncultured bacterium clone HDBW-WB38	deep subsurface groundwater	AB237701	97.03
TR29_R_361		1			Uncultured bacterium clone HDBW-WB38	deep subsurface groundwater	AB237701	97.17
TR29_R_348		1			Bacteroidetes bacterium clone GalB60	oxidized iron deposits	AY193184	91.15
TR29_R_381		1			Uncultured bacterium clone 08N210B	hypersaline microbial mat	DQ331017	90.24
TR29_R_365		1			Uncultured bacterium clone TSAC21	polychlorinated dioxin microcosm	AB186809	89.22
TR29_R_363		1			Bacteroidetes bacterium clone MVS-31	suboxic freshwater-pond sediment	DQ676447	93.38
TR29_R_255		2	1		Uncultured bacterium clone F7	river water	FJ230934	94.15
TR29_R_302		4	1		Uncultured bacterium clone H2SRC235	biotrickling filter removing H_2S	FM213016	96.42
TR30_R_440			1	3	Porphyromonadaceae bacterium JN18_A107_G	PCB-dechlorinating enrichment culture	DQ168658	98.35
TR30_R_492			1		Uncultured bacterium clone 008G11	municipal wastewater treatment plant	CR933177	99.09
TR30_R_479			1		Uncultured bacterium clone 008E10	municipal wastewater treatment plant	CR933186	90.86

	TR30 R 656			1		Uncultured bacterium clone 008E10	municipal wastewater treatment plant	CR933186	95.89
	TR8 R 748				1	Uncultured bacterium clone 053H05	municipal wastewater treatment plant	CR933247	99.16
	TR8 R 736				1	Uncultured bacterium clone 061F06	municipal wastewater treatment plant	CT574109	94.3
	TR30 R 533			1		Uncultured bacterium clone A42	sediment	DQ080187	92.91
	TR30 R 659			1		Uncultured bacterium clone WM69	sulfidic cave stream biofilm	DQ415777	91.77
	TR30 R 663			1		Uncultured bacterium clone 118ds10	water 10 m downstream of manure	AY212569	96.38
	TR8 R 793				1	Uncultured bacterium clone LS4-227	sediment and soil	AB234247	92
	TR8 R 824				1	Bacteroidaceae bacterium WK042	methanogenic reactor	AB298727	99.32
	TR8 R 676				13	Uncultured bacterium clone: WU75	anaerobic digester sludge	AB494358	98.57
	TR8 R 703				2	Uncultured bacterium clone: WU75	anaerobic digester sludge	AB494358	98.16
	TR8 R 709				3	Uncultured bacterium clone: WU75	anaerobic digester sludge	AB494358	96.34
	TR8 R 735				1	Uncultured bacterium clone: WU75	anaerobic digester sludge	AB494358	98.46
	TR8 R 789				1	Uncultured bacterium clone: WU75	anaerobic digester sludge	AB494358	98.67
	TR8 R 752				1	Uncultured bacterium clone D25	tar-oil contaminated aquifer sediments	EU266907	93.11
	TR8 R 802				1	Iron-reducing bacterium enrichment clone HN9	paddy soil from mining area	FJ269049	88.12
	TR8 R 730				1	Iron-reducing bacterium enrichment clone HN126	paddy soil from mining area	FJ269080	93.59
	TR8 R 771				3	Iron-reducing bacterium enrichment clone HN126	paddy soil from mining area	FJ269080	94.98
	TR8 R 687				1	Uncultured bacterium clone TS27 a03g07	feces	FJ366781	88.12
	TR29 R 234		1			Uncultured bacterium clone FCPP558	grassland soil	EF515956	92.46
	TR29 R 339		1			Uncultured Flexibacter sp. clone TM7	truffle	DQ279363	90.73
	TR29 R 387		1			Uncultured bacterium clone Eb26	anaerobic methanogenic UASB reactor	EF063613	89.27
	TR30 R 434			1		Uncultured bacterium clone D12	tar-oil contaminated aquifer sediments	EU266838	88.28
	TR8 R 832				1	Uncultured organism clone MAT-CR-M7-E09	hypersaline microbial mat	EU245877	86.32
Clostridia									
	TR27 R 2	3		2		Unidentified eubacterium clone vadinHB04	anaerobic digester fed by vinasses	U81750	99.26
	TR27 R 12	1				Acetobacterium carbinolicum		X96956	98.97
	TR27 R 172	1				Unidentified eubacterium clone vadinHB04	anaerobic digester fed by vinasses	U81750	98.25
	TR30 R 484			1		Unidentified eubacterium clone vadinHB04	anaerobic digester fed by vinasses	U81750	95.7
	TR30 R 626			1		Unidentified eubacterium clone vadinHB04	anaerobic digester fed by vinasses	U81750	98.5
