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Diversity of archaea and eubacteria in constructed wetland in California

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DIVERSITY OF ARCHAEA AND EUBACTERIA IN A CONSTRUCTED WETLAND IN CALIFORNIA

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by
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ABSTRACT

DIVERSITY OF ARCHAEA AND EUBACTERIA IN A CONSTRUCTED WETLAND IN CALIFORNIA

by Paula B. Matheus-Carnevali

Wetlands harbor microorganisms that make significant contributions to global warming. In the anoxic niche, methanogens and sulfate-reducing bacteria (SRB) compete for the same substrates. Although approaches have been developed to study microbial diversity in natural wetlands, knowledge of the microbial diversity of constructed wetlands is scarce. This study was conducted to survey the eubacterial and archaeal diversities in a constructed wetland and to establish the functional groups of methanogens and SRB predominant under disturbance and restoration conditions. Phylogenetic analyses of the 16S rRNA gene indicated that the archaeal community was dominated by members of the phylum Crenarchaeota and that the eubacterial community was dominated by the phylum Proteobacteria. A difference in the diversity of the microbial communities from an agriculturally polluted input zone and a downstream natural site was observed. A small number of sequences corresponded to methanogens and SRB. Non-acetoclastic methanogenesis appeared to be the dominant methanogenic pathway, and SRB were either complete or incomplete acetate oxidizers. Novel groups of both methanogens and SRB may exist in this wetland.

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Introduction

Wetlands contribute to global warming by releasing greenhouse gases such as methane (CH_4). Wetland CH_4 emissions are estimated to be between 110 and 260 Tg $year^{-1}$ (Tg = 10^{12} g) [6, 15, 29] comprising about 30% of all sources of atmospheric methane. Other sources of methane include rice fields (~ 60 Tg $year^{-1}$), domestic sewage (~ 25 Tg $year^{-1}$), exploration and combustion of fossil fuels (~ 100 Tg $year^{-1}$), enteric fermentation in ruminants and animal wastes (~ 105 Tg $year^{-1}$), biomass burning, methane hydrates, freshwater and coastal marine sediments, and decomposition in landfills [7, 22, 29].

Wetlands, rice fields, and peat fields provide the most favorable conditions for microbial CH_4 production, a process known as methanogenesis [13, 15, 22, 48]. This process is carried out by methanogenic Archaea, which utilize decomposed organic matter under low redox potentials as substrate (Eh < -200 mV) [22]. Methanogenesis constitutes the main natural source (70 to 80%) of CH_4 in the atmosphere [22, 36, 48].

Methane increases the greenhouse effect of carbon dioxide (CO_2) and nitrous oxide (N_2O) by 20%. Although the concentration of CO_2 is 224 times that of CH_4 (380 ppm of CO_2 versus 1.7 ppm of CH_4) [7], methane has a 20-to-30-fold greater greenhouse effect than CO_2 [18, 22]. On a molar basis, CH_4 is more

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effective than CO_2 as a greenhouse gas because it shows strong absorption at 7.7 μm and does not share absorption bands with other gases [48]. According to Torres *et al.* [44], 1 kg of atmospheric CH_4 retains more of the heat emitted from the planet's surface than 1 kg of CO_2.

Methane is released either directly to the atmosphere or to surrounding environments, where it may be oxidized to carbon dioxide and water ($CO_2 + H_2O$) by methane-assimilating bacteria and autotrophic ammonia-oxidizing bacteria [7]. Bacterial uptake of CH_4 accounts for less than 10% of methane consumption. Other major sinks of CH_4 are the breakdown of the molecule in the stratosphere, and, more importantly, breakdown in the troposphere by OH^- radicals (accounting for > 85% of CH_4 consumption) [7].

Methanogenesis. Methane production relies on a syntrophic association between different metabolic groups of organisms. First, hydrolytic bacteria (aerobic, facultatively or strictly anaerobic) initialize the process by decomposing complex organic matter (microbial biomass, roots, and root exudates) into smaller molecules (fatty acids and amino acids) that can be used by other prokaryotes [11]. Second, fermentative bacteria (facultatively or strictly anaerobic) release substrates such as highly volatile fatty acids, methanol and other primary and secondary alcohols, methylated compounds, hydrogen (H_2), and CO_2, which are then used by proton-reducing acetogens or by homoacetogenic bacteria [22]. Third, obligate proton-reducing bacteria use H^+

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as an electron acceptor to produce H_2, formate, acetate, and CO_2 when electron acceptors are a limiting factor. In this situation, there is a syntrophic association between methanogens and proton-reducing bacteria, where methanogens rely on the electrons provided by these bacteria via interspecies transfer. The resulting low partial pressures of H_2 are thermodynamically favorable for the proton-reducing bacteria [48]. In acidic environments homoacetogenic bacteria may replace the methanogens as H_2 scavengers to produce acetate [11, 22, 48]. Substrates such as acetate, H_2, and CO_2, as well as formate, methanol, and other methylated compounds, can be used for methanogenesis [7].

The four main pathways of methane production are summarized as:

i) $CH_3COOH \dashrightarrow CO_2 + CH_4$ $\Delta G^{o\prime} = -31$ KJ reaction^{-1}

ii) $CO_2 + 4H_2 \dashrightarrow CH_4 + 2H_2O$ $\Delta G^{o\prime} = -131$ KJ reaction^{-1}

iii) $4CH_3OH \dashrightarrow 3CH_4 + CO_2 + 2H_2O$ $\Delta G^{o\prime} = -319$ KJ reaction^{-1}

iv) $4CH_3NH_3 + 2H_2O \dashrightarrow 3CH_4 + CO_2 + 4NH_4$

Reaction (i) represents the acetate fermentation pathway, where the methyl group of acetate is transformed to methane. The carbon dioxide reduction pathway summarized in reaction (ii) requires hydrogen (H_2) or formate as electron acceptors for the reduction of CO_2. Finally, reactions (iii) and (iv) correspond to the utilization of methylated compounds in the production of methane [5, 11, 44].

Methanogenesis may be carried out only by members of the phylum Euryarchaeota (domain Archaea) [11, 18]. This phylum includes the orders Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanococcales and Methanopyrales [11]. Twenty-six genera and more than 60 species of methanogens have been identified [22], the majority of which are associated with the families Methanosarcinaceae and Methanomicrobiaceae.

Methanogens from different orders are often grouped according to substrate utilized for methanogenesis. Methanogens known as acetotrophs (utilizing the acetate fermentation pathway) produce 2/3 of the methane, and the remaining 1/3 is produced by the group known as hydrogenotrophs (utilizing the carbon dioxide reduction pathway) [7, 11, 22]. A small portion of methanogens, including the genera *Methanosarcina* and *Methanosaeta* are acetotrophs, whereas the vast majority of species (77%) are hydrogenotrophs (*e.g., Methanobacterium spp., Methanobrevibacter spp.,* and *Methanogenium spp.*) [44]. Acetoclastic methanogenesis produces less energy under normal conditions than hydrogen/formate-based methanogenesis [22], but given fresh water natural conditions this relationship may be reversed [5]. Table 1 describes the main orders of methanogens, their families, and the carbon and energy sources utilized as well as other relevant features from each group.

Table 1. Orders of methanogens and their main characteristics. Adapted from Ferry and Kastead [11].

Characteristics	Order *Methanobacteriales*		Order *Methanosarcinales*		Order *Methanomicrobiales*			Order *Methanococcales*		Order *Methanopyrales*
Families within the order	Methano-bacteriacea	Methano-thermaceae	Methano-sarcinaceae	Methano-saetaceae	Methano-microbiaceae	Methano-corpusculaceae	Methano-spirillaceae	Methano-coccaceae	Methano-caldo-coccaceae	Methano-pyralaceae
No genera, No species	4, 32	1, 2	8, 26	1, 2	7, 23	1, 4	1,1	2, 5	2, 6	1,1
Energy source/ Electron donor	In general CO_2/H_2	CO_2/H_2	Methylated compounds, acetate, CO_2/H_2	Acetate	Acetate, CO_2/H_2, formate, secondary alcohols	CO_2/H_2, formate, secondary alcohols	CO_2/H_2, formate	CO_2/H_2, formate	Mostly CO_2/H_2	CO_2/H_2
Carbon source	Mostly autotrophic some heterotrophic	Autotrophic	Metabolically versatile	_____	_____	Acetate	_____	Autotrophic	Autotrophic	Autotrophic
Nitrogen and sulfur sources	_____	_____	Ammonium and sulfide	Ammonium and sulfide	_____	Peptones	Nitrogen fixation	Ammonium, sulfide, elemental sulfur	Ammonium, sulfide, may also need selenium	Ammonium, sulfide
Temperature affinity	Mostly mesophilic	Thermo-philic	Mostly mesophilic	Mesophilic, thermo-philic	Mostly mesophilic	Mesophilic	Mesophilic	_____	Hyper-termophilic	Hyperthermophilic
pH affinity	Mostly neutralophilic (6.8-8.0)	Neutralo-philic	Mostly neutralo-philic (6.5-7.5)	Neutralo-philic (6.0-7.0)	_____	Neutralophilic	Neutralophilic (6.6-7.4)	_____	Slightly acidophilic	Slightly acidophilic
Salt affinity	_____	_____	_____	_____	Some are holotolerant	_____	_____	Mostly halotolerant	Some are halotolerant	_____

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Other Microbial Processes Relevant to the Biogeochemistry of Wetlands.
Methanogens use the same substrates as denitrifiers and sulfate-reducing bacteria (SRB) as electron donors in anaerobic respiration [9, 32, 36]. Competition for these substrates may thus occur, altering the dynamics of the different biogeochemical cycles that take place in wetlands. An important reaction of SRB is dissimilatory sulfate reduction, in which organic matter is oxidized using sulfate (SO_4^{2-}) as an electron acceptor [5]. This process can take place at redox potentials between -120 and -150 mV. A typical reaction carried out by the SRB from group II is shown below.

$$CH_3COO^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \qquad \Delta G^{o\prime} = -57.5 \text{ KJ reaction}^{-1}$$
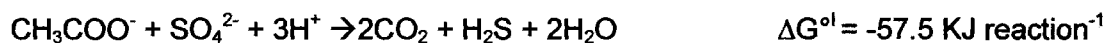
According to Madigan *et al.* [24] sulfate-reducing bacteria from the phylum Proteobacteria are classified into two groups:

- ❖ Group I (nonacetate oxidizers): This group includes genera such as *Desulfovibrio, Desulfomicrobium, Desulfobotulus, Desulfotomaculum,* and *Desulfobulbus.* Besides H_2, lactate, and pyruvate, they utilize malate, sulfonates, and alcohols such as methanol, ethanol, propanol, and butanol as energy sources, which are reduced to the level of acetate.

- ❖ Group II (acetate oxidizers): Representative genera from this group include *Desulfobacter, Desulfobacterium, Desulfococcus, Desulfonema,* and *Desulfoarculus.* These organisms have the ability to oxidize fatty acids, lactate, succinate, and, infrequently, benzoate to CO_2.

When SO_4^{2-} is abundant, SRB out-compete methanogens. This has been observed in the uppermost layers of coastal marine sediments where up to 50% of the degradation of organic matter occurs through the action of SRB [32, 36]. SO_4^{2-} reduction is the favored mineralization pathway of organic matter (conversion of an organic carbon into an inorganic, gaseous form of carbon) in marine and coastal environments such as brackish wetlands. Methanogenesis assumes this role in freshwater sediments [5, 44], where the availability of carbon is greater than the availability of electron acceptors [44]. However, in freshwater wetlands, where there is a shift from anoxic to increased oxygenated conditions, the activity of SRB is favored over that of methanogenic Archaea possibly due to the re-oxidation of some of their substrates [9, 32].

Under anoxic conditions – when the redox potential is between +350 and +100 mV – nitrate (NO_3^-) is preferred over SO_4^{2-} as electron acceptor, because NO_3^- is a better oxidant that could be used as an alternative electron acceptor by denitrifying bacteria [5, 46]. Some SRB also utilize NO_3^- as an electron acceptor during growth and the reduction of SO_4^{2-} to H_2S [24]. Denitrifying, anaerobic Gram-negative bacteria include the genera *Pseudomonas, Clostridium, Bacillus* and *Alcaligenes* [44]. Nitrates are chemically transformed to elemental nitrogen according to the general series:

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2.$$
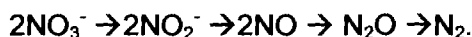
The balanced reactions are shown below [46]:

$$4(CH_2O) + 4NO_3^- \rightarrow 4HCO_3^- + 2N_2O + 2H_2O$$

$$5(CH_2O) + 4NO_3^- \rightarrow H_2CO_3 + 4HCO_3^- + 2N_2 + 2H_2O$$

During NO_3^- reduction, nitrous oxide (N_2O) and nitrogen gas (N_2) are formed. Under certain environmental conditions such as high moisture levels, the transformation of N_2O to N_2 may not occur [5, 7].

Distribution of Wetlands Around the World. In general, wetlands are areas where inflow of water surpasses the outflow, resulting in temporal or permanent water saturation of the soil. The accumulation of a water column over the soil substrate, combined with the depletion of oxygen (diffusion rates of O_2 in soil is ~10,000 times slower than in an aqueous solution), and the decrease of the redox potential with depth [44] create an anaerobic zone suitable for methanogenic Archaea.

Other strata or zones may also be found in the sediment. In the first few millimeters or centimeters of the sediments, a gradient from oxic to anoxic conditions is usually found [44], although the upper sediment is still generally thought of as the 'oxic zone'. The 'rhizosphere' is another well-documented ecological zone: many nitrogen-fixing microorganisms grow in symbiosis with the roots of the wetland plants [44].

