ABSTRACT

As the importance of oceanic environments to the regulation of global energy and nutrient dynamics is better understood, the critical role marine microbial communities play in cycling nutrients and regulating gas flux from oceans is becoming more apparent. With the prediction that rising atmospheric temperatures will cause an overall decrease in marine oxygen levels, the oxygen minimum zones (OMZ’s) of today may represent oceanic environments of the future. To better understand the microbial dynamics of these environments, I undertook a spatial and temporal study of the microbial community in the water column of Saanich Inlet, a model OMZ. This inlet is seasonally anoxic, and is notable for microbial regulation of methane flux to the atmosphere. The microbial community was sampled in February 2006 at 4 depths spanning the oxycline, and molecular taxonomic analysis revealed that the bacterial groups present were very similar to other OMZ’s. Overall, the proteobacteria were most abundant and sequences similar to Type-I and Type-II methanotrophs were detected at 10 m and 100 m. Relatives of sulfur-oxidizing bacterial endosymbionts, commonly found in other OMZ’s almost completely dominated the community at 215 m. However, the absence of epsilon proteobacteria linked to sulfide oxidation was in contrast to other OMZ’s. Members of the crenarchaea dominated the water column; and no methanogens or ANME groups belonging to the euryarchaea were detected. However, novel pmoA phylotypes detected at all 4 depths in Saanich Inlet, showed a depth distribution suggesting that aerobic methane oxidation is the primary component of the methane cycle occurring in the water column. Temporal data suggested that the microbial community is relatively stable and primarily distributed according to depth, and that methane and oxygen concentrations contribute to this distribution. Notably, a late summer deep-water turnover event in Saanich Inlet did affect the distribution of the microbial communities at 200 m.
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INTRODUCTION

Look at any map or globe and you will see a world dominated by water. In some ways it might be most accurate to call our planet “ocean”. Indeed, the processes unfolding in the deep blue sea help create and sustain the common conditions for life itself. From time immemorial the sea has been a source of inspiration, fascination, livelihood and fear, and for those who would listen, a call to both discovery and catastrophic failure (17). We can see this call manifested in the search for new trade routes, the riches of new worlds for old, and in the mapping of continental margins. For many societies, oceanic exploration has been the seed for economic, cultural, political and scientific transformations. Beyond the more tangible elements of discovering new life forms, landmasses or shipping lanes, the scientific community has become increasingly aware that ocean processes play a critical role in the regulation of global energy and nutrient dynamics. And although not recognized until recently, it is the invisible community of microorganisms and viruses that appears to be one of the primary causative agents for this regulation (36).

Microorganisms thrive in virtually every imaginable region of the ocean, including the dark waters of the abyss, deep subsurface sediments, hydrothermal vent surfaces, and the nutrient-rich photic zone. In sheer numerical terms bacteria and archaea are estimated to account for more than $6 \times 10^{30}$ cells planet-wide (78) and in most marine ecosystems there is an estimated $1 \times 10^6$ cells per ml of water (79). Given their abundance and global distribution patterns, the effort to profile marine microbial communities has intensified, most notably on recent large-scale oceanic surveys (13, 63, 72).
OXYGEN MINIMUM ZONES

Oxygen minimum zones (OMZ’s) are areas of low dissolved oxygen concentration that presently account for over one million square kilometers of oceanic surface area (28). Within open ocean and coastal waters the most extensive OMZ systems are found in the eastern and northwest Pacific Ocean (including the Chilean and British Columbia coastlines), the southeast Atlantic off West Africa, and the Northern Indian Ocean (Arabian Sea) (16, 28). In addition, the Cariaco Basin, Baltic Sea, and Black Sea also support well established and, in some cases, permanently stratified OMZ systems (16). OMZ’s are defined by their level of oxygen depletion (\( \leq 0.5 \text{ ml L}^{-1} \text{ dissolved oxygen} \)) (28) and the Baltic Sea, Black Sea, and northwest Pacific Ocean represent some of the most oxygen-depleted systems on the planet (16). High primary productivity in surface waters, combined with poor water circulation within the underlying water mass (or the absence of deep water contact with the surface in the case of the NW Pacific Ocean OMZ), is the primary mechanism for oxygen depletion within OMZ systems, although exact details will vary from open ocean to enclosed basins (45, 62).