	TR27 R 153	1				Unidentified eubacterium clone vadinBB35	anaerobic digester fed by vinasses	U81761	92.28
	TR27 R 13	7	5	8		Uncultured bacterium clone AKAU3538	uranium contaminated soil	DQ125557	96.63
	TR27 R 43	1				Uncultured bacterium clone AKAU3538	uranium contaminated soil	DQ125557	93.7
	TR29 R 380		1			Uncultured bacterium clone AKAU3538	uranium contaminated soil	DQ125557	94.74
	TR30 R 504			1		Uncultured bacterium clone AKAU3538	uranium contaminated soil	DQ125557	88.24
	TR30 R 646			1		Uncultured bacterium clone AKAU3538	uranium contaminated soil	DQ125557	96.43
	TR27 R 26	1				Uncultured rumen bacterium clone BF264	rumen	EU850495	87.91
	TR30 R 552			1		Uncultured rumen bacterium clone BF66	rumen	EU850478	94.7
	TR27 R 45	1				Uncultured bacterium clone R62	clay wall material	AB307641	97.32
	TR30 R 625			1		Uncultured bacterium clone R62	clay wall material	AB307641	88.97
	TR27 R 166	1				Uncultured bacterium clone R70	clay wall material	AB307642	93.89
	TR30 R 462			1		Uncultured bacterium clone R70	clay wall material	AB307642	95.07
	TR30 R 642			1		Uncultured bacterium clone R70	clay wall material	AB307642	93.69
	TR27 R 16	1				Uncultured bacterium clone RL388 aao93b08	feces	DQ801264	89.79
	TR30 R 464			1		Uncultured bacterium clone RL203 aai64a05	feces	DQ805543	92.04
	TR27 R 67	1				Uncultured Clostridiales bacterium clone D10	tar-oil contaminated aquifer sediments	EU266794	89.56
	TR30 R 649			1	1	Uncultured Clostridiales bacterium clone D10	tar-oil contaminated aquifer sediments	EU266794	96.9
	TR27 R 69	1				Uncultured bacterium clone 1103200826632	rumen	EU842568	94.78
	TR8 R 708				2	Uncultured bacterium clone 1101352040671	rumen	EU842993	91.82
	TR8 R 814				1	Uncultured bacterium clone 1101352040671	rumen	EU842993	96.55
	TR8 R 811				1	Uncultured bacterium clone 1103200821650	rumen	EU843138	91.6
	TR8 R 828				1	Uncultured bacterium clone 1103200820022	rumen	EU844061	92.3
	TR8 R 721				3	Uncultured bacterium clone 1103200949801	rumen	EU844641	95.34
	TR8 R 818				1	Uncultured bacterium clone 1103200831996	rumen	EU845546	92.62
	TR27 R 47	1				Uncultured Clostridia bacterium clone S-F26	subsurface soil	AY622268	98.98
	TR27 R 36	1				Uncultured bacterium clone 2E7	anaerobic wastewater treatment system	EF688166	88.05
	TR27 R 82	1				Uncultured bacterium clone 1A11	anaerobic wastewater treatment system	EF688146	86.93
	TR30 R 515			1		Uncultured bacterium clone 2E7	anaerobic wastewater treatment system	EF688166	85.97
	TR30 R 537			1		Uncultured bacterium clone 2E7	anaerobic wastewater treatment system	EF688166	96.25
	TR8 R 725				1	Uncultured bacterium clone 4D10	anaerobic wastewater treatment system	EF688194	91.41
	TR29 R 357		1			Uncultured bacterium clone 5E11 cons	anaerobic wastewater treatment system	EF688230	96.99
	TR27 R 52	1				Uncultured bacterium clone C61	river receiving penicillin wastewater	EU234230	99.12
	TR30 R 426			1		Uncultured bacterium clone C61	river receiving penicillin wastewater	EU234230	93.15
	TR27 R 76	2				Uncultured bacterium clone LJ3	Siwhaho sediment	AY756592	94.12
	TR29 R 290		1			Uncultured bacterium clone LJ3	Siwhaho sediment	AY756592	96.27
	TR8 R 702				1	Uncultured bacterium clone LJ6	Siwhaho sediment	AY756595	92.36
	TR29 R 269		1			Uncultured bacterium clone LJ8	Siwhaho sediment	AY756597	92.64
	TR30 R 570			3		Uncultured bacterium clone LJ8	Siwhaho sediment	AY756597	93.52
	TR27 R 89	1				Uncultured eubacterium clone BSV51	anoxic bulk soil	AJ229203	94.77
	TR27 R 147	1				Uncultured eubacterium clone BSV51	anoxic bulk soil	AJ229203	97.09
	TR27 R 201	1				Uncultured eubacterium clone BSV51	anoxic bulk soil	AJ229203	97.6
	TR30 R 450			1		Uncultured eubacterium clone BSV51	anoxic bulk soil	AJ229203	97.4