The classification systems used to study wetlands are quite complex and

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based on different factors, such as hydrology, topography, and vegetation. Torres *et al.* [44] and Mitsch and Gosselink [30] have proposed the following major wetland types:

❖ Freshwater and salt marshes in temperate regions that are periodically saturated with water.

  ➢ Freshwater marshes (20°-45° N, 30°-50°S) comprise a mixture of organic and inorganic sedimentary matter under alkaline or acid pH, and show high rates of mineralization with little or no peat deposition.

  ➢ Salt marshes (20°N-30°S) are brackish environments found along coastlines, and are influenced by the inflow of nutrients and organics tides from ground water, fresh water runoff, and rivers.

❖ Mangroves, alluvial floodplains, swamps, and rice paddy fields in tropical and subtropical regions.

  ➢ Rice paddy fields (20°N-30°S) are freshwater, agricultural wetlands in which rice straw is utilized as the main fertilizer.

❖ Freshwater bogs and fens in boreal regions (45°-60°N).

  ➢ Bogs (histosols) are characterized by spongy peat deposits and are usually found in glaciated areas. Their water supply is mainly from precipitation and the average pH is acidic.

> Fens also tend to occur in glaciated areas, but have ground and superficial runoff supplies of water. Peat is formed, but the pH may be alkaline, neutral, or moderately acidic.

❖ Arctic wetlands (>70°N), which exist as freshwater tundra and receive 1/3 of its annual water inflow as snowfall.

Antarctica is the only continent on Earth in which wetlands have not been observed.

Each type of wetland contributes differently to the input of methane to the atmosphere. Most studies indicate that tropical wetlands are the main contributor, followed by boreal and arctic wetlands, and then by temperate wetlands [44]. However, other studies indicate that temperate wetlands are of more importance than arctic wetlands as methane contributors [6].

Physicochemical Parameters of Wetlands. Methanogenesis, sulfur-reduction, and denitrification are tightly linked processes that occur primarily in the anoxic compartment of wetland sediments. These processes are influenced by the different environmental factors intrinsic to the ecology of these ecosystems. Substrate availability may be the most important characteristic, but other relevant factors include hydrology, temperature, pH, redox potential, soil texture, and plant composition [17, 22].

The ecology of wetlands depends strongly on hydrological factors – the water source and the quality of the water, frequency and amount of water influx, duration of flooding, and the wetting-drying cycles [4, 5, 16]. The water source is the most important element in classification of wetlands, and thus determines the wetland type. For example, the main difference between bogs and fens is the origin of the input water, which can be rainfall or an underground stream, determining the nutrient content and the contribution to the wetland [5]. The quality of the water is also influenced by its source.

Biogeochemical processes including methane production [19, 41] are dependent on the depth of the water column during different seasons. Johansson *et al.* [19] used linear regression to explain variations in the methane flux rates measured at a constructed wetland, finding that the water level was negatively correlated to the flux rates. Dowrick *et al.* [9] confirmed that a lowered water table is unfavorable to methanogens, and suggested that this is due to aeration of peat surfaces which increases methane oxidation.

The wetting-drying cycles of a wetland establish the anoxic-oxic conditions of the sediments. When the water table rises, the oxygen is rapidly exhausted from the sediments, creating anaerobic conditions that would favor processes such as methanogenesis, denitrification, and sulfate reduction. A decrease in the water table increases the rate of diffusion of gases, thus favoring oxidation of methane (methanotrophy) and nitrification [5, 7]. The duration of the submersion

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period also affects these processes. It has been observed that prolonged submersion supports methanogenesis over denitrification because of a lack of oxygen and NO_3^- [40]. In a wetting-drying cycle, the NO_3^- accumulated during the dry period promotes N_2O production [7] and a high redox potential resulting in a decrease in CH_4 production and an increase in oxidation of CH_4 coupled to denitrification [40].

Temperature is considered to be important for all biochemical reactions, and many methanogens are mesophilic, undergoing optimal methanogenesis at 30 and 40°C [48]. Relatively, tropical wetlands have higher average CH_4 emissions than temperate, boreal, and arctic wetlands. Boreal and arctic peatlands exhibit an increased CH_4 emission during the thaw season [48]. It has also been observed that at higher temperatures, the preferred energy source for methanogenesis is CO_2/H_2, but at lower temperatures, acetate is preferred [7, 44].

A few observations should be mentioned regarding pH, soil texture and redox potential. Although methanogens and denitrifiers can adapt to acidic pH, most of them are neutrophiles, and optimal activity is observed under neutral or slightly alkaline conditions [7, 22, 48]. Soil texture is correlated with rate of mineralization, which in turn determines the availability of substrates. Soils most favorable for methanogenesis are those that retain water, allowing for the

mineralization of organic matter in conditions of low redox potential fluctuation and relatively neutral pH (*e.g.*, swelling clays) [22].

As previously mentioned, redox potentials less than -200 mV favor methanogenesis. For example, a decrease from -200 mV to -300 mV showed an increase in methane production and emission [22]. Soils with high Fe content also favor methanogenesis due to the rapid decrease in redox potential after submersion [22]. Methanogenesis requires Ni, Co, and Cu, [3] plus a flow of carbon and electrons. However, carbon and electrons may be restricted due to competition for H_2 and C with electron acceptors such as Fe^{3+}, Mn^{4+}, SO_4^{2-}, and NO_3 [7].

Methanogenesis, sulfate reduction, and denitrification are all pathways for mineralization of the labile organic matter. The availability and the nature of the organic matter in combination with the availability of electron acceptors, determine the redox potential and the likelihood of the processes of mineralization in the sediments. Because these are biochemical reactions mediated by enzymes, they are dependent on the concentration of the substrates [7], which usually decreases with sediment depth [22]. Consequently, availability of substrates is a key issue for the occurrence of any of these processes, more so than competition for these substrates.

The processes of mineralization of organic matter have been extensively studied in environments that bear a resemblance to natural wetlands due to their

flooded conditions (*e.g.*, paddy field soils) [1, 18, 37]. In recent years, more interest has turned to the study of constructed wetlands [17, 45, 46], because of the effect of the biochemical activities of microbial communities on the nutrient cycling (biogeochemistry), and its possible role in bioremediation of polluted runoff waters [46].

Constructed Wetlands. Constructed wetlands are man-made systems that resemble natural wetland-ecosystems where resident microbial community mediates multiple nutrient transformations. These processes are used to treat wastewaters from different origins [46] at a lower cost than other alternatives [16, 19]. Constructed wetlands are often built close to agricultural fields to remove contaminants, such as fertilizer, from runoff waters, and have also been used as primary, secondary or tertiary treatment areas for municipal and industrial wastewaters [10]. Combined, these functions of constructed wetlands reduce the input of nutrients to major water bodies and thus prevent eutrophication [39].

Mechanisms driving the removal of contaminants from wastewaters include sedimentation, microbial degradation, precipitation and plant uptake [23]. Wastewater from agricultural runoff usually contains fertilizers with phosphates that can sorb to plant surfaces or sediments, or precipitate with metals at alkaline pH. The main mechanism of removal of soluble inorganic phosphates is uptake by plants [23].

Many questions arise surrounding the ecological effects of using constructed wetlands to remediate polluted waters. How can the remediation potential of a constructed wetland be established? What variables can be monitored to determine this potential? For how long must the constructed wetland be monitored? Does the bioremediation of polluted water sources become the central driving force of the ecosystem? How long should this process be sustained? What is the role of the vegetation associated with the wetland? What role has the microbial community in bioremediation?

While constructed wetlands are designed to resemble natural wetlands, there are important differences, including hydrology, substratum, and biodiversity of the system [16]. The hydrology of constructed wetlands is less variable because the water level is usually maintained through the year by external inputs. Biodiversity of constructed wetlands is lower in comparison to natural wetlands [16], where the biota is relatively self-sustainable and does not depend on artificial input of nutrients.

The difference in diversity makes constructed wetlands an interesting place to study the succession of the system from the point of origin to the establishment of specific populations [16]. The focus of this process is the transformation from a man-made or artificial system to a sustainable ecosystem, oriented towards the use of particular nutrients and contaminants, and thus preventing them from contaminating the surrounding natural environments.

Duncan and Groffman [10] recognized the necessity of understanding the biogeochemistry of constructed wetlands, microbial processes, and their relationship to environmental parameters. Research pertaining to these aspects would allow comparisons with natural wetlands to determine if constructed wetlands harbor a functionally relevant microbial community [10]. A study comparing denitrification enzyme activity, microbial biomass C content, and soil respiration between constructed and natural wetlands showed that these parameters fell within the same range of variation in both types of wetlands [10]. As a result the authors concluded that constructed wetlands have a potential for pollutant attenuation [10].

Probably due to their anaerobic conditions, natural wetlands have shown high rates of CH_4 and N_2O emissions, though low turnover rates of organic matter are common [39]. When constructed wetlands are used for purification of wastewaters, high emissions of these gases have been observed [25-27, 41] due to the increased input of nutrients and organic matter [39]. Furthermore, anaerobic conditions determine the diversity of plants that can thrive in such wetlands, contributing further to the rate of these emissions. Johansson *et al.* [19] assessed the effect of plant composition and nutrient load on methane flux in different wetlands, and found little significant difference between constructed wetlands and their natural counterparts with similar vegetation.

Plants that grow in these anaerobic environments have adapted by developing aerenchymous tissues that transport oxygen to their roots. Consequently, these plants not only release soluble organic compounds that may become substrates for methanogenesis, but their aerenchymous tissues become conduits that release the methane to the atmosphere [19]. In the same study, Johansson et al. [19] used linear regression to determine that plant composition, temperature, and water level greatly influenced the extent of CH_4 emissions in constructed wetlands.

Different approaches have been used to study methanogenesis, sulfur reduction and denitrification processes in both natural wetlands and constructed wetlands. Most of these studies are oriented toward the overall contribution to global warming rather than their involvement in bioremediation of polluted waters. Many of these approaches consider the chemical aspect of the mineralization processes by quantifying production rates [33, 34]. However, microorganisms responsible for these activities have also been examined.

Biological and Chemical Approaches for the Study of Wetlands. Recent studies have implemented culture-based methods combined with molecular techniques to study bacterial diversity [43]. Strict anaerobes, such as methanogens and sulfate-reducing bacteria (SRB), are difficult to grow. Given the advantages of conducting diversity and richness surveys of microorganisms by extracting DNA from environmental samples, a variety of molecular

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techniques have been developed. Terminal fragment length polymorphism (t-RFLP) [2, 37], Northern blots using oligonucleotide probes [12], and phospholipid fatty acid analysis (PLFA) [20, 47] are often utilized. PCR-based techniques have predominated, and the 16S rRNA gene has been the primary choice for conducting diversity studies [14, 28, 42, 45, 47].

Group-specific genes have been utilized to provide more specific probes for identification of microorganisms. Primers have been designed for each of the methyl coenzyme M reductase (MCR) subunits, such as the MCR alpha subunit codified by the *mrcA* gene of methanogens [18], and the dissimilatory (bi)sulfate reductase, *dsr* gene in SRB [1, 35, 37]. The PCR-amplified fragments are then cloned and sequenced. Successful cloning requires precision and control over the entire process, and long processing times. Therefore, innovative techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) have been implemented in microbial identification efforts [8, 21, 31, 47].

DGGE provides a relatively fast method for conducting diversity studies, which subjects the PCR-amplified sequence of interest to electrophoretic separation based on an electric field, temperature, and the presence of a denaturing reagent [31]. By using specific primers (which include a GC-clamp on one end of one primer), relatively small fragments (between 300-700 bp) of the 16S rRNA gene may be amplified, and becomes possible to detect single nucleotide differences within the gene sequence [38]. DGGE makes it possible

to survey microbial diversity at first glance, and then identify the fragments by sequencing.

Although numerous approaches other than culturing have been developed to conduct diversity studies, basic knowledge of the microbial diversity of constructed wetlands is scarce, thus limiting the availability of comparisons with natural wetlands [16]. Microbial communities involved in the pathways of mineralization of organic matter might be an excellent indicator of the functionality of wetland ecosystems under restoration, providing information about the substrates that are available and the kind of nutrient cycling that is taking place.

In a functional man-made system closely resembling a natural wetland, thriving populations of methanogens, SRB and/or denitrifiers would be expected. If so, the degradation of organic matter must have occurred to the point where the substrates for C mineralization pathways have been released to the environment and the constructed wetland becomes considered less "artificial".

At the same time, if any one of these populations of microorganisms becomes dominant during a particular season, the kind of substrates present in the environment may be inferred to a specific level, and the functional role of the wetland in nutrient transformation may be predicted. Finally, this data could be supported directly by quantifying substrates, enzymes, products, or rates of production.

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For constructed wetlands receiving runoff waters from agricultural fields, a program to monitor diversity and richness of the microbial community would be useful to address a range of questions, including the following: (a) Is the functional structure of the microbial community determined to some extent by the pollutants present in the input water? (b) How does the community change along nutrient gradients? (c) What functional groups predominate under restoration conditions?

Specific Goals of This Study

The goal of this study is to gain an insight into the microbial community inhabiting a constructed wetland as indicator of the functional status of the system by performing the following analysis:

1. Create a clone library of the 16S rRNA gene to survey the Eubacteria and Archaea diversities.

2. Determine how the community changes along a nutrient gradient generated by its interaction with the polluted runoff waters.

3. Determine whether methanogens or SRB play a dominant role in this environment.

The final goal of this research is to be able to use the microbial structural composition of this system as a reference point for monitoring the progression of the restoration program of this previously farmed environment.

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The approach combines traditional cloning methods and DGGE. The present study is composed of two stages: (i) the creation of clone libraries of the 16S rRNA gene to survey the diversity of Eubacteria and Archaea, and (ii) the use of phylogenetic analyses to infer the functional role of the different populations. Further research requires the use of group-specific primers for methanogens and SRB that amplify smaller regions within the 16S rRNA gene to determine diversity by DGGE.