BIOGEOCHEMICAL CYCLING WITHIN OMZ SYSTEMS

OMZ’s play a major role in biogeochemical cycling within the world’s oceans. They are major sinks for nitrogen (7), minor sinks for methane (66), and sources of carbon dioxide (66) and nitrous oxide (67). Within OMZ systems, reduced levels of oxygen, the most energetically favorable electron acceptor for oxidation of organic matter, selects for alternative modes of respiration similar to those found in stratified sediment environments (55, 69). Directly related to their potential energy yields, modes of respiration preferentially occur in a defined order: denitrification, followed by manganese and iron reduction, sulfate reduction and, finally
methanogenesis (20). Denitrification is of particular interest within OMZ systems, because it, along with the recently discovered process of anaerobic ammonium oxidation (anammox) (42), depletes biologically useful nitrogen species (7). Given the widespread nature of OMZ systems, the cumulative effect of denitrification and anammox reactions likely has a significant impact on the global nitrogen cycle (5, 7, 55). In addition to nitrogen cycling, recent trends in atmospheric warming have made OMZ’s and microbial carbon cycling of the greenhouse gases carbon dioxide and methane of special interest (33, 37, 66). It is hypothesized that atmospheric warming will cause a decrease in the oxygen inventories in oceanic waters (37), making current OMZ’s excellent model systems in which to study microbial carbon cycling under anoxic conditions so that the future impacts of oxygen loss in major marine environments can be better predicted. Overall, the biological activity associated with microbial communities present in OMZ systems impacts oceanic productivity and the balance of greenhouse gases in the atmosphere. For this reason, studies aimed at evaluating the phylogenetic composition and metabolic capacity of microbial communities within these systems have great promise to enhance our mechanistic understanding of the patterns and processes that drive global biogeochemical phenomena in both aquatic and atmospheric compartments of the biosphere.

**Denitrification**

Nitrogen cycling, unlike other nutrient cycles that occur in marine environments, can result in the net loss of nitrogen from the water (32). This loss occurs primarily through denitrification (32), which is a multi-step process whereby nitrate is converted to nitrogen gas as per the following redox reaction:

\[ 2\text{NO}_3^- + 10 \text{e}^- + 12\text{H}^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O} \]
A number of marine microorganisms are capable of denitrification, including a variety of facultative aerobes belonging to the proteobacteria (32). These microorganisms can alter the redox state of nitrogen such that it takes a gaseous form, which results in its loss to the atmosphere and its removal from the available stores (32). Since the enzymes that catalyze the steps in denitrification (nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase) are extremely sensitive to oxygen (38), denitrification is almost completely limited to anoxic sediments and anoxic waters (7) such as the Black Sea, Cariaco Basin, and Arabian Sea (32). Denitrification in sediments occurs on a depth scale of millimeters to centimeters, while in OMZ’s it can occur over meters of water (73), making them excellent study sites. Denitrification typically occurs just below the oxic/anoxic interface as nitrate is the next preferential electron acceptor after oxygen (59).

Anammox

Anammox is the most recently discovered component of the nitrogen cycle, and is an anaerobic process by which the enzyme hydroxylamine oxidoreductase (65) catalyzes the reduction of ammonium to nitrogen gas using nitrite (32) according to the reaction below:

\[ \text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \]

This process, first discovered in anaerobic bioreactors (34), has now been studied in the Black Sea, where it has also been found to occur just below oxic/anoxic interface (42). The relationship between aerobic ammonia-oxidizers, ‘annamox bacteria’ (of the phylum Planctomycetes) (42), and nitrogen cycling is a complex one, and is continuing to be resolved (32, 44; 57).
Nitrous Oxide

Nitrous oxide cycling in suboxic marine environments is believed to be responsible for a great deal of the nitrous oxide flux to the atmosphere (67). Similar to denitrification, the production of nitrous oxide results in the loss of biologically useful nitrogen to the oceanic environment (67). However, its production can also affect atmospheric temperatures as nitrous oxide is a powerful greenhouse gas (33). Produced as an intermediate during both nitrification and denitrification, elevated levels of nitrous oxide are found in OMZ’s (7).

Carbon Dioxide

Carbon dioxide is classically thought of as a byproduct of respiration in oxic environments (59). However, this greenhouse gas (33) is also a product of fermentations and anaerobic respiration (e.g. the anaerobic oxidation of methane) conducted by a wide variety of organisms (59). Acting as a major substrate and byproduct of bacterial and archaeal metabolism, carbon dioxide cycling is critical to the overall regulation of carbon flux to the atmosphere (33).