	TR8 R 707				1	Uncultured eubacterium clone BSV34	anoxic bulk soil	AJ229194	97.64
	TR27 R 141	2				Uncultured bacterium clone YWB34	deep coal seem groundwater	AB294303	90.7
	TR27 R 168	2				Clostridiaceae bacterium clone Rs-117	termite gut	AB100481	88.62
	TR8 R 790				2	Clostridiales bacterium clone MgMjR-046	termite gut	AB234489	90.2
	TR8 R 823				1	Clostridiales bacterium clone MgMjR-046	termite gut	AB234489	91.54
	TR27 R 162	2				Uncultured bacterium clone p-2448-18B5	swine intestine	AF371780	94.2
	TR27 R 129	1				Uncultured bacterium clone BS37	mesophilic biogas digester treating pig manure	EU358712	94.55
	TR8 R 846				1	Uncultured bacterium clone BS39	mesophilic biogas digester treating pig manure	EU358714	88.17
	TR27 R 164	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	86.31
	TR27 R 219	1				Uncultured bacterium clone GW-32	household biogas digester	EU407215	86.63
	TR29 R 241		1			Iron-reducing bacterium enrichment clone HN3	paddy soil from mining area	FJ269045	89.3
	TR27 R 152	1	1			Iron-reducing bacterium enrichment clone HN109	paddy soil from mining area	FJ269072	99.39
	TR29 R 257		1	1		Iron-reducing bacterium enrichment clone HN109	paddy soil from mining area	FJ269072	95.75
	TR29 R 393		1			Iron-reducing bacterium enrichment clone HN109	paddy soil from mining area	FJ269072	93.45
	TR8 R 820				1	Iron-reducing bacterium enrichment clone HN-HFO75	paddy soil from mining area	FJ269100	91.07
	TR27 R 180	1				Uncultured bacterium clone RB13C12	reactor treating monochlorobenzene	AF407415	95.63
	TR29 R 344		1			Uncultured bacterium clone RB13C12	reactor treating monochlorobenzene	AF407415	92.13
	TR30 R 665			1		Uncultured bacterium clone RB13C12	reactor treating monochlorobenzene	AF407415	86.96
	TR27 R 193	1	2			Uncultured bacterium clone Chun-s-14	freshwater sediment	EF632747	97.71
	TR29 R 287		2			Uncultured bacterium clone Chun-s-14	freshwater sediment	EF632747	97.64
	TR29 R 318		2			Uncultured bacterium clone Chun-s-14	freshwater sediment	EF632747	94.54
	TR27 R 194	1				Clostridium sp. strain RCel1	anoxic rice field soil	Y15986	93.78
	TR27 R 197	1				Clostridium sp. strain RCel1	anoxic rice field soil	Y15986	93.64
	TR27 R 203	1				Uncultured bacterium clone B9	bioreactor treating paper mill wastewater	AY426453	93.07
	TR27 R 223	1				Uncultured bacterium clone AE3 aaa04g09	feces	EU771385	87.53
	TR8 R 680				2	Uncultured bacterium clone AFBAB	feces	EU771471	91.85

TR30_R_439			1		Uncultured bacterium clone AS2_aao34d01	feces	EU772158	89.58
TR29_R_261		1			Uncultured bacterium clone HDBW-WB47	deep subsurface groundwater	AB237710	93.58
TR29_R_316		2			Uncultured bacterium clone HDBW-WB52	deep subsurface groundwater	AB237715	96.19
TR29_R_243		1			Uncultured bacterium clone HDBW-WB58	deep subsurface groundwater	AB237721	94.9
TR29_R_231		2			Peptostreptococcaceae bacterium clone D12	tar-oil contaminated aquifer sediments	EU266824	98.99
TR30_R_483			1		Peptostreptococcaceae bacterium clone D12	tar-oil contaminated aquifer sediments	EU266824	98.95
TR30_R_571			1		Peptostreptococcaceae bacterium clone D12	tar-oil contaminated aquifer sediments	EU266824	98.64
TR29_R_330		1			Uncultured Clostridiales bacterium clone D12	tar-oil contaminated aquifer sediments	EU266838	97.88
TR8_R_759				1	Uncultured Clostridiales bacterium clone D12	tar-oil contaminated aquifer sediments	EU266838	90.38
TR29_R_250		3			Acetobacterium woodii		X96954	91.9
TR29_R_301		1			Uncultured bacterium clone SMQ95	composting sample	AM930337	99.64
TR29_R_354		1			Uncultured bacterium clone SMQ95	composting sample	AM930337	87.64
TR29_R_282		1		5	Acidaminococcus sp. DJF_RP55	intestine	EU728758	94.37
TR8_R_815				1	Uncultured bacterium clone Eb26	anaerobic methanogenic UASB reactor	EF063613	95.37