Studying only a compartment of a constructed wetland allows only a glimpse of the complex ecosystem. A full understanding of the physical and chemical dynamics is not yet possible, particularly considering the multiplicity of the interactions between the biota and the environmental factors. Many other processes that take place in wetlands such as methane oxidation, nitrification, and ammonification, are not in the scope of the present study, but their importance is being acknowledged.

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CHAPTER II

Phylogenetic analysis of the diversity of Eubacteria and Archaea in the sediments of a constructed wetland in Monterey County, CA: A study of community structure and function

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Short version of the title: Diversity of Archaea and Eubacteria in a constructed wetland in CA

Abstract

Wetlands harbor a variety of microorganisms that contribute to global warming. In the anoxic compartment of wetland sediments, methanogens, sulfate-reducing bacteria (SRB), and denitrifiers compete for substrates. Most wetlands studies describe mineralization of organic matter but do not describe the microbial populations responsible. There are a few studies about microbial diversity in constructed wetlands. The present study compares microbial populations in a polluted (Site 1) and a clean site (Site 3) of a constructed wetland, more specifically to (i) survey the diversity of Eubacteria and Archaea, (ii) determine differences between the microbial communities at the two sites, and (iii) describe predominant microbial guilds present in the polluted and clean sites, focusing on methanogens and SRB. A clone library of the 16S rRNA gene was constructed, and phylogenetic analysis was performed. The archaeal community in both sites was dominated by members of the phylum Crenarchaeota, and Proteobacteria was dominant among Eubacteria. Only a small portion of these communities were methanogens and SRB, and phylogeny indicated that novel groups of both may be present in this wetland. The microbial community in Site 1 appeared to be more diverse than the community in the downstream clean site, suggesting selection by pollutants. Sequence analysis demonstrated differences between the microbial populations at each site. Non-acetoclastic methanogenesis appeared to be the dominant pathway for methanogenesis in this wetland, indicating acetate could be produced via

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hydrogenotrophic methanogenesis. Families primarily responsible for the hydrogenotrophic methanogenesis were identified to be Methanobacteriaceae and Methanomicrobiaceae. Site 3 SRB from the phylum Deltaproteobacteria were either complete acetate oxidizers or incomplete oxidizers from the order Desulfobacterales. This finding suggested that at Site 3 competition for acetate as substrate for sulfate-reduction and methanogenesis may explain the absence of acetoclastic methanogenic Archaea.

Introduction

Metabolic activities of microorganisms in wetlands contribute to global warming through their role in geochemical cycles of carbon, nitrogen, and sulfur. Dissimilatory metabolism of organic carbon substrates, such as acetate, result in the production of methane. Methane emissions from wetlands comprise about 30% of atmospheric methane, and are estimated to be between 110 and 260 Tg year^{-1} (Tg = 10^{12} g) [14, 20]. Atmospheric methane adds 20% to the greenhouse effect of carbon and nitrogen dioxide (CO_2 and N_2O), justifying concern about this greenhouse gas and its sources [12].

Accumulation of a water column over a soil substrate, oxygen depletion, and decrease in redox potential with depth make wetlands suitable environments for methanogenic Archaea [5, 28, 46]. In the anoxic compartment of wetland sediments, methanogenesis, sulfate-reduction, and denitrification can occur [12]. Denitrifiers, SRB, and methanogens compete for substrates as electron donors in

anaerobic respiration [13, 39, 30]. Environmental factors intrinsic to wetland ecosystems influence which of these metabolic pathways predominate.

Methanogenesis by Archaea of the phylum Euryarchaeota is the sole biological source (70-80%) of atmospheric methane (1.5 to 1.7 ppm) [28, 39, 54] [14, 20]. Methane production relies on a syntrophic association between different metabolic groups of microorganisms [14]. Organic matter is first decomposed by hydrolytic and fermentative bacteria. Under low redox potentials (Eh < -200 mV) the end products of this degradation are utilized for methanogenesis [28]. Two thirds of this methane is produced by the acetate fermentation pathway (acetoclastic methanogenesis), and the remaining 1/3 comes from the carbon dioxide reduction pathway (hydrogenotrophic methanogenesis) [12, 14, 28].

Mineralization pathways of organic matter have been extensively studied in environments such as rice paddy fields, which bear a resemblance to natural wetlands because of their flooded conditions [3, 20, 40]. In recent years, more interest has turned to the study of constructed wetlands [19, 48, 49], due to the role of microbial communities in bioremediation of polluted runoff waters [49].

Numerous culture-independent approaches have been used to study microbial diversity. However, comparative research on microbial communities in natural and constructed wetlands is scarce [17]. Identifying the microbial communities involved in mineralization of organic matter could lead to predicting wetland restoration success. If constructed wetlands play a role in

bioremediation of polluted waters, these systems should eventually resemble natural wetlands. If true, populations of methanogens, SRB and/or denitrifiers should thrive in constructed wetland sediments.

The present study was conducted to gain insight into the microbial communities inhabiting the Sea Mist constructed wetland located in Monterey, CA. This wetland receives fertilizer- and pesticide-polluted runoff water from adjacent agricultural fields. The purpose of this study was to compare two sites of this wetland in terms of: (i) Eubacteria and Archaea diversities, (ii) differences between the microbial communities at each site, and (iii) the metabolic pathways utilized by methanogens and SRB under disturbed and restored conditions.

The information about microbial communities in this study will contribute to understanding the microbial pathways for mineralizing organic matter during bioremediation of polluted waters, and thus the overall contribution to geochemical cycles and global warming.

Methods

Study Site and Samples Collection. The study site is on the Sea Mist property, a former agricultural field, part of which was transformed to a restoration wetland. The wetland is located adjacent to the Monterey Bay, California, USA (Fig. 1, Lat: 36.795230 N Long: 121.763772 W). It was constructed in 2006 as part of a restoration program led by the Elkhorn Slough

Foundation (http://www.elkhornslough.org), in order to reduce the pollution in the runoff waters from the surrounding agricultural fields.
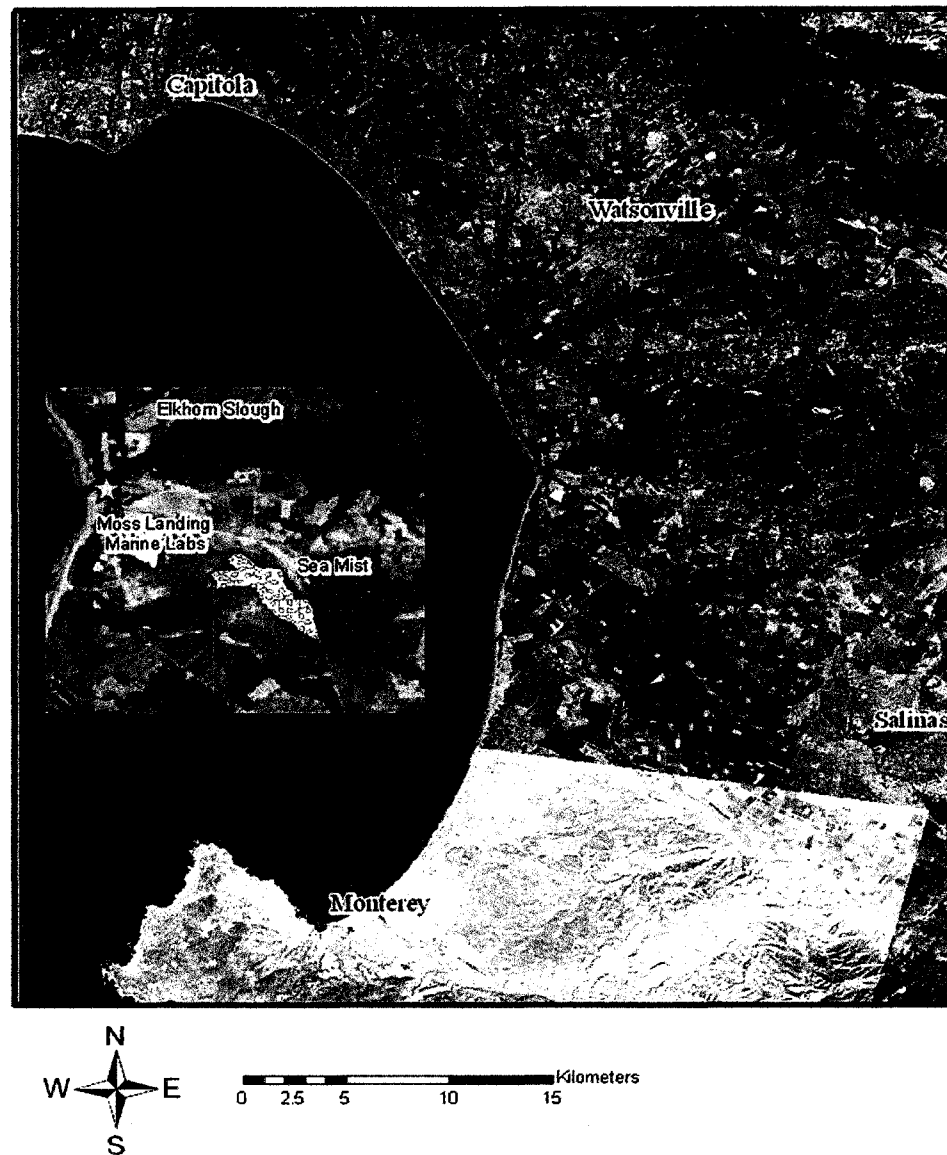
Figure 1. Geographic location of the Sea Mist constructed wetland in the Monterey County, CA. Extracted from the National Map Seamless Server (USGS) based on NASA's LANDSAT imagery. Courtesy of Leonardo Hernandez.

This constructed wetland was installed to reduce contaminants flowing to Monterey Bay through the adjacent Moro Cojo Slough. Moro Cojo Slough is a brackish estuarine system that has gone through extensive modification since the 1800's, and now is being contaminated with pesticides, excess sediments, and nutrients from the surrounding fields (Greening J. 2007. A baseline study of soil characteristics and vegetation at the Sea Mist wetland along Moro Cojo Slough [Bachelor's thesis]. [Monterey (CA)]: California State University, Monterey Bay).

The Sea Mist wetland has an area of 4.85 ha. A pump transports runoff water from the adjacent agricultural fields to a water channel leading to a small pond (Fig. 2). The water then flows through a channel to a second pond located about 100 m away. Core sediment samples were collected 2 m downstream from the outlet of the pump (Site 1). This is considered the most disturbed site of the wetland. Core samples were also collected 1 m from the shore of the second pond (Site 3) which is the least disturbed site because microbial processes have removed contaminants from the runoff water.

In July 2007, six cores comprising the first 10 cm of sediment were obtained using a sterile polypropylene coring device. At each site, three replica core samples were collected at least 0.5 m apart. The cores were immediately saved in sterile sample bags (18 oz, Nasco Whirl-pak®, Fort Atkinson, WI), and kept on ice during transport to the laboratory (within four hours to prevent DNA degradation).
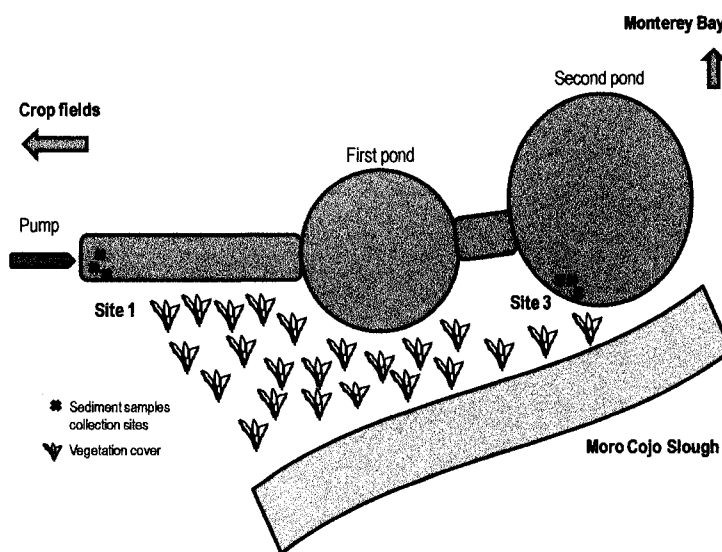
Figure 2. Diagram illustrating the collection sites on the Sea Mist constructed wetland

At the time of collection, environmental variables such as temperature of the water column, salinity, dissolved oxygen, and pH were measured with an YSI 556 multiprobe (Forestry suppliers Inc, Jackson, MS) at the chosen sites. In March of 2008 the same variables were measured, and a particle size analyzer (Beckman Coulter, Fullerton, CA) was used to determine the mean particle size of the sediments. In 2008 the measurements were not performed at the beginning of the dry season as in 2007, but even so they were considered as point of reference. Nitrate (NO_3^-) and PO_4^{3-} were measured with an Alpkem series 300 Rapid Flow Analyzer (OI Corporation, College Station, TX). NO_2^- and NH_3^- were manually analyzed with an Ocean Optics USB 200 Spectrometer (Ocean Optics, Dunedin, Fl).

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DNA Extraction. Duplicate samples consisting of 1 gm of sediment were obtained from each field sample. Community genomic DNA was extracted from each sample using the UltraClean™ Soil DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. In some cases slightly longer centrifugation times were required to obtain more supernatant. The quality of the genomic DNA was checked by electrophoresis on 0.8 % analytical grade agarose gels stained with ethidium bromide (0.5 μg/ml). The bands were visualized using a GelDoc ™ XT 170-8171 (BioRad Laboratories, Hercules, CA). The quantity and purity of the extracted DNA was determined on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). All samples were sheared 30 times using 16G needles and diluted 10 fold.