Methane Oxidation

Three greenhouse gases are produced in suboxic zones: nitrous oxide (7), carbon dioxide (59), and methane (6). Methane is a potent greenhouse gas, with a radiative capacity 25 times that of carbon dioxide (6, 33). With atmospheric levels of methane at the highest levels ever recorded (33), calls have been made to reduce the production of this gas to mitigate atmospheric warming (33). Three groups of microbes are presently known to be involved in methane cycling: methanogens (11) and anaerobic methane oxidizers (ANME) (4) are both members of the euryarchaea, while the methanotrophs Types I and II, respectively, are members of the gamma
and alpha proteobacteria (76). Methane cycling mediated by the archaea is an anaerobic process
with low free energy yields (24) (77) whereas bacterial methanotrophy is an oxygen-dependent
process with relatively high free energy yields (77). Both methanogenesis and aerobic methane
oxidation are well-studied processes, but the reaction by which anaerobic oxidation of methane
occurs is still hypothetical (4). The reactions for these processes are given below:

\[
\text{Methanogenesis:} \\
\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O} \text{ (from carbon dioxide)} \\
\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \text{ (from acetate)}
\]

\[
\text{Aerobic methane oxidation} \\
\text{CH}_4 + 2\text{O}_2 \rightarrow \text{CO}_2 + 2\text{H}_2\text{O}
\]

\[
\text{Anaerobic methane oxidation} \\
\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}
\]

Given the links between methane production, atmospheric warming, and increased
anoxia of marine environments, OMZ’s are excellent environments in which to study microbial
methane cycling in the attempt to better determine how microbial regulation controls methane
flux from oceans to the atmosphere (Fig. 1).
MICROBIAL COMMUNITY STRUCTURE OF OMZ SYSTEMS

Most of what we know about the microbial community structure of OMZ systems has been born from studies focused on identifying the microorganisms responsible for nitrogen cycling (8, 21, 44, 58, 81). Comparative analysis between studies is confounded by variation in physical and chemical parameters, poor depth and time resolution in sampling, and the choice of analytic methods which can ultimately bias the number and types of small subunit (SSU) rDNA sequences recovered and the types of correlations made. For instance, alternative methods ranging from traditional SSU (most often 16S) rDNA clone library screening, DNA fingerprinting and cell sorting coupled to reverse-transcription PCR have been used to evaluate community diversity associated with the Cariaco Basin, Black Sea and Arabian Sea respectively (22, 51, 73). Given these caveats it is still possible to draw some general conclusions about the structure of OMZ systems based on the pervasiveness of certain microbial groups and the cumulative absence of others in relation to observed geochemical conditions. The following paragraphs outline some of the major trends identified among and between some of the best described OMZ systems in the current literature including community surveys of the Cariaco Basin (51), Black Sea (73), Arabian Sea (Northern Indian Ocean) (22), Baltic Sea (43) and, most recently, the Namibian Upwelling system (81).

The Cariaco Basin, the second largest anoxic basin on earth, is stably anoxic below 320 m and this anoxia extends to the seafloor at approximately 1,400 m (61). Members of the proteobacteria were found to dominate the SSU rDNA clone libraries used to profile microbial community structure, with epsilon proteobacteria related to the autotrophic denitrifier *Thiomicrospira denitrificans* (capable of fixing carbon dioxide at the expense of sulfide or nitrate), by far the most prevalent ribotype recovered between 320 and 500 m (51). Changes in
community composition were observed between 320, 500 and 1,310 m, with the greatest ribotype diversity found at the 1,310 m depth interval (51). In addition to the strong proteobacterial signal, ribotypes for OP3 and OP8 candidate divisions, *Planctomycetes*, *Verrucomicrobiun*, *Fibrobacter* and *Bacteroides* groups were also recovered in the clone libraries (51). Archaeal ribotypes represented less than 5% of sequences recovered in the clone libraries, and although both planktonic group I crenarchaea and euryarchaea were detected, the majority of these sequences were related to uncultivated euryarchaeal groups associated with the anaerobic oxidation of methane (51). The low representation of archaeal ribotypes likely resulted from the choice of universal SSU primers selected for environmental DNA screening although little is known in general about the overall archaeal contribution to planktonic biomass in OMZ systems (51).

The Black Sea represents the largest enclosed anoxic basin on earth (8). It is completely and permanently anoxic from 100 m to the seafloor at 2,200 m (8). Similar to the Cariaco Basin, microbial assemblages along the oxycline between 10 m to 500 m were dominated by members of the proteobacteria based on the combined results of SSU rDNA clone library and terminal restriction fragment length polymorphism (T-RFLP) analysis (73). *Pseudoalteromonas*-like species and uncultured members of the epsilon proteobacteria related to *Thiomicropsira denitrificans* were very abundant in the upper part of the anoxic water column, while gamma proteobacteria related to methanotrophs and sulfide-oxidizing endosymbionts of hydrothermal vent invertebrates were abundant between 100 m and 300 m (73). In addition to the candidate division OP11, ribotypes representing *Planctomycetes* and *Bacteroides* groups were also recovered in clone libraries. Archaeal diversity through the oxycline was similar to the Cariaco Basin with typical planktonic marine group sequences dominating the upper water column and...
euryarchaeal sequences dominating deeper anoxic layers (73). Euryarchaeal ribotypes fell within three groups, one related to known methanogens, one related to planktonic group II within the *Thermoplasmatales*, and one related to the ANME-1 group of uncultivated methane oxidizing archaea (73).