TR30_R_576			1	2	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	96.99
TR8_R_694				2	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	90.99
TR8_R_734				1	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	94.78
TR8_R_757				2	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	89.98
TR8_R_839				1	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	94.46
TR8_R_860				1	Uncultured bacterium clone Eb64	anaerobic methanogenic UASB reactor	EF063614	89.81
TR29_R_407		1			Uncultured eubacterium WCHB1-54	contaminated aquifer	AF050582	99.44
TR30_R_451			2		Clostridiaceae bacterium WN011	methanogenic reactor	AB298726	97.24
TR30_R_530			1		Clostridiaceae bacterium SK082	methanogenic reactor	AB298754	98.31
TR30_R_603			1		Uncultured bacterium clone PL-38B10	low-temperature biodegraded oil reservoir	AY570570	98.24
TR30_R_469			2		Uncultured bacterium clone PL-37B6	low-temperature biodegraded oil reservoir	AY570630	94.48
TR30_R_535			1		Uncultured bacterium CA19	anaerobic digester	AF129866	91.78
TR30_R_588			1		Uncultured bacterium HB69	anaerobic digester	AF129867	96.08
TR30_R_582			1		Uncultured bacterium clone SJA-29	trichlorobenzene-transforming consortium	AJ009459	96.29
TR30_R_611			1		Uncultured bacterium clone tios61a	sulphide-oxidizing bioreactor	AM950260	87.87
TR30_R_442			1		Uncultured Clostridium sp. clone AC036	methanogenic landfill leachate bioreactor	AY330126	88.6
TR29_R_360		1			Uncultured bacterium clone FTLM45	treatment of trichloroethene-contaminated site	AF529124	87.13
TR30_R_564			2	2	Uncultured Clostridiaceae bacterium clone IRB11	Au mine	DQ069191	95.31
TR8_R_822				2	Uncultured Clostridiales bacterium clone SRB47	Au mine	DQ069222	90.31
TR30_R_489			1		Uncultured bacterium clone CI75cm.2.20	sandy carbonate sediment	EF208712	88.81
TR30_R_544			1		Uncultured bacterium clone SJTU_D_02_70	feces	EF401864	91.16
TR8_R_829				1	Uncultured bacterium clone SJTU_B_12_36	feces	EF402779	94.47
TR8_R_819				1	Uncultured bacterium clone SJTU_G_05_90	feces	EF405016	89.44
TR30_R_488			1		Uncultured bacterium clone horsem_aai93d03	feces	EU463410	95.45
TR8_R_825				1	Uncultured bacterium clone KO1_aai41h01	feces	EU460994	93.1
TR8_R_698				1	Uncultured bacterium clone orang1_aai53f02	feces	EU462390	95.64
TR8_R_850				1	Uncultured bacterium clone HRX_O09	feces	EU465206	93.18
TR8_R_866				1	Uncultured bacterium clone AS2_aao35h07	feces	EU465808	93.78
TR8_R_795				1	Uncultured bacterium clone D242_27F_BAC_012	anaerobic granule sludge	AB447694	87.58
TR8_R_678				1	Uncultured bacterium clone TSCOR003_B08	rice paddy soil	AB486861	92.53
TR8_R_851				1	Uncultured bacterium clone TSCOR003_B08	rice paddy soil	AB486861	92.18
TR8_R_849				1	Uncultured bacterium clone TANB107	TCE-dechlorinating groundwater	AY667265	94.9
TR8_R_863				1	Uncultured bacterium clone 054F09_B_DI_P58	municipal wastewater treatment plant	CR933212	91.49
TR8_R_855				1	Uncultured bacterium clone 053G03	municipal wastewater treatment plant	CR933253	90.8
TR8_R_753				1	Sedimentibacter sp. JN18_A14_H	PCB-dechlorinating enrichment culture JN18	DQ168650	99.27
TR8_R_859				1	Sedimentibacter sp. JN18_A14_H	PCB-dechlorinating enrichment culture JN18	DQ168650	99.03
TR8_R_781				1	Clostridiales bacterium JN18	PCB-dechlorinating enrichment culture JN18	DQ168652	93.04
TR8_R_854				1	Clostridiales bacterium JN18	PCB-dechlorinating enrichment culture JN18	DQ168652	91.38
TR8_R_722				1	Uncultured bacterium clone R-1167	feces	DQ777889	94.14
TR8_R_720				1	Uncultured bacterium clone B55	thermophilic anaerobic solid waste bioreactor	DQ887967	93.05
TR8_R_792				2	Clostridiaceae bacterium JN18_V56_P	PCB-dechlorinating enrichment culture	EF059534	94.58
TR8_R_861				1	Uncultured bacterium clone NED5C6	dairy cow rumen	EF445248	95.09