16S rRNA Amplification by PCR. Primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, Al). Near full-length eubacterial rDNA (1500 bp) was amplified using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') designed for most Eubacteria [53]. The PCR reaction mixture consisted of 1.5 mM MgCl$_2$, 250 μM of each dNTP, 1 μM of forward and reverse primers, 2 μl of template DNA (5-136 ng μl^{-1} optimized for each reaction), 2.5 U of GoTaq® DNA polymerase and 1X GoTaq® reaction buffer (Promega, Madison, WI), in a 20 μl final reaction volume. Purified *E. coli* DH5-α (100 ng) genomic DNA was obtained following instructions by QIAGEN Genomic DNA Isolation Kit (Valencia, CA) and was used as the positive control.

Forward primer A109f (5'-ACKGCTCAGTAACACGT-3') and reverse primer A934b (5'-GTGCTCCCCGCCAATTCCT-3') [18, 42] were used to amplify an 825 bp fragment of rDNA from Archaea 16S rRNA genes. The PCR reaction mixture consisted of 20 nmol each dNTP, 30 pmol each primer, 10 µg/µl BSA, 2 µl template DNA (5-136 ng µl^{-1}, optimized for each reaction), 2.5 U GoTaq$^®$ DNA polymerase, and 1X GoTaq$^®$ reaction buffer, in a 100 µl final reaction volume. The positive control for PCR was *Methanosarcina mazei* (ATCC$^®$ BAA-159D^{TM}, Manassas, VA) genomic DNA (50 ng in 1 µl).

Amplification of the DNA fragments was performed on a MJ Research PTC 100 thermal cycler (Global Medical Instrumentation Inc, Ramsey, MN). For PCR of eubacterial DNA, samples were denatured at 95°C for 2 min, before 35 PCR cycles (95°C for 15 s, 55°C for 30 s, 72°C for 2 min), and a final incubation at 72°C for 6 min. For PCR of archaeal DNA, samples were denatured at 94°C for 5 min, before 38 cycles (94°C for 1 min, 52°C for 1 min, 72°C for 90 s), and a final incubation at 72°C for 6 min. PCR products were electrophoresed on 1 % analytical grade agarose gels, stained with ethidium bromide (0.5 µg ml^{-1}), and visualized (GelDoc ^{TM} XT 170-8171).

Cloning and Sequencing. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN), and quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific). Amplification products were then cloned into pGEM$^®$-T Easy plasmid vectors (Promega), according to manufacturer's instructions and

used to transform competent JM109 *E. coli* cells. Plasmids were purified using a Wizard Plus SV Minipreps kit (Promega). The isolated plasmids were stored at -20°C for further applications, such as DNA quantification. Cloned plasmid DNA was screened by restriction analysis using *Eco*RI (12 u µl⁻¹). Plasmids containing 1.5 Kb fragments of eubacterial DNA, and plasmids containing 825 bp fragments of archaeal DNA were sequenced by Sequetech (Mountain View, CA). Forward primer M13F was used for unidirectional sequencing of 1000 nucleotides.

Phylogenetic Analyses. Sequences were visually screened for deficiencies using BioEdit 7.0.9 [21] and edited with the help of Vector Screening (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to remove the unnecessary fragments. Chimera Detection (http://35.8.164.52/html/index.html) [9] and Bellerophon Chimera Detection (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) [23] programs were utilized to determine the presence of chimerical sequences. Only one sequence was eliminated as a result of these analyses.

Tools from the NCBI and the Ribosomal Database Project (RDP) release 10 were utilized to determine the taxonomic affiliation of each member of the clone library. Closest relatives to the clones were chosen using the Basic Local Alignment Search Tool (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and sequences from GenBank were selected based on the following criteria in the same order of relevance: sequence identity ≥ 97%, highest percentage of sequence coverage, *E* value = 0, and highest score assigned. RDP's Classifier

(http://rdp.cme.msu.edu/classifier/classifier.jsp) [51] was used with the preset

bootstrap confidence estimate (80%) to determine the hierarchical taxonomic

affiliation of each of the clones.

Seven hundred and forty-nine base pairs of the Archaea sequences, and

680 bp of the Eubacteria sequences were aligned with their closest relatives from

GenBank using ClustalX version 1.83 [8, 45]. Phylogenetic trees were initially

constructed using MEGA 4.0 [44] by Neighbor Joining (NJ), with the Kimura two-

parameter model to compute the evolutionary distances. The robustness of

nodes previously determined by bootstrap (1000 replicas) was improved based

on the best nucleotide substitution model selected using Modeltest 3.8

(http://darwin.uvigo.es/software/modeltest_server.html) [33] according to AIC

criterion, and PAUP 4.0 beta 10 win [43]. The Tamura-Nei equal transversion

frequencies, gamma distributed variations (TrNef+G) model was selected using

the hierarchical likelihood ratio tests (hLRTs). Branch-support probabilities were

inferred by Bayesian analysis using MrBayes version 3.1.2 [24, 37], and four-

chain metropolis-coupled Markow-chain Monte-Carlo (MCMCMC) analysis [1].

Methanogens consensus tree searches were run for 2,000,000 generations (until

chains converged), sampling every 1000 generations. Sampling began at

generation 500 by which time parameters had reached equilibrium. Posterior

probabilities were based on 1500 trees, and posterior probability values ≥ 0.80

are considered significant. A similar procedure was followed to create the

phylogenetic tree for Deltaproteobacteria, except that tree searches were run for

500,000 generations, sampling every 100 generation. Sampling began at generation 1000 by which time parameters had reached equilibrium. The Tamura-Nei, gamma distributed variations (TrN+G) model was selected using the hierarchical likelihood ratio tests (hLRTs). Trees were visualized using Treeview 1.6.6 [32].

Diversity Analyses. Rarefaction curves were obtained using Analytic Rarefaction 1.3 by Steven Holland (http://www.uga.edu/~strata/software/), to represent the richness of the microbial community and determine the degree of species saturation. The Shannon-Wiener function was utilized to estimate diversity of Archaea and Eubacteria at both sites according to:

$$H = - \sum_{i=1}^{s} (Pi)(LogPi)$$

H = information content of the sample (bits/individual) or index of species diversity, *S* = number of species, and *pi* = proportion of total sample belonging to the *i* th species [27].

Results

Wetland Physicochemistry. The most significant chemical difference between collection sites 1 and 3 is the NO_3^- concentration (Table 1).

Table 1. Physicochemical values of water samples from two sites (1 and 3) of Sea Mist wetland taken at two time points (2007 and 2008).

Physicochemical value	2007		2008	
	Site		Site	
	1	3	1	3
Temperature (°C)	19.25	27.20	12.90	17.84
DO_2 (mg/L)	10.46	14.93	> 17.00	8.88
Salinity (mg/L)	1.44	7.77	1.76	4.3
pH	7.84	9.66	7.50	9.52
NO_3^- (mg/L)	78.8	6.9	194.5	4.3
NO_2^- (mg/L)	0.64	0.01	5.76	0.0
NH_3^- (mg/L)	0.04	0.50	------	------
PO_4^{3-} (mg/L)	0.09	0.45	------	------
Mean particle size (μm)	------	------	38.74	12.21

In 2007 the concentration of NO_3^- in Site 1 was 11 times higher than in Site 3, whereas in 2008 it was about 50 times higher (Table 1). The NO_2^- concentration was lower at Site 1 than Site 3 for both years and the concentration of NO_2^- was much lower than NO_3^-.

Measures of NH_3^- and PO_4^{3-} were not obtained the second year, but the 2007 values were higher at Site 3 compared to Site 1. The water temperature, salinity and pH were also different between the two sites (Table 1). In 2007 there was a temperature difference of about 10°C at the time of collection (mid-day).

The 2008 difference was lower. Site 3 was about two pH units above that of Site 1 in both years, displacing the pH conditions from neutrality to alkalinity. Salinity was also higher at Site 3 in both years. Dissolved oxygen (DO_2) measurements showed no trend. Finally, the mean particle size of the sediments was three times smaller in Site 3.

Archaeal 16S rRNA Gene Sequences. GenBank BLAST search results using a criterion of ≥ 97% similarity suggested that 23 of 135 (17%) clones from Site 1 matched an "uncultured bacterium" instead of an "archaeon". These 23 sequences were not considered in subsequent analyses, which resulted in 111 clones in the first library. The clones left in the library were related to a phylum of Archaea based on data from GenBank (Fig. 3). Using the criterion of > 97% similarity, 47% were Crenarchaea, 7 % were unidentified, and 5% were Euryarchaeota. Almost half (41%) of 111 clones were only identified to the level of "uncultured archaeon", therefore these sequences were considered unidentified.

The Classifier tool from RDP largely confirmed these results. This tool relies on a naïve Bayesian rRNA classifier to provide a bootstrap confidence estimate for 100 trials. It was preset to an 80% threshold of confidence in the genus assignment and all taxonomic categories are displayed. Very few clones were identified to the level of genus with 95% bootstrap confidence. Therefore, higher order assignments were only made to the level of phylum.
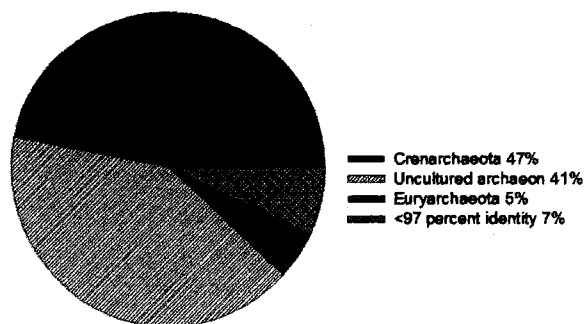
Figure 3. Phylum identity of 111 sequences obtained from Site 1 using GenBank (≥97% similarity).

RDP Classifier analysis showed results relatively comparable to GenBank

(Fig. 4). Most sequences were assigned to the phylum Crenarchaeota, and only

19% of them were assigned to a particular phylum with less than 80% bootstrap

confidence. Slightly more sequences (2%) were assigned to the phylum

Euryarchaeota, which includes methanogenic Archaea.
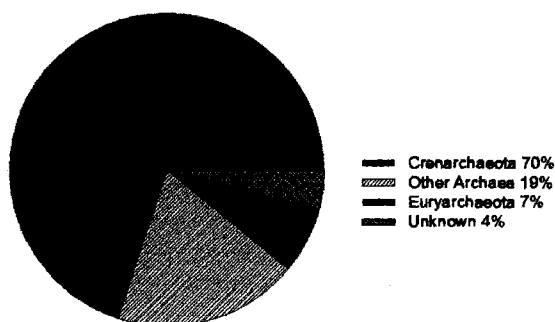
Figure 4. Phylum identity of 111 sequences obtained from Site 1 using the naïve Bayesian rRNA Classifier (RDP).

Of the 145 sequences from Site 3 none matched a GenBank "bacterium". BLAST results indicated that 78% of the sequences from Site 3 were "uncultured archaeon", and only 2% were not identified to the species level using the ≥ 97% identity criterion (Fig. 5). According to the RDP Classifier only one of 145 clones was assigned to the domain Eubacteria, and 53% of the 144 Archaea sequences in this library belong to the phylum Crenarchaeota (Fig. 6).
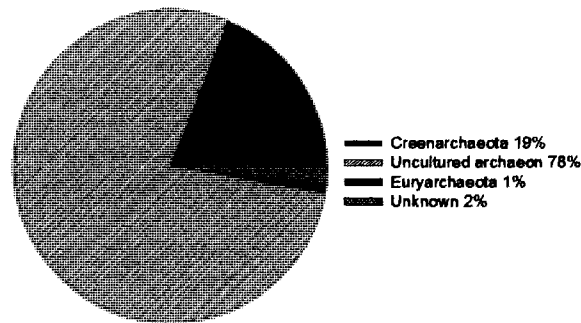
Figure 5. Phylum identity of 145 sequences obtained from Site3 using GenBank (≥97% similarity).

In comparison to GenBank, RDP Classifier analysis showed that Crenarchaeota increased from 19% to 53%, unclassified Archaea decreased from 78% to 41%, and Euryarchaeota increased from 1% to 4%. Fewer sequences were assigned to the phylum Euryarchaeota compared to Site 1.
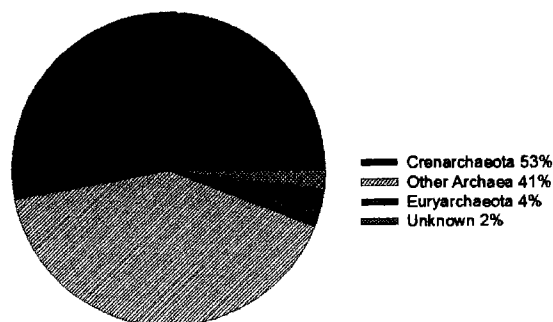
Figure 6. Phylum identity of 144 sequences obtained from Site 3 using the naïve Bayesian rRNA Classifier (RDP).

Eubacterial 16S rRNA Gene Sequences. The similarity search for the closest relatives in GenBank to the 124 clones from Site 1 did not provide many matches using ≥ 97% similarity. Fifty-five percent (55%) of the matches had less than 97% similarity to the clones, and 29% of the clones were identified only as an "uncultured bacterium".

According to the RDP Classifier almost half of the sequences (44%) were assigned to the phylum Proteobacteria (Fig. 7A), 16% to Acidobacteria, and 8% each to the phyla Verrucomicrobia and Gemmatimonadetes. The subphylum Betaproteobacteria (33%) predominated among the Proteobacteria, whereas Deltaproteobacteria had the smallest representation within this phylum (11%) (Fig. 7B). Only 4 clones of 128 in this library were Archaea.