The Arabian Sea, enclosed on three sides by segments of the Indian subcontinent, Arabian Peninsula and the Somalian coast, harbors a relatively stable OMZ between 200 m and 1000 m depth (55). Similar to other OMZ systems, proteobacteria were found to dominate the oxycline based on a combination of flow cytometry, SSU rDNA library screening, and fluorescence in situ hybridization (FISH) (22). Although this study focused on the community of heterotrophic picoplankton such as the ubiquitous SAR11 group, ribotypes representing sulfate-reducing delta proteobacteria related to uncultivated members of the *Desulfofrigus* and *Desulfosarcina* clusters and a variety of gamma proteobacteria related to methanotrophs and sulfide-oxidizing endosymbionts of hydrothermal vent invertebrates were frequently recovered (22). Epsilon proteobacteria were conspicuously absent from the survey (22). In addition to the strong proteobacterial signal, the candidate division OP11 and ribotypes representing *Planctomycetes, Verrucomicrobium, Fibrobacter* and *Bacteroides* groups were also recovered in the clone libraries. Archaeal groups were not evaluated in this study (22).

The Baltic Sea harbors an extensive halocline from 60 m to 80 m that limits oxygen renewal, leading to the formation of a stable brackish OMZ (43). Similar to the Black Sea, *Pseudoalteromonas*-like species and gamma proteobacteria related to sulfide-oxidizing endosymbionts of hydrothermal vent invertebrates dominated suboxic intervals between 80 m to 100 m, while epsilon proteobacteria related to *Thiomicrospira denitrificans* were recovered in the sulfide-rich bottom layer of the basin based on the denaturing gradient gel electrophoresis
(DGGE) fingerprints and selective band sequencing used to evaluate community composition (43). DGGE bands associated with Type I methanotrophs and sulfate-reducing delta proteobacteria related to Desulfobacula were also recovered (43). In addition to the strong proteobacterial signal, bands associated with the Bacteroides, Actinobacteria and Nitrospina groups were also recovered (43). Due to methodological limitations, archaea were not well resolved, although a single band representing marine group I Crenarchaeota was recovered (43).

The Namibian upwelling system is an open ocean OMZ harboring a highly productive surface layer with significant oxygen depletion in deeper layers extending to the seafloor at 140 m (5, 28). Similar to other OMZ studies, members of the proteobacteria were abundant in both free and particle-associated phases using SSU rDNA clone libraries used to profile microbial community structure. Gamma proteobacteria related to methanotrophs and sulfide-oxidizing endosymbionts of hydrothermal vent invertebrates were abundant between 100 to 130 m. Alpha proteobacteria related to the SAR11 cluster were also prevalent as were sequences related to those of sulfate-reducing delta proteobacteria of the SAR324 cluster (81). Beta and epsilon proteobacteria ribotypes were also recovered but at much lower frequency. In addition, ribotypes representing Bacteroides, Nitrospina, Actinobacteria, particle-associated Planctomycetes and members of the SAR406 cluster were also recovered (81). A PCR screen targeting anammox bacteria identified numerous particle-associated sequences closely related to the planctomycete Candidatus Scalindua sorokinii (81). With respect to archaeal representation, although several euryarchaeal ribotypes were recovered, Group I crenarchaeota closely related to ammonia-oxidizer Nitrosopumilus maritimus dominated the 130 m SSU rDNA clone library (81).
COMMON THEMES AND QUESTIONS

Several common themes and questions emerge from this review of microbial diversity found within OMZ systems. A subset of groups, including crenarchaeae related to known ammonia-oxidizers, gamma proteobacteria related to methanotrophs and sulfide-oxidizing endosymbionts of hydrothermal vent invertebrates, Bacteroides, and Planctomycetes are associated with the majority of OMZ systems. Epsilon proteobacteria appear more restricted to basin habitats where sulfide levels accumulate in anaerobic bottom waters (48). Anammox bacteria are likely widespread. However standard universal SSU rDNA primer sets do not efficiently target this enigmatic group, and directed screens are necessary to properly recover them. As well, bacteria in OMZ systems appear to partition between free-water and particle-associated phases (81). The interplay