TR8_R_749				2	Uncultured bacterium clone G35	mesophilic anaerobic digester	EF559143	95.13
TR8_R_681				2	Uncultured Clostridiales bacterium clone A1435	Anderson Lake	EU283551	90.84
TR8_R_739				1	Uncultured bacterium clone AS2_aao35h07	feces	EU465808	90.58
TR8_R_742				1	Iron-reducing bacterium enrichment clone HN-HFO75	paddy soil from mining area	FJ269100	97.02
TR8_R_767				2	Uncultured bacterium clone C-s	saturated C horizon soil aggregate	EU307095	88.24
TR8_R_782				1	Uncultured Ruminococcaceae bacterium clone EMP	rumen	EU794101	93.25
Bacillales								
TR29_R_355		1			Uncultured bacterium clone E2-2	leaf litter	EF600582	98.94
TR29_R_414		1			Uncultured bacterium clone TSAT08	polychlorinated dioxin microcosm	AB186876	99.59
TR8_R_705				3	Uncultured rumen bacterium clone YNRC86	rumen	EF686597	92.28
TR8_R_791				2	Uncultured bacterium clone CAP_aai00f03	feces	EU459584	89.56
TR8_R_760				1	Bacillus pycnus		AB271739	90.2
Mollicutes								
TR30_R_458			1		Uncultured bacterium clone C81B	landfill soil, 5.5 ft. depth	EU219944	90.25
TR8_R_778				1	Uncultured bacterium clone C105B	landfill soil, 5.5 ft. depth	EU219952	88.53
TR8_R_693				1	Erysipelotrichaceae bacterium clone EMP_K38	feces	EU794282	86.94
TR8_R_838				1	Uncultured bacterium clone R3ENDE9	digester of toluene wastewater	DQ401524	94.42
TR8_R_794				1	Unidentified eubacterium clone vadinHA31	anaerobic digester fed by vinasses	U81729	93.94
TR8_R_799				1	Uncultured bacterium clone A55	thermophilic anaerobic digester	EF559039	90.01
TR8_R_853				1	Uncultured bacterium clone A55	thermophilic anaerobic digester	EF559039	90.67
TR8_R_695				1	Uncultured bacterium clone 68	anaerobic fermentation reactor	FJ535013	98.85
Alphaproteobacteria								
TR27_R_35	1				Uncultured bacterium clone Er-LLAYS-12	sediment and soil slurry	EU542494	98.86
TR29_R_323		1			Sphingomonas chungbukensis	PAH-contaminated soil	AY151392	98.32
TR29_R_369		1			Uncultured bacterium clone 290cost002-P3L-1606	termite hindgut	EF454079	85.98
TR29_R_371		1			Uncultured bacterium clone Chun-s-2	freshwater sediment	EF632760	98.65
TR29_R_377		1			Allorhizobium undicola		Y17047	87.29
TR30_R_522			1	1	Uncultured bacterium clone LYC075	oil-contaminated soil	DQ984605	96.1
TR8_R_728				1	Uncultured bacterium clone YK	oil-contaminated soil	DQ984620	98.07
TR8_R_683				1	Uncultured bacterium clone p36k10ok	undisturbed tall grass prairie, top 5 cm	FJ478981	98.76
TR8_R_690				1	Uncultured bacterium clone p36k10ok	undisturbed tall grass prairie, top 5 cm	FJ478981	97.76
TR8_R_692				1	Uncultured organism clone EME109	ice	EF127604	99.21
TR8_R_745				1	Candidatus Alysiosphaera europeae	activated sludge	AY428766	91.64
TR8_R_758				1	Uncultured sludge bacterium A39	sludge	AF234724	98.3
TR8_R_848				1	Uncultured bacterium clone SC-48	corroded concrete sample	AB255094	99.31
TR8_R_738				2	Uncultured bacterium clone tios61a	sulphide-oxidizing bioreactor	AM950260	89.11
Betaproteobacteria								
TR27_R_140	1				Hydrogenophaga sp. CL3	oil refinery collecting lagoon	DQ986320	98.66
TR27_R_212	1				Uncultured bacterium clone WM95	sulfidic cave stream biofilm	DQ415788	97.45
TR27_R_44	1				Uncultured bacterium clone WM95	sulfidic cave stream biofilm	DQ415788	99.1
TR30_R_622			1		Uncultured bacterium clone 191up	water 20 m upstream of manure	AY212644	98.93

TR8_R_833				1	Petrobacter succinimandens BON4	oil well	AY219713	99.6
Gammaproteobacteria								
TR29_R_333		2			Uncultured bacterium clone 290cost002-P3L-812	termite hindgut	EF454967	90.85
TR29_R_413		1			Uncultured bacterium clone 290cost002-P3L-2527	termite hindgut	EF454681	91.29
TR30_R_441			1		Uncultured bacterium clone TP51	geothermal spring mat	EF205569	86.15