Samples from Site 3 showed a similar picture. Forty six percent of 116 sequences had less than 97% similarity to sequences in GenBank, and 22%

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were similar to an "uncultured bacterium". However, as many as 19% were a match to some archaeal clone, as also predicted by the RDP Classifier. Therefore, the remaining 94 sequences were assigned to different phyla using the Classifier (Fig. 8).

Proteobacteria was the predominant phylum, assigned to 63% of the sequences in this library, followed by Gemmatimonadetes at 9% (Fig. 8A). Among the Proteobacteria, Betaproteobacteria sequences predominated (36%), followed by Deltaproteobacteria (30%), and Gammaproteobacteria (14%) (Fig. 8B). Since there was agreement between GenBank and RDP data, and the RDP Classifier provided more detail, only RDP Classifier results are presented here.

A

- Proteobacteria 46%
- Acidobacteria 17%
- Verrucomicrobia 8%
- Gemmatimonadetes 8%
- Planctomycetes 3%
- Actinobacteria 2%
- Firmicutes 2%
- Cyanobacteria 1%
- Spirochaetes 1%
- Unidentified Eubacteria 12%

B

- Betaproteobacteria 33%
- Alphaproteobacteria 28%
- Gammaproteobacteria 28%
- Deltaproteobacteria 11%
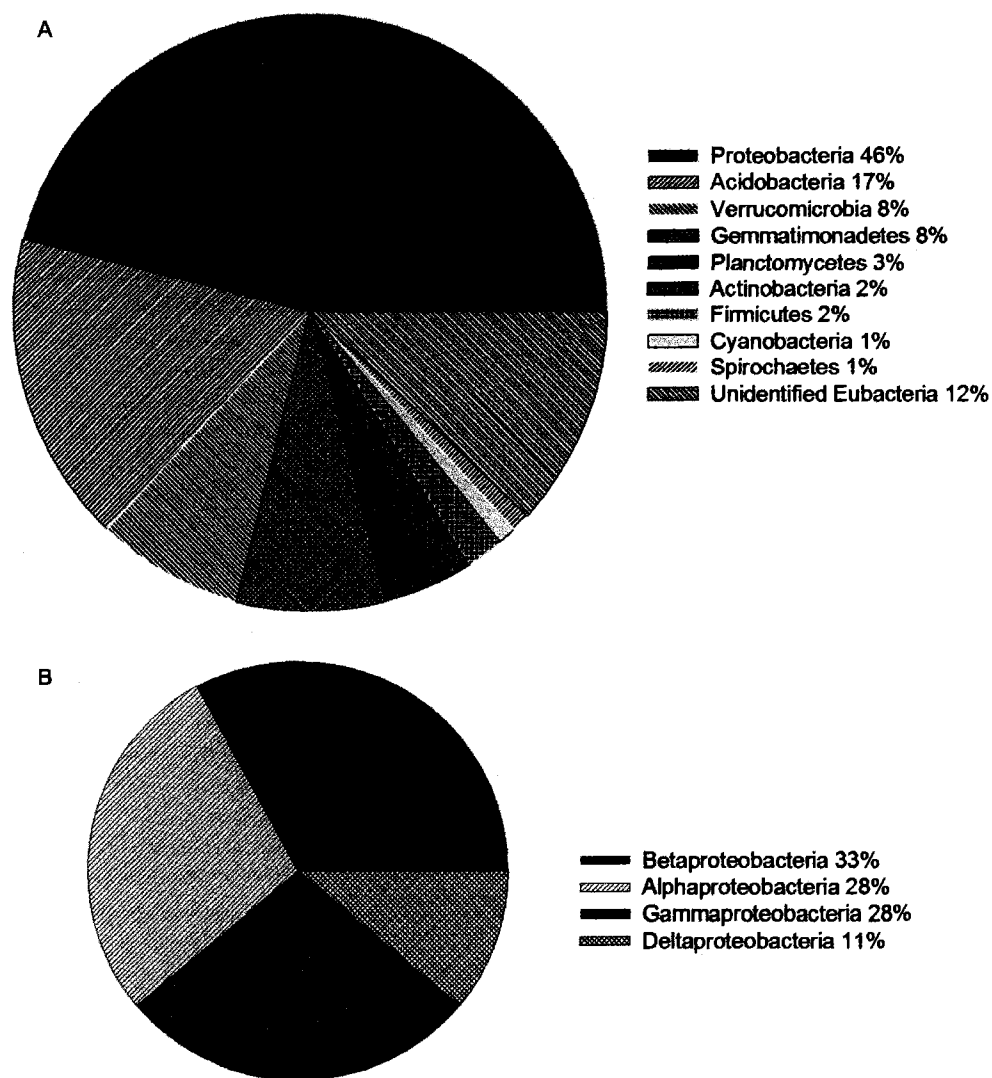
Figure 7. **(A)** Phylum identity of 124 sequences obtained from Site 1 using the naïve Bayesian rRNA Classifier (RDP), **(B)** The percent distribution of subphyla among the 57 Proteobacteria sequences from Site 1.
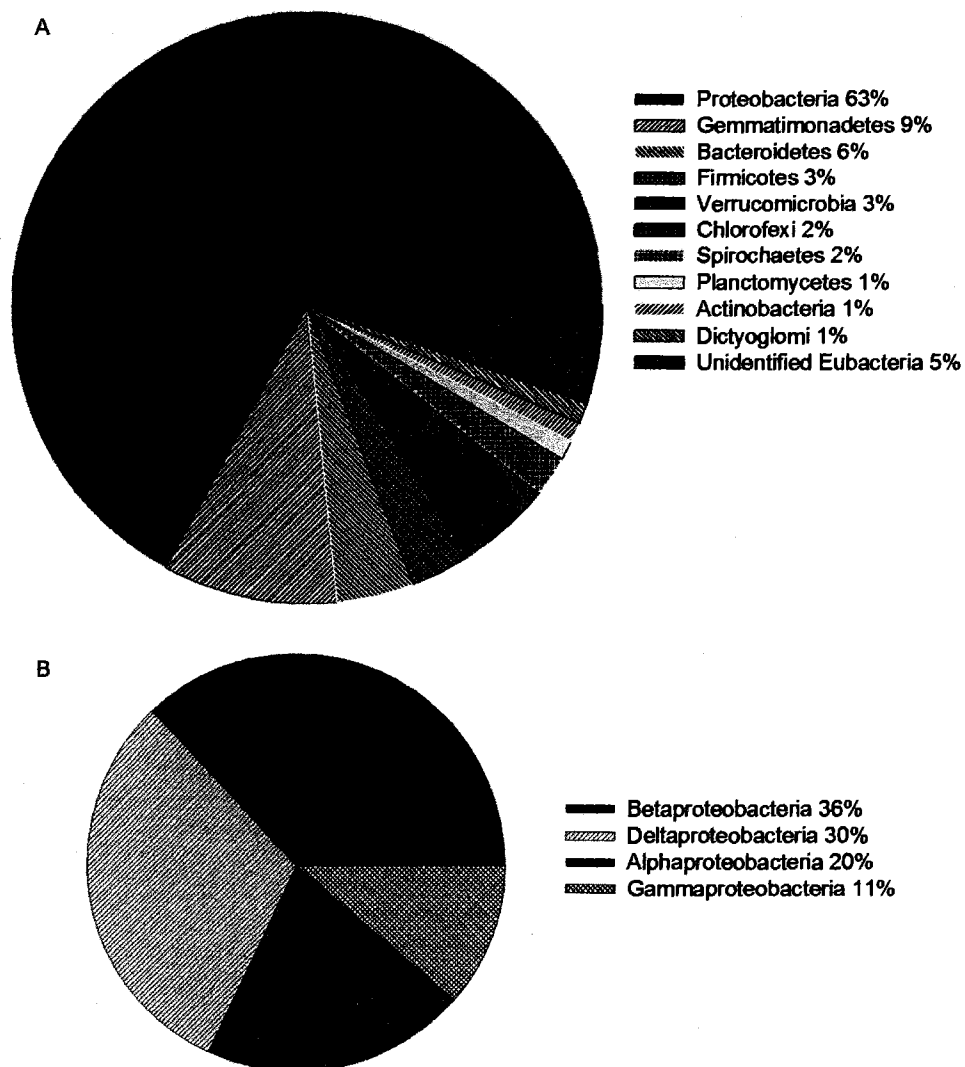
Figure 8. (A) Phylum identity of 94 sequences obtained from Site 3 using the naïve Bayesian rRNA Classifier (RDP), **(B)** The percent distribution of subphyla among the 59 Proteobacteria sequences from Site 3.

Diversity Analyses. The 245 Archaea sequences isolated from both sites were identified by matches to GenBank (Fig. 9). Not all of the GenBank sequences were present at both sites.

Different sequences dominated each site. For instance, 43 clones from Site 1 were similar to the sequence "Crenarcheote clone A172" from GenBank, but only 17 were similar to it in Site 3. The second dominant sequence in Site 1, "Archaeon Elev_16S_arch_944", is represented by 13 clones only. At Site 3 the sequence "uncultured archaeon clone archaea_19" was dominant, with 42 similar clones, but the same sequence is underrepresented in Site 1. Two other sequences, "Archaeon CAP3-44" and "Archaeon SCA1175" were co-dominant in Site 3 and represented a minor portion of the Site 1 community.

Although most of these GenBank clones were not associated with a particular phylum, the RDP Classifier analysis showed that different Crenarchaea populations thrive in each site, dominating to different extent the archaeal community. A more detailed identification of these sequences obtained using the RDP Classifier was not within the accepted estimate of confidence for taxonomic categories other than phylum.
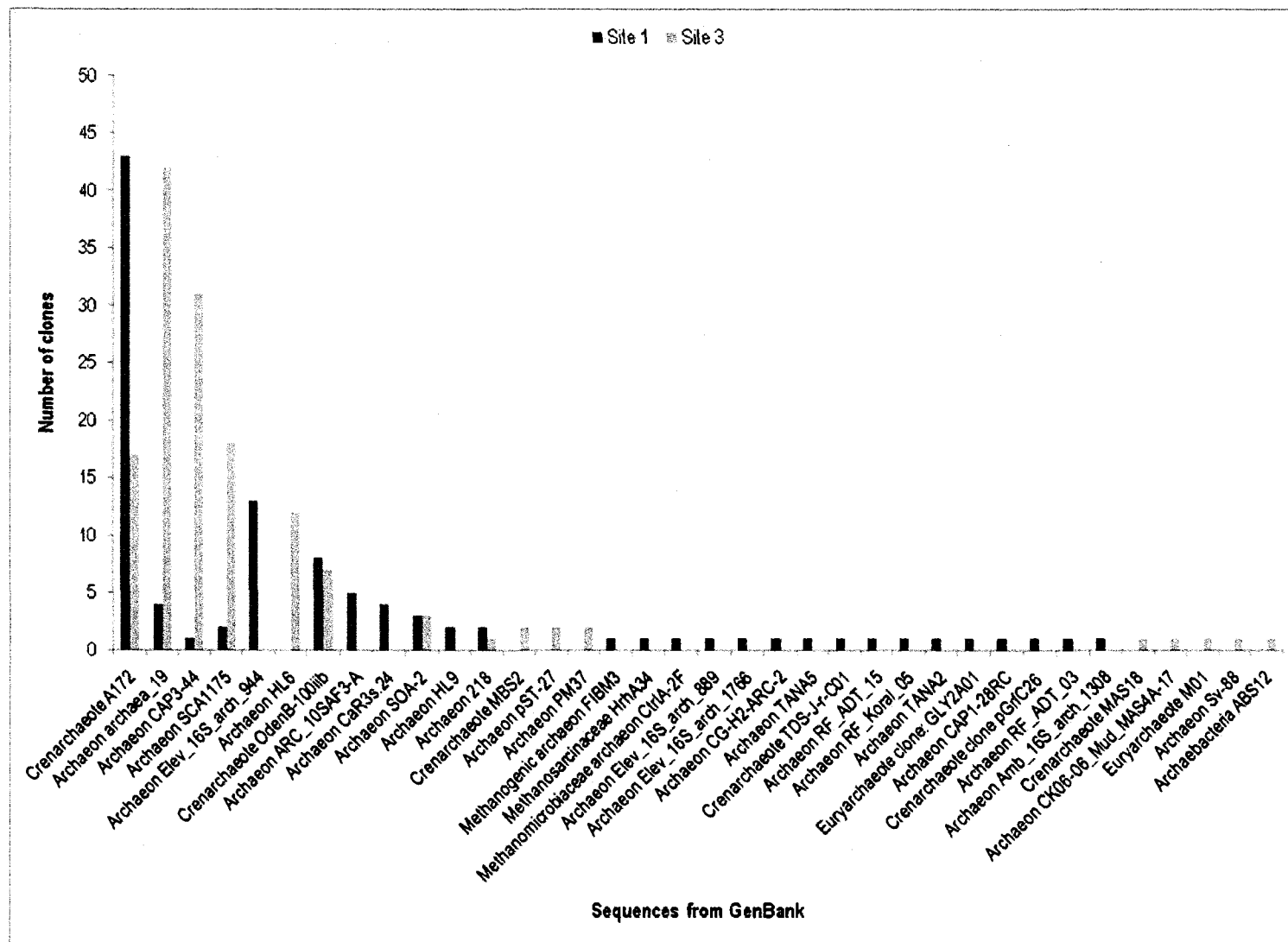
Figure 9. Abundance of Archaea sequences identified by GenBank (≥ 97% similarity) for Site 1 and Site 3 (GenBank names shortened to fit available space).