TR30_R_459			1		Uncultured gamma proteobacterium clone G07	tundra wet meadow soil 0-20 cm	DQ450792	94.44
TR8_R_697				1	Uncultured Xanthomonadaceae bacterium BF21		AM691113	99.8
TR8_R_723				1	Rhodanobacter sp. CC-JY-1	oil-contaminated site	DQ239766	99.8
TR8_R_785				2	Uncultured bacterium clone G3DCM-154	chromium contaminated soil	EU037342	98.01
Deltaproteobacteria								
TR27_R_15	1	1	1		Desulfomicrobium baculatum strain DSM 1742		AJ277896	99.87
TR27_R_108	1				Desulfomicrobium baculatum strain DSM 1742		AJ277896	99.92
TR30_R_666			1		Desulfomicrobium baculatum strain DSM 1742		AJ277896	93.26
TR27_R_99	2				Desulfomicrobium norvegicum strain DSM 1741T		AJ277897	99.5
TR27_R_202	1				Uncultured bacterium clone Er-LLAYS-12	sediment and soil slurry	EU542494	98.79
TR27_R_131	1				sulfate-reducing bacterium R-LacA1	rice field soil	AJ012593	99.3
TR27_R_171	1				sulfate-reducing bacterium R-LacA1	rice field soil	AJ012593	99.69
TR27_R_98	2				sulfate-reducing bacterium R-LacA1	rice field soil	AJ012593	99.37
TR30_R_584			1		sulfate-reducing bacterium R-LacA1	rice field soil	AJ012593	94.43
TR27_R_167	1				Desulfobacter vibrioforme B54		U12254	98.74
TR27_R_188	1				Uncultured bacterium clone HDBW-WB25	deep subsurface groundwater	AB237688	91.81
TR27_R_208	1				Desulfuromonadales bacterium JN18_A94_J	PCB-dechlorinating enrichment culture	DQ168651	99.12
TR29_R_256		2			clone TNR-I-16	rice paddy soil	AB255987	96.61
TR29_R_384		1			clone TNR-I-16	rice paddy soil	AB255987	95.21
TR29_R_336		1			Desulfobulbus sp. RPf35L17	reactor treating acid mine drainage	AY548775	96.9
TR29_R_263		1			Uncultured Desulfobacteraceae bacterium clone D25	tar-oil contaminated aquifer sediments	EU266914	96.35
TR29_R_296		1			Uncultured bacterium clone C-s	saturated C horizon soil aggregate	EU307093	99.27
TR29_R_311		1			Uncultured delta proteobacterium clone MVP-3	suboxic freshwater-pond bacterioplankton	DQ676338	94.46
TR30_R_470			1		Uncultured bacterium clone SJA-29	trichlorobenzene-transforming consortium	AJ009459	87.45
TR30_R_498			1		Uncultured bacterium clone 290cost002-P3L-1827	termite hindgut	EF454232	88
TR30_R_519			1		Uncultured Syntrophorhabdaceae clone SJA-162	trichlorobenzene-transforming consortium	AJ009498	98.81
TR30_R_627			1		delta proteobacterium clone HMMVBeg-12	marine sediment	AJ704684	91.27
TR30_R_500			1		Uncultured bacterium partial clone 311	chemocline of meromictic Lake Cadagno	AJ831749	97.47
TR30_R_580			1		Uncultured organism clone MAT-CR-M3-E12	hypersaline microbial mat	EU245604	93.79
TR30_R_565			1		Uncultured bacterium clone 2E7	anaerobic wastewater treatment system	EF688166	85.76
TR8_R_747				1	clone TNR-I-16	rice paddy soil	AB255987	92.85
Spirochaetes								
TR27_R_11	1				Uncultured spirochete clone C6_2	paper pulp column	EF562545	95.96
TR27_R_57	1				Uncultured spirochete clone C6_2	paper pulp column	EF562545	94.65
TR27_R_71	1				Uncultured spirochete clone C6_2	paper pulp column	EF562545	97.49
TR29_R_307		1			Uncultured spirochete clone C6_2	paper pulp column	EF562545	89.71
TR8_R_689				4	Uncultured spirochete clone C6_2	paper pulp column	EF562545	96.3
TR8_R_775				1	Uncultured spirochete clone C6_2	paper pulp column	EF562545	97.67
TR8_R_779				2	Uncultured spirochete clone C6_2	paper pulp column	EF562545	96.36
TR8_R_841				1	Uncultured spirochete clone C6_2	paper pulp column	EF562545	97.47
TR8_R_869				1	Uncultured spirochete clone C6_2	paper pulp column	EF562545	96.62
TR27_R_34	1				Uncultured bacterium clone SR_FBR_E2	SRB reactor treating acid mine drainage	AY340818	93.06
TR27_R_61	2				Uncultured bacterium clone SR_FBR_E2	SRB reactor treating acid mine drainage	AY340818	92.87
TR27_R_72	2				Uncultured bacterium clone SR_FBR_E2	SRB reactor treating acid mine drainage	AY340818	87.23
TR27_R_94	2				Spirochaeta caldaria strain DSMZ7334		EU580141	93.32
TR27_R_156	1				Spirochaeta zuelzerae		M88725	95.54
TR30_R_575			1		Spirochaeta stenostrepta		M88724	95.37
Fibrobacteres								
TR29_R_390		1			Fibrobacteres bacterium clone ADK-MOh02-63	acid-impacted lake	EF520549	91.88
TR30_R_485			1		Fibrobacteres bacterium clone ADK-MOh02-63	acid-impacted lake	EF520549	88.79
TR8_R_685				1	Fibrobacteres bacterium clone ADK-MOh02-63	acid-impacted lake	EF520549	91.83
TR8_R_732				1	Fibrobacteres bacterium clone ADK-MOh02-63	acid-impacted lake	EF520549	91.45
TR30_R_465			1		Uncultured Fibrobacter sp. clone Brom F17	landfill	EF190827	92.69
TR30_R_495			1		Uncultured Fibrobacter sp. clone Brom F17	landfill	EF190827	94.39
Verrucomicrobiae								
TR27_R_138	1				Uncultured bacterium clone P13-67	arctic surface sediment	EU287160	94.11
TR8_R_675				3	Uncultured Opitutus sp. clone Y145	moderate saline soil	EU328081	91.05
TR8_R_677				3	Uncultured Opitutus sp. clone Y145	moderate saline soil	EU328081	90.86
TR8_R_763				1	Uncultured Opitutus sp. clone Y145	moderate saline soil	EU328081	89.17
Acidobacteria								
TR29_R_326		1			Uncultured bacterium clone PS-Ba63	phenol-degrading sludge	EU399666	91.5
Actinobacteria								
TR8_R_797				1	Uncultured bacterium clone PR35	rhizosphere root	DQ298352	96.97
TR8_R_840				1	Uncultured bacterium clone FCPT456	grassland soil	EF516030	97.16
Lentisphaerae								
TR8_R_712				1	Uncultured eubacterium AA08	anaerobic reactor	AF275917	97.75
TR8_R_718				3	Uncultured eubacterium AA08	anaerobic reactor	AF275917	96.2
Planctomycetes								
TR27_R_19	1	1			Uncultured organism clone MAT-CR-M8-E11	hypersaline microbial mat	EU245949	86.88
TR27_R_64	2				Uncultured bacterium clone B86	sphagnum peat bog	AM162476	91.23
Cyanobacteria								
TR30_R_433			2		Uncultured bacterium clone ADK-CSe02-18	acid-impacted lake	EF520515	95.83
Chlorobi								
TR30_R_541			1		Uncultured bacterium clone 655949	contaminated sediment	DQ404821	95.74
Nitrospirae								
TR27_R_104	2				Uncultured bacterium clone tios61a	sulphide-oxidizing bioreactor	AM950260	91.17
TR27_R_137	1	2	5		Uncultured bacterium clone tios61a	sulphide-oxidizing bioreactor	AM950260	91.52
Deferribacteres								
TR29_R_248		1			Uncultured bacterium clone G3DCM-41	chromium contaminated soil	EU037327	95.54
TR30_R_449			1		Uncultured bacterium clone lcfa	anaerobic sludge	DQ339709	91.58
TG3								
TR27_R_86	1						EU358689	95.35
TR29_R_230		15	18	2	Candidate division TG3 clone TNR-I-16	rice paddy soil	AB255987	96.53
TR29_R_268		1			Candidate division TG3 clone TNR-I-16	rice paddy soil	AB255987	92.39
TR30_R_624			1		Candidate division TG3 clone TNR-I-16	rice paddy soil	AB255987	91.48
TG1								
TR8_R_755				1	Uncultured rumen bacterium clone P5	rumen	EU381925	95.1
OD1								
TR27_R_92	1				Uncultured bacterium clone FW17	forested wetland	AF524026	89.11
TR29_R_305		1			Uncultured bacterium clone TUM-dMbac-MR4-B1-KC-	anaerobic reactors fed with silage	FJ234919	88.88
TM7								
TR8_R_852				1	Uncultured bacterium clone FCPT530	grassland soil	EF516872	94.27

SR1							
	TR29 R 356	1		Uncultured bacterium clone Pav-SR15	Lake Pavin water column	FJ482227	96.62
unidentified							
	TR30 R 551		1	Uncultured eubacterium WCHB1-01	contaminated aquifer	AF050597	91.31
	TR30 R 604		1	Uncultured bacterium clone FFCH2415	soil	EU134965	89.2
	TR30 R 647		1	Uncultured bacterium clone PL-11B10	low-temperature biodegraded oil reservoir	AY570581	98.42
	TR30 R 486		1	Uncultured bacterium clone TSCOR003 O20	rice paddy soil	AB486974	92.3

Table D.2: Abundance and nearest phylogenetic neighbors of all OTUs obtained from complex carbon material *dsr* clone libraries.