Analysis of Eubacteria libraries using the RDP Classifier assigned 72% of the clones from Site 1 and 66% of the clones from Site 3 to different phyla and different families, with 80% bootstrap confidence (Fig. 10).

A few families dominated each of the sites. Each of these had fewer clones than the dominant archaeal groups. Acidobacteriacea (Phylum Acidobacteria), and Xantomonadaceae (Gammaproteobacteria) predominated in Site 1. Families Comamonadaceae (Betaproteobacteria), Gemmatimonadaceae (Gemmatimonadetes) and Xantomonadaceae (Gammaproteobacteria) were dominant in Site 3. The family *Desulfuromonadaceae* (Deltaproteobacteria) was more abundant at Site 3 than Site 1.

Some families were unique for each site. Cystobacteraceae, Caldilineaceae, Desulfobacteraceae, Hydrogenophilaceae, Opitutaceae, Mycrobacteriaceae, Clostridiaceae, Geobacteraceae, Clostriales Family XIII Incertae Sedis, Shewanellaceae, and Polyanginaceae were unique to Site 3. Neisseriaceae, Caulobacteraceae, Phyllobacteriaceae, Rubrobacteraceae and Verrucomicrobia Subdivision 3 were unique to Site 1.
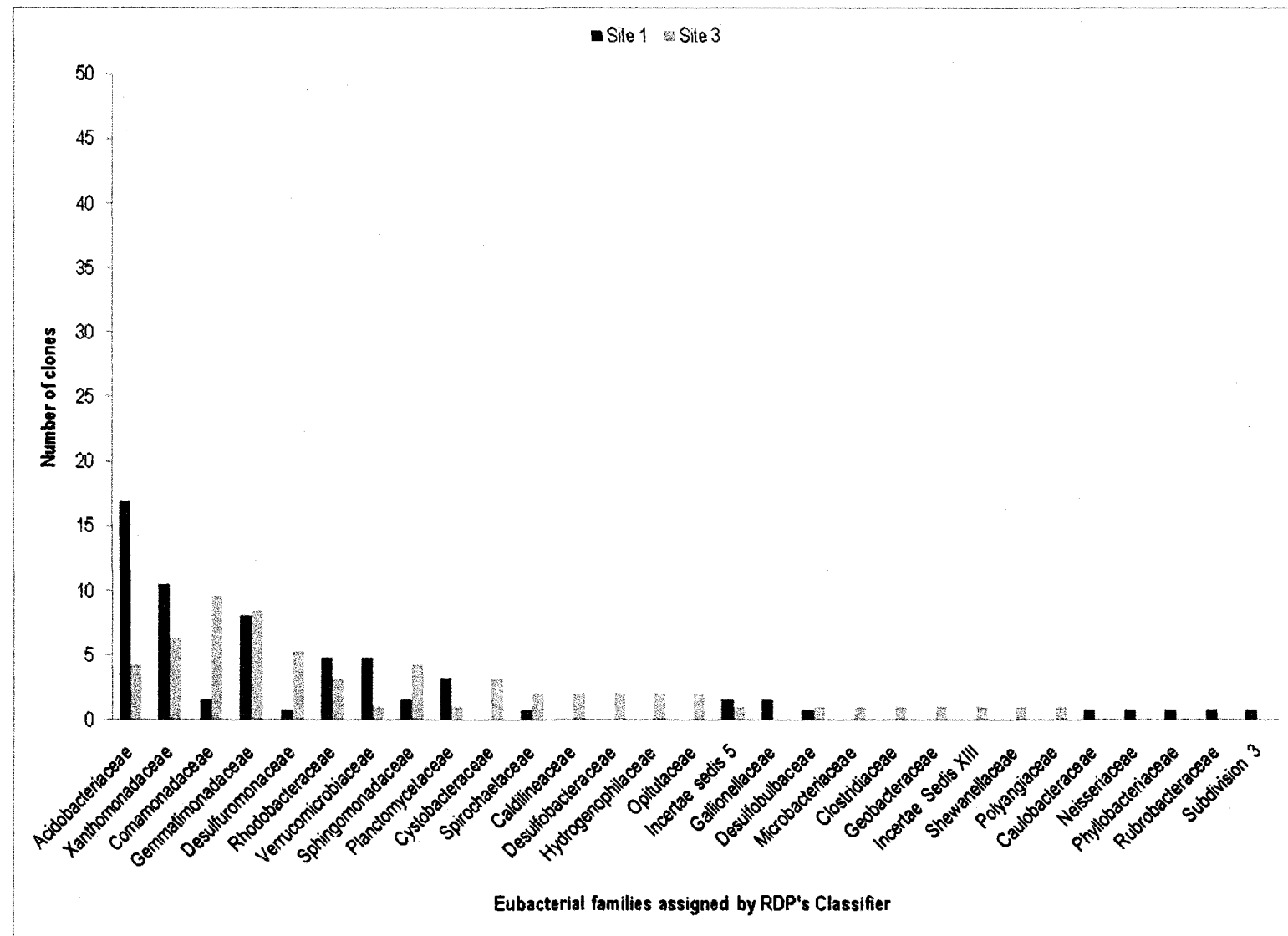
Figure 10. Abundance of Eubacteria families at Site1 and Site 3 assigned by the RDP Classifier with a bootstrap estimate of confidence ≥ 80.

Microbial diversity at both sites was also analyzed by rarefaction curves

(Fig. 11 and Fig. 12). Rarefaction is a standardization technique that permits

comparisons between samples with different numbers of individuals, to estimate

their species richness. This technique also allows the estimation of the size of

the sample that would represent the total number of species.

Analyses of both all Archaea sequences, and Archaea sequences that

met the ≥97% similarity criterion are presented for Site 1 (Fig. 11A), and Site 3

(Fig. 11B). The slopes of the curves from Site 3 indicated that approximately 140

clones adequately represent the archaeal species, while species richness at Site

1 will require a larger sample.

Analyses of both all Eubacteria sequences, and Eubacteria sequences

that met the ≥97% similarity criterion are presented for Site 1 (Fig. 12A), and Site

3 (Fig. 12B). In both cases curves indicated that a larger sample would be

needed to capture the sequence richness.
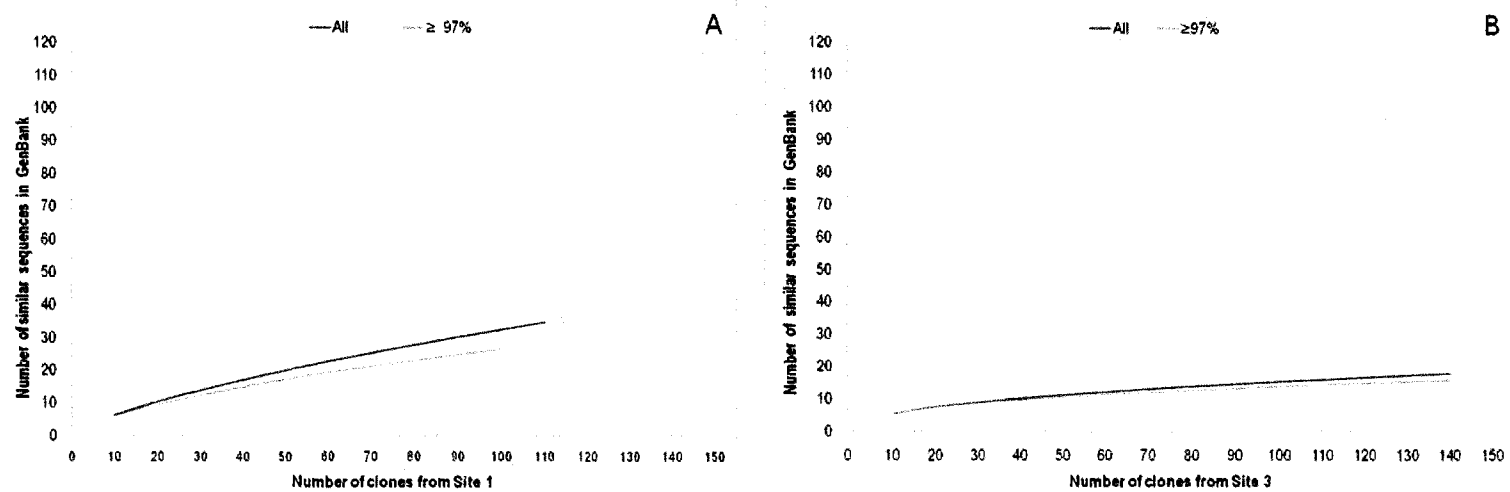
Figure 11. All Archaea sequences and Archaea sequences that met the ≥97% similarity criterion for Site 1 (Fig. 11A) and Site 3 (Fig. 11B).
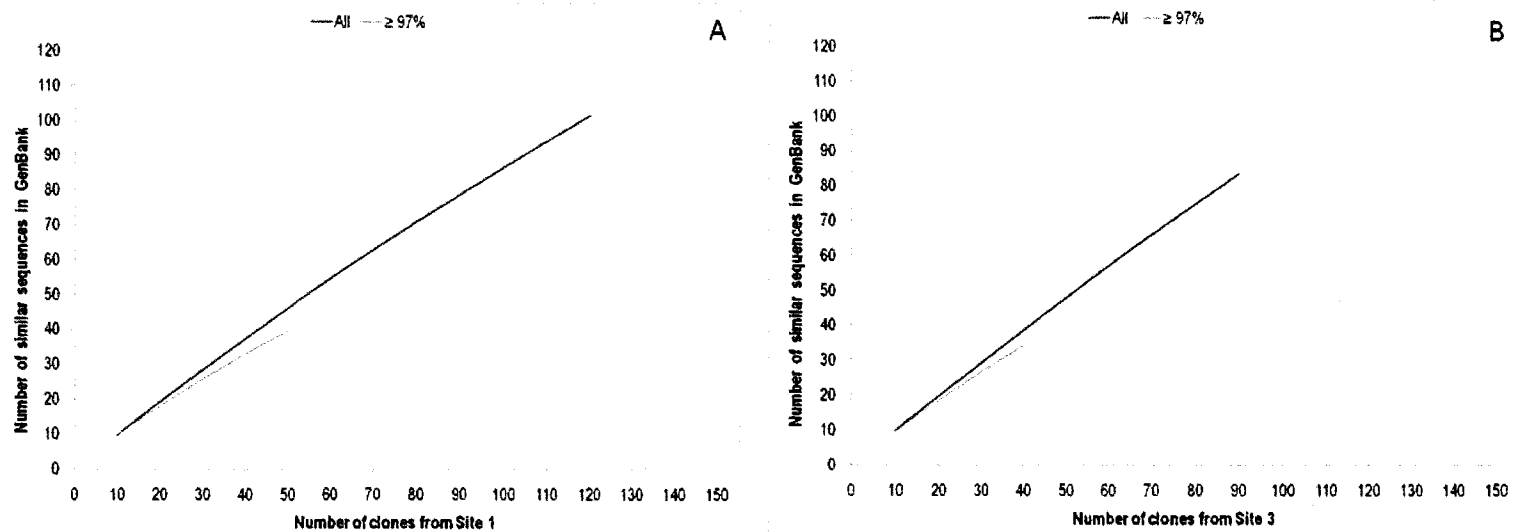
Figure 12. All Eubacteria sequences and Eubacteria sequences that met the ≥97% similarity criterion for Site 1 (Fig. 12A) and Site 3 (Fig. 12B).

To quantitatively determine the diversity of the sequences found in GenBank, the Shannon-Wiener function (H) was also used. This function combines the two components considered to measure species diversity in ecology: number of species and evenness of individuals among species [27]. This function is based on a premise borrowed from information theory. According to this premise information content is a measure of the amount of the uncertainty of correctly predicting what the next species would be if randomly drawn from a sample [27]. When H is closer to 0, the uncertainty of correctly predicting the next species decreases, therefore the lower the diversity of the sample.

This analysis was performed with the sequences from GenBank at ≥ 97% similarity threshold. The index of species diversity (H) or information content of the sample (bits/individual) increases with species diversity. The index was $H =$ 1.008 for the Archaea clones from Site 1, and $H = 0.084$ for the Archaea clones from Site 3. In the case of the Eubacteria libraries, in Site 1 the index was $H =$ 1.587, while in Site 3 it was $H = 1.506$. Based on the data obtained the microbial community in Site 1 is more diverse than the microbial community in Site 3.

Phylogenetic Analyses of Methanogens and Sulfate-reducing Bacteria. Sequences that were related to methanogens or SRB, and their correspondent hits (≥ 97% similarity) from the BLAST analysis, were aligned with sequences from known isolates deposited in GenBank. To choose the 16S rRNA gene

sequences of the known isolates the possible ID assigned by the RDP's

Classifier was used as a guideline. Two different phylogenetic trees were

constructed, one for Archaea and one for Eubacteria, in order to determine likely

evolutionary relationships of the sequences and identify them.

Little was known about the sequences that appeared to be related to

methanogens, except that they were members of different orders and families

according to the RDP Classifier. Therefore, 16S rDNA sequences of known

methanogens from GenBank were included in the phylogenetic tree for reference

(Fig. 13).

The majority of the sequences from Site 1 were grouped with the two

major families of methanogens: Methanomicrobiaceae from the order

Methanomicrobiales, and Methanosarcinaceae from the order

Methanosarcinales. Most of the sequences from Site 3 belonged to the family

Methanobacteriaceae, from the order Methanobacteriales. A cluster of clones

that were not closely related to any group in the tree was also observed. The

only known sequence associated with this cluster was that of *Methanoculleus*

marisnigri JR1 (gil126177952:1822651-1824115) from the family

Methanomicrobiaceae.

The phylogenetic tree constructed with the sequences identified as

Deltaproteobacteria included only one hit from GenBank (Fig. 14). Sequences

were mainly related to either SRB or to sulfur-reducing bacteria, rather than to

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any other group of Deltaproteobacteria. The only exceptions to this grouping are the sequences affiliated with the order Myxococcales.

The phylogenetic tree for Deltaproteobacteria (Fig. 14) also shows sequences from both sites that formed a separate cluster, with the highest posterior probability support. Most of the Deltaproteobacteria sequences in Site 1 are included in this cluster.
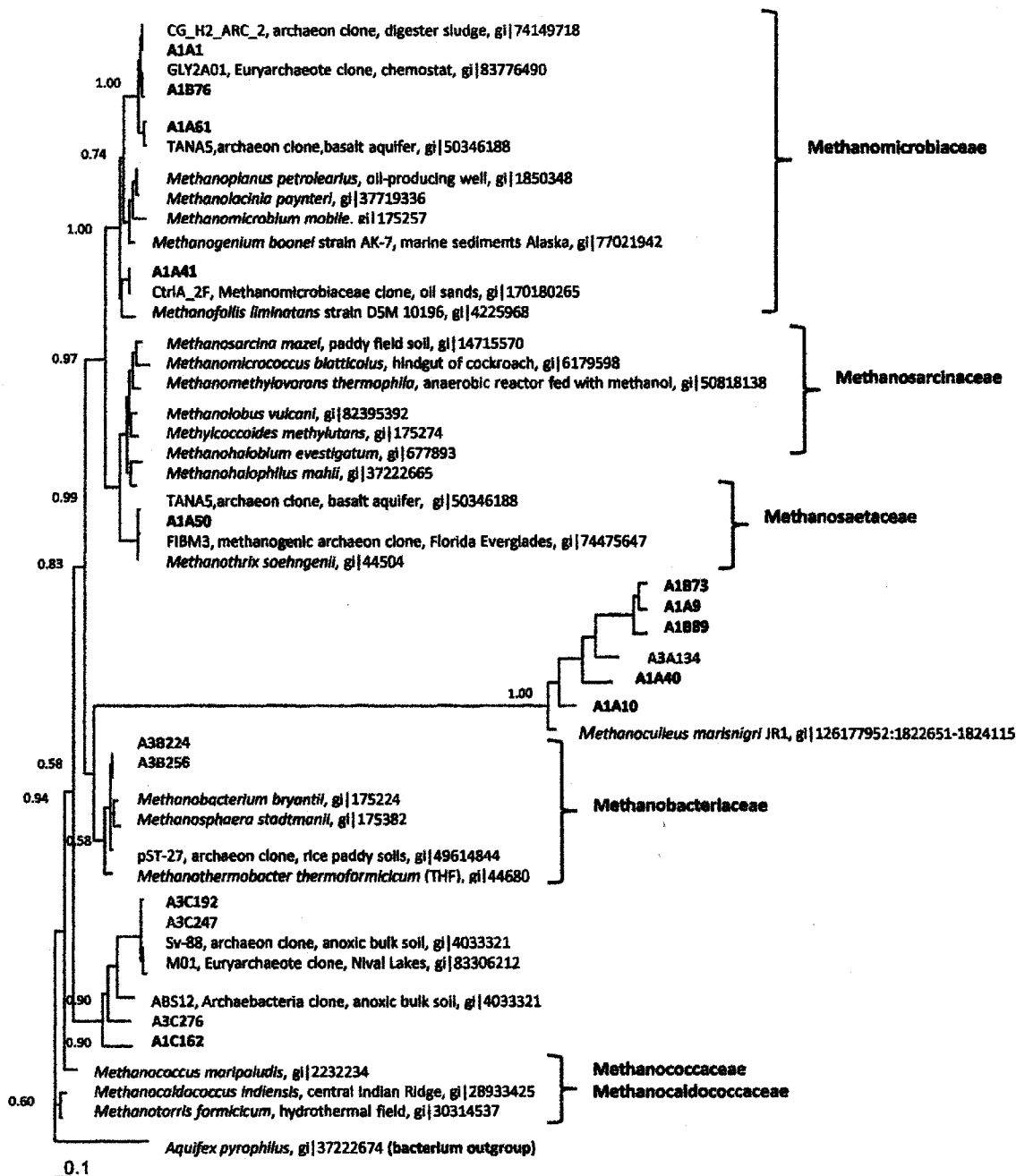
Figure 13. Phylogenetic analysis of archaeal 16S rRNA gene partial sequences from Sites 1 (bold) and 3 (gray). The tree was built by Bayesian analysis using MrBayes version 3.1.2. Posterior probabilities ≥ 0.80 are considered significant.

B3A84
B1A51
B3B48
B3B77
B3B49
B1B92
B1B72
B3B134
B1A16
B3B132

1.00

B3B53
Desulfofustis glycolicus, gi|1490230
0.92
B1C19
Desulfocapsa sp. Cad626,chemocline of meromictic lake, gi|23954310
Desulforhopalus vacuolatus, temperate estuary, psychrophilic, gi|2072388
0.74
B3C39
0.87
Desulfobulbus rhabdoformis, gi|3046327
Desulfovibrio aespoeensis clone Aspo3, gi|189039238

Desulfobacula toluolica, gi|20146087
Desulfobacter vibrioforme B54, water-oil separation system, gi|2501751
0.70
0.72
B3B34
Desulfobacterium vacuolatum DSM 3385, gi|18034281
Desulfococcus biacutus, gi|11558175
Desulfonema magnum, gi|1399438
0.72
0.85
Desulfosarcina variabilis, gi|174346
B3B63

B3B56
0.72
Geobacter uraniireducens Rf4, sediment undergoing uranium bioremediation, gi|145408212

B3B120
B3C150
Pelobacter acetylenicus, gi|397558
0.97
Desulfuromonas thiophila *, anoxic freshwater sediment, gi|2326420
Geothermobacter ehrlichii, hydrothermal vent, gi|28627990
0.98

Anaeromyxobacter dehalogenans strain FRC-W 16S, pristine and radionuclide impacted environments, gi|206598473
B3B76
B3B92
0.90
Myxococcus sp. HBUM WH091t-1, gi|158348038
0.98
Cystobacter gracilis strain DSM 14753, gi|110564210
0.85
B1B210
0.63
B3B89
Desulfuromusa sp *. Fe30-7C-S, gi|148524648
Desulfurella multipotens *, thermal environments, gi|3341427
Methanosarcina mazei Go1, gi|21226102:c236779-235306 (Archaea Outgroup)

0.1

Desulfobacteraceae

Desulfobulbaceae

Desulfobacteriales
(Sulfate-reducing Bacteria)

Desulfuromonadales
(*Sulfur-reducing Bacteria)

Myxococcales
(Other Deltaproteobacteria)

Figure 14. Phylogenetic analysis of eubacterial 16S rRNA gene partial sequences from Sites1(bold) and 3 (gray). The tree was constructed by Bayesian analysis using MrBayes version 3.1.2. Posterior probabilities ≥ 0.80 are considered significant.

Discussion

Microbial Diversity in the Sediments of the Sea Mist Constructed Wetland.

The structure of the microbial community inhabiting the sediments of Sea Mist

was investigated by a combination of bioinformatics tools. BLAST identified the

closest relatives in the GenBank database by aligning the sequences and

comparing their nucleotides. RDP assigned the sequences to different taxa

using a Naïve Bayesian classifier, relying on a database that combines Bergey's type strain sequences and the full-length 16S rRNA sequences from GenBank [51]. These tools were complementary in analyzing the microbial community and helped delineate differences in the microbial populations at the two sites.

Identifying new sequences is difficult. If the sequences meet the ≥ 97% similarity standard they may be grouped in the same taxon or the same operational taxonomic unit (OTU). However, the definition of species in Eubacteria and Archaea is an ongoing subject of controversy, due to the fact that neither group exhibits sexual reproduction. Additionally, several of the environmental sequences deposited in GenBank are partial sequences that have not been fully identified.

Phylogenetic analysis using molecular techniques provide an efficient means of rapidly identifying new sequences. The RDP Classifier is designed to facilitate this process by determining the hierarchical taxonomic affiliation of unidentified sequences with some level of certainty. Using both NCBI and RDP tools enhances the ability to assign sequences to at least the phylum level and reduces uncertainty regarding "uncultured archaeon" or "uncultured bacterium" matches obtained from GenBank.

BLAST search results indicated that the majority of clones in the Archaea libraries are related to an "uncultured archaeon". About 70% of the archaeal community in Site1, and 53% of the community in Site 3, could be identified as

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members of the phylum Crenarchaeota. This phylum encompasses mostly thermophilic microorganisms that metabolize elemental sulfur and could either be chemoorganotrophs or chemolithotrophs [29]. This result was surprising, since methanogens from the phylum Euryarchaeota were expected to dominate this environment.

Identifying lower taxonomic categories within the phylum Crenarchaeota was not possible under appropriate estimates of confidence. Nevertheless, a few members of the phylum Euryarchaeota were identified at multiple taxonomic categories with high bootstrap levels of confidence. Most of the members of this group were related to methanogens.

The isolation of few clones belonging to the phylum Euryarchaeota could be due to bias in the method utilized for extracting genomic DNA that might have favored extraction of more abundant sequences such as Crenarchaea [48, 52]. Also, multiple sequences from Site 1 were eliminated because they were related to Eubacteria, decreasing the number of clones available for diversity analysis of Archaea at this site. There is no explanation for why Eubacteria from Site 1 were amplified using the archaeal primers if DNA from both sites was amplified at the same time. Although the primer pair 109F-934b may anneal more easily with the rDNA sequence of Crenarchaea under the PCR conditions used, the proportions of Crenarchaea clones identified by GenBank at both sites should have been equal. Großkopf *et al.* [18] successfully utilized the same PCR protocol to

describe the diversity of methanogens in anoxic rice paddy soil microcosms. Several sequences were unique to each site indicating that there were differences in the composition of the archaeal communities.

The rarefaction curves and the Shannon-Wiener function demonstrated a difference in the microbial diversity at Sites 1 and 3. The archaeal community at Site 1 appeared to be more diverse than at Site 3. Although Crenarchaea dominated Site 1, a variety of other matches from other phyla were obtained from GenBank. Utsumi *et al.* [48] used the Shannon-Wiener function to compare the archaeal sequence diversity in a wetland in Siberia, in Japan, and in United States (n=161, n=134 and n=131 respectively). Their results were based on numbers of sequences similar to the numbers used in this study. They concluded that the highest diversity (H = 2.84) among the three wetlands was present in an acidic wetland in United States, followed by Japan (H = 1.38), and Siberia (H = 1.20). These results suggested a relationship between wetland latitude and temperature, and methanogen diversity [48].

At Site 1 none of the rarefaction curves reached a plateau. Increased disturbance at Site 1 may result in increased microbial diversity. The archaeal rarefaction curves for Site 3 plateaued. The archaeal rarefaction curves were not as steep as the curves for Eubacteria at either site. These findings suggest that the archaeal community was adequately sampled, whereas more samples would be required to adequately describe the Eubacteria diversity.

The bacterial community is highly diverse at both sites. Taxonomic affiliations were made to the level of family with 88% of the sequences from Site 1 and 95% of the sequences from Site 3. At Site 1 almost half of the sequences were affiliated with Proteobacteria (44%), followed by Acidobacteria (16%), Verrucomicrobia, and Gemmatimonadetes (8% each). Of the Proteobacteria, the subphylum Betaproteobacteria was dominant. These findings are consistent with Kraigher et al. [26], who studied the community composition of drained fen soils. They demonstrated that among 114 clones, 53% corresponded to Proteobacteria, 23% to Acidobacteria, and ≤7% to other groups. At Site 3, a higher percentage of the sequences were affiliated with Proteobacteria (63%). The subphylum Betaproteobacteria was dominant (36%). The prevalence of the subphylum Deltaproteobacteria was 30% at Site 3, although it was the least represented proteobacteria in Site 1.

The microbial populations within Archaea and within Eubacteria at the two sites were different. Within the phylum Proteobacteria sequences from Alphaproteobacteria and Gammaproteobacteria were lower at Site 3. Bacteroidetes, a phylum that was not represented at Site 1, constituted 6% of the sequences from Site 3. At Site 3 only 4% of the sequences were related to Acidobacteria compared to the 17% in Site 1.

Any conclusions drawn regarding the diversity of Eubacteria need to be made with caution, considering the fact that the diversity of this group has not

been fully determined. Primers fD1 and rD1 were analyzed with Probe Match, an RDP tool that determines the percentage of sequences covered by primer sets in the different domains of life. Primer fD1 was found to target 17% of bacterial sequences while primer rD1 targeted 2%, perhaps explaining why the diversity of Eubacteria was insufficiently sampled in this study. Because eubacterial sampling was incomplete, interpretations regarding metabolic activities of the eubacterial families must be more conservative than for Archaea.

Phylogenetic Analysis of the Diversity of Methanogens and their Function. A phylogenetic tree was constructed to determine the evolutionary relationships of the Euryarchaeota sequences found in the clone libraries. Four clones from Site 1 and their correspondent hits from GenBank were closely related to genera in the family Methanomicrobiaceae, order Methanomicrobiales. Methanomicrobiaceae is comprised of hydrogenotrophic members, thus almost half of the 10 clones from Site 1 were suspected to be H_2-CO_2-utilizing methanogens. Two other clones were likely members of the order Methanosarcinales, but only one of them was proven to belong to the family Methanosaetaceae.

Großkopf *et al.* [18] suggested that *Methanosaeta spp.* and *Methanosarcina spp.* could be indicators of steady-state concentrations of acetate in rice paddy soils. The species of *Methanosaeta* are acetate-utilizing specialists, and thus have a lower threshold of acetate concentration compared

to *Methanosarcina spp.*, which are generalists (capable of utilizing various substrates). Galand *et al.* [15] also determined that in some minerotrophic fens (high nutrient content), *Methanosaeta spp.* can out-compete *Methanosarcina spp.*, possibly explaining why no members of the family Methanosarcinaceae were found. Minerotrophic fens receive groundwater and runoff water as inputs. In moderate minerotrophic fens pH values range between 5.5 and 7.0, however, the pH is typically >7 in calcareous fens [7]. At Site 1 the recorded pH value was between 7.5 and 7.84, making it comparable with calcareous fens.

The high concentration of NO_3^- at Site 1, compared to Site 3 may indicate that methanogenesis is inhibited by N-oxides [36, 47]. The denitrification intermediates, particularly nitric oxide (NO), inhibit acidogenesis, fermentation, methanogenesis, and also denitrification at low initial concentrations [47]. Tugtas and Pavlostathis [47] determined that when NO_2^- is added to nitrite-amended cultures, acetoclastic methanogenesis is inhibited due to transient NO accumulation. Starmark and Leonardson [41] however determined that neither NO_3^- concentration nor temperature of incubated sediment samples has an effect on methanogenesis, but the interaction of the two did. They also found that at concentrations of NO_3^- between 8 and 16 mg/L, CH_4 production was inhibited even though there was an increased temperature [41]. Thus, the low diversity of methanogens at Site 3 might have been due to inhibition by N-oxides formed during denitrification, or the interaction between these oxides and a higher temperature.

One sequence was identified as *Methanotrix sp.* (Methanosaetaceae) with 100% certainty by RDP's Classifier. However, its sister group on the phylogenetic tree created by MrBayes was *Methanoculleus marisnigri* from the family Methanobacteriaceae. This sequence formed part of a cluster of sequences that did not have any matches in GenBank. The closest relatives in the database were between 87% and 92% similarity. The posterior probability (1.00) of this branch in the tree strongly supports the affiliation between the sequences. However, the relationship with other members of the family Methanobacteriaceae is not well supported (posterior probability = 0.56).

Since the similarity scores to known sequences were low for this cluster, they may represent a novel monophyletic group with the family Methanobacteriaceae. It can be inferred that multiple base changes differentiated them from their relatives in the family Methanobacteriaceae over time. Alternatively, slightly different regions of the 16S rRNA gene could have been inadvertently cloned, possibly resulting in different secondary structures and therefore different functions (Joshua Mackie, personal communication). Unassociated clusters of sequences have been previously reported. Cadillo-Quiroz *et al.* [6] found Methanomicrobiales-related phylotypes whose physiologies have not been elucidated among the methanogenic populations in Northern acidic peatlands using 16S rRNA gene clone libraries and T-RLFP profiles.

Two other sequences from Site 3 were directly affiliated with the family Methanobacteriaceae, another hydrogenotrophic group of methanogens. This family was only observed at Site 3, either because it was the dominant group of methanogens or because it was rare in Site 1. One sequence from Site 1 and three other sequences from Site 3, and their corresponding matches from GenBank, formed a completely separate cluster in the phylogenetic tree that could only be identified as Euryarchaea. This cluster might represent novel sequences found in this wetland, as indicated by the GenBank match "ABS12 Archaebacteria", which was within the cluster of sequences. ABS12 was obtained during the study of novel euryarchaeotal lineages in flooded rice microcosms.

The distribution of Archaea sequences in this study generally correlated with the findings of Rooney-Varga *et al.* [38] in Alaskan peatlands. They conducted phylogenetic analyses of denaturant gradient gel electrophoresis (DGGE) bands, indicating that 70% of their sequences were related to unidentified Crenarchaeota, while only 30% were related to methanogens. The hydrogenotrophic families Methanomicrobiaceae, and Methanobacteriaceae were identified, as well as the acetoclastic family Methanosaetaceae. No members of the family Methanosarcinaceae were detected, although acetate was considered more relevant in cold than in temperate environments [11]. Ganzert *et al.* [16] studied Siberian Arctic permafrost affected soils, obtaining sequences

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from DGGE bands that were affiliated to Methanomicrobiaceae, Methanosarcinaceae and Methanosaetaceae [16].

This study is consistent with data using a different gene, the functional methyl coenzyme M reductase gene (mcrA). Galand et al. [15] reported that an oligotrophic fen and an ombrotrophic bog in Finland had a high proportion of methane being produced by the hydrogenotrophic pathway. Characteristic vegetation (Sphagnum mosses) of the ombrotrophic bog does not introduce labile carbon substrates to the peat layers, thus explaining the small proportion of acetoclastic methanogenesis observed [15]. The authors speculate that the lower diversity compared to other studies in northern peatlands was due to low pH (< 4) [4]. Horn et al. [22] also studied an acidic peat bog, obtaining sequences related to Methanobacteriaceae and Methanomicrobiales from most probable number (MPN) dilutions and enrichments.

Acetoclastic methanogenesis is thought to be the dominant methanogenic pathway in freshwater wetlands [2, 10, 18]. Although, Sea Mist is considered a freshwater environment due to its low salinity compared to that of the adjacent Monterey Bay, non-acetoclastic methanogenesis appeared to be the dominant pathway for methanogenesis. Acetate appeared to be produced via hydrogenotrophic methanogenesis rather than consumed. Site 1 sequences affiliated with hydrogenotrophic methanogens may supply permissive amounts of acetate for Methanosaeta spp. survival. Site 3 did not appear to support

acetoclastic methanogenesis since only Methanobacteriaceae were found despite low NO_3^- levels.

Phylogenetic Analysis of Sulfate-reducing Bacteria and its Role in the Biogeochemistry of the Wetland. It is not surprising that the diversity of Eubacteria supersedes that of Archaea, and that Proteobacteria dominate the bacterial community. This phylum is the largest and most physiologically diverse among Eubacteria. Of the four subphyla, Deltaproteobacteria is the second dominant group in Site 3 while it is the least represented in Site 1.

The phylogenetic tree identified two metabolically different groups of sulfate-reducing bacteria (order Desulfobacterales) primarily in Site 3. Two sequences from this site were affiliated with the sulfate-reducing family Desulfobacteraceae (genera *Desulfobacterium* and *Desulfosarcina*), characterized by acetate oxidation (SRB, Group II). The metabolically versatile family Desulfobacteraceae proved to be important in *Spartina*-dominated salt marsh sediments along the Atlantic coast of United States [3, 25].

Klepac-Ceraj *et al.* [25] created a 16S rRNA gene library using specific primers for SRB. They found that over 80% of the deltaproteobacterial ribotypes clustered with the complete oxidizers *Desulfosarcina, Desulfococcus,* and *Desulfobacterium.* The presence of acetate oxidizers among SRB at Site 3 suggests that they may deplete some of the acetate released to the sediments

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before it can be used by methanogenic Archaea. This may explain why no acetoclastic methanogens were detected at Site 3.

Two sequences from Site 3 clustered within the family Desulfobulbaceae. One was closely related to the genus *Desulfobulbus*, which utilizes propionate to produce acetate and CO_2 during sulfate reduction [29]. According to Purdy *et al.* [34] bacteria from this genus appear to require sulfate in marine sediments but not in freshwater sediments. The other sequence was affiliated with *Desulfofustis*. Both genera are known for being incomplete oxidizers that produce acetate (SRB, Group I). Only one sequence from Site 1 was affiliated with the same family and was associated with the genus *Desulfocapsa*.

Four more clones from Site 3 were affiliated with members of the order Desulfuromonadales, represented by the families Desulfuromonadaceae, Geobacteraceae and Pelobacteraceae. Species of *Desulfuromonas* are incapable of reducing sulfate, but utilize substrates such acetate or ethanol to reduce elemental sulfur to hydrogen sulfide [29]. The presence of sulfur-reducing bacteria exclusively at Site 3 suggests that elemental sulfur is readily available.

The majority of sequences from Site 1, and 7 clones from Site 3 remained unidentified, though the RDP Classifier placed them in the phylum Deltaproteobacteria. None of the sequences were identified as chimerical, suggesting that novel uncultured bacteria are present. Schmalenberger *et al.*

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[40] suggested that novel sulfate-reducing prokaryotes colonize low sulfate minerotrophic fens, in which dissimilatory sulfate reduction contributes to the retention of sulfur.

The emergence of this cluster of novel sequences may be the result of basing their identification solely on the 16S rRNA gene. Although 16S rRNA genes evolve slowly, bacteria harbor multiple operons, and their sequences can diverge (< 1%) generating microdiversity in the clone libraries [25]. Molecular methods based on the phylogeny of a single gene may not be sufficiently accurate to identify unknown sequences. Phylogenetic reconstruction might be more reliable if genes specific to certain groups of microorganisms or group-specific primers are used.

The Sea Mist wetland was constructed two years prior to this study. Organic matter mineralization may be limited at this stage of the restoration process, perhaps explaining why a small portion of the archaeal community corresponds to methanogens and why fewer sulfate-reducing bacteria were found. The increased microbial diversity in Site 1 may be a result of the polluted runoff water that reaches the wetland. Site 3 was less disturbed, more alkaline and displayed lower microbial diversity. Site 3 is adjacent to the Moro Cojo Slough. This proximity may allow underground interaction between the pond and the estuarine environment, generating different geochemical conditions at this

site. Other environmental factors which differentiate the two sites should be investigated.

Nitrogen may be a determining factor on the biogeochemistry of the sediments. Ammonia concentration was higher in pore water from Site 3 than from Site 1. Some bacteria prefer ammonia as a nitrogen source for assimilative uptake [55]. This ammonia may be formed during bacterial sulfide oxidation when NO_3^-, rather than O_2, is utilized to produce S and SO_4^- [55]. Anaerobic oxidation of methane coupled to sulfate reduction or denitrification [31, 35, 50] may have depleted NO_3^- at Site 3. Raghoebarsing *et al.* [35] demonstrated that a microbial consortium from anoxic freshwater sediments, which had been polluted by agricultural runoff, carried out the oxidation of methane coupled to denitrification.

Fertilizers and pesticides in runoff water deposited at Site 1 may also explain differences in biogeochemistry between sites. Le Mer and Roger [28] summarized the effect of fertilizers and pesticides on rice fields where organic and chemical fertilizers are primarily used. Reports on the effects of chemical N-fertilizers on CH_4 emission are contradictory. Organic fertilizers usually increase methane production because of the higher labile C content [28]. Pesticides, such as acetylene, have been shown to decrease methanogenesis, methanotrophy and nitrification [28]. Similar interactions may have contributed to low numbers of methanogens throughout the wetland.

The phylum Crenarchaeota was the predominant Archaea found in the sediments of this constructed wetland based on the 16S rRNA gene, but their concentration was not determined. Crenarchaea usually require elemental sulfur as an electron acceptor in anaerobic respiration, or an electron donor for chemolithotrophy [29]. Sulfur-reducing bacteria were detected only at Site 3, indicating possible competition for sulfur at the disturbed site.

Differences in diversity were observed between the Archaea and Eubacteria communities from the disturbed and the restored site. In freshwater sediments of this wetland, methanogens may be playing a more important role in the mineralization of organic matter than sulfate-reducing bacteria.

Research that includes microbial and chemical quantification, more extensive analysis of the physical environment (*e.g.*, redox potential), and evaluation of changes over time, will provide important data that can enable better understanding of this dynamic wetland environment.

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CHAPTER III

Conclusion

Information about microbial character of the sediments from two sites at the Sea Mist constructed wetland was derived from a combination of bioinformatics tools. Although methanogens from the phylum Euryarchaeota were expected to be dominant, members of the phylum Crenarchaeota dominated the archaeal community in Sites 1 and 3. Proteobacteria from different subphyla were dominant among Eubacteria at each site. The relatively low numbers of sulfate-reducing bacteria (SRB) and methanogenic Archaea may be due to the recent construction of the Sea Mist wetland. Organic matter mineralization may be limited at this stage in the restoration process.

Although the Sea Mist wetland is considered a freshwater environment where acetoclastic methanogenesis should be dominant, non-acetoclastic methanogenesis predominated. It appeared that acetate was produced via hydrogenotrophic methanogenesis, probably by Methanobacteriaceae and Methanomicrobiaceae.

Phylogenetic analyses of Deltaproteobacteria sequences showed that sequences from Site 3 were related to two metabolically different groups of SRB in the order Desulfobacterales. One of the groups produces acetate from incomplete oxidation (SRB, Group I). The other group was SRB that perform

complete acetate oxidation (SRB, Group II). This finding suggested that some of the acetate released to the sediments might have been depleted by SRB before utilization by acetoclastic methanogenic Archaea.

Distinct clusters of sequences were observed in the phylogenetic analyses of methanogens and SRB. These clusters comprised sequences that did not match GenBank sequences given the ≥97% standards of similarity, but the posterior probability of the branches of the trees strongly supported the affiliation among the sequences in each cluster. Since the similarity scores in GenBank were low, it was inferred that novel groups of methanogens and SRB exist in this wetland.

The distribution of archaeal and bacterial sequences at Site 1 and Site 3 were different. Rarefaction curves and the Shannon-Wiener function showed that the microbial community at Site 1 may be more diverse than at Site 3. Considering that Site 1 was more disturbed than Site 3, multiple adaptations to this disturbance might increase microbial diversity. The rarefaction curves also suggested that our sampling of the archaeal community was more complete than of the bacterial community.

Site 3 was adjacent to the Moro Cojo Slough that ultimately connects to the Monterey Bay, suggesting that a different set of geochemical conditions due to underground water exchange exist there. Nitrate, fertilizers and pesticides in

the runoff water that reaches Site 1 may play important roles in the

biogeochemistry of this site.

Evaluation of changes over time that include microbial and chemical

quantification, and more extensive analysis of the physical environment will

enable better understanding of this dynamic wetland environment.