OTU	Frequency in library				Nearest phylogenetic neighbor			
	Silage	Compost mix	Molasses + hay	Wetland biosolids	Sequence	Source	Accession no.	Similarity %
TR27_1	2	1			Desulfitobacterium hafniense Y51		AP008230	72
TR27_3	2				Uncultured bacterium	solid waste digester	AB114346	100
TR27_5	1		2		Uncultured bacterium clone NTUA-5A-DSR16	fixed-bed reactor	EF645671	98
TR27_6	8	3	8		Desulfotomaculum thermosapovorans		AF271769	81
TR27_12	2				Desulfobacter vibrioformis		AJ250472	93
TR27_13	17				Desulfomicrobium escambiense		AB061531	94
TR27_20	2				Desulfovibrio burkinensis		AB061536	87
TR27_22	18	10	13		Uncultured sulfate-reducing bacterium clone P4D-20	near-surface sediment	AY725433	91
TR27_33	8	2			Uncultured sulfate-reducing bacterium	sheath of Thioploca sp. 'Lake Biwa	AB263672	92
TR27_38	4	6	3		Uncultured sulfate-reducing bacterium clone GranDSR5	UASB bioreactor	AY929599	79
TR27_43	1				Uncultured sulfate-reducing bacterium clone GranDSR10	UASB bioreactor	AY929609	88
TR27_44	2	3	7		Uncultured bacterium clone NTUA-5A-DSR22	fixed-bed reactor	EF645675	96
TR27_46	1				Desulfomicrobium baculatum		AB061530	89
TR27_47	4				Desulfomicrobium apsheronum		AB061529	99
TR27_51	4	1			Desulfomicrobium escambiense		AB061531	93
TR27_65	1				Uncultured sulfate-reducing bacterium clone LGWI05	groundwater polluted by leachate from landfill	EF065044	79
TR27_72	1				Uncultured sulfate-reducing bacterium clone P4D-20	near-surface sediment	AY725433	91
TR29_80		1			Desulfotomaculum aeronauticum		AF273033	93
TR29_90		2	1	2	Desulfovibrio desulfuricans isolate SRDQC		DQ450464	99
TR29_93		1			Uncultured Desulfotomaculum sp. clone DSR_Irb1	mafic sill	DQ415718	98
TR29_94		2			Uncultured sulfate-reducing bacterium clone GranDSR16		AY929604	90
TR29_99		25	31		Uncultured bacterium clone NTUA-5A-DSR22	fixed-bed reactor	EF645675	96
TR29_136		3			Uncultured sulfate-reducing bacterium	sediment of Lake Biwa	AB263656	90
TR29_139		1			Uncultured prokaryote clone GSL_27_18	Great Salt Lake sediment	EF158463	81
TR29_140		3	2		Uncultured sulfate-reducing bacterium clone 25H-0D-46	cold seep sediment	FJ403729	82
TR29_143		2			Uncultured sulfate-reducing bacterium clone 15-169	sediment	EU199880	91
TR29_145		1			Uncultured sulfate-reducing bacterium clone LGWG22	groundwater polluted by leachate from landfill	EF065032	85
TR29_146		1	1		Uncultured sulfate-reducing bacterium clone LGWG22	groundwater polluted by leachate from landfill	EF065032	86
TR30_156			1		Uncultured bacterium clone NTUA-5A-DSR16	fixed-bed reactor	EF645671	87
TR30_162			1		Desulfatibacillus olefinivorans strain LM2801		DQ826725	85
TR30_171			2		Uncultured bacterium clone NTUA-5A-DSR22	fixed-bed reactor	EF645675	91
TR30_196			2		Uncultured sulfate-reducing bacterium clone P4D-20	near-surface sediment	AY725433	91
TR8A_211					Desulfovibrio aerotolerans		AY749039	90
TR8A_212					Uncultured sulfate-reducing bacterium clone Mont3F	acid mine drainage	EU189171	87
TR8A_213				16	Uncultured bacterium clone NTUA-1A-DSR3	fixed-bed reactor	EF645667	98
TR8A_214				96	Uncultured bacterium clone NTUA-1A-DSR3	fixed-bed reactor	EF645667	98
TR8B_264				1	Uncultured sulfate-reducing bacterium clone BV11F	sludge	EU552479	92
TR8B_265				3	Uncultured sulfate-reducing bacterium clone Mont25F	acid mine drainage	EU189179	99
TR8B_268				1	Uncultured bacterium clone NTUA-1A-DSR14	fixed-bed reactor	EF645665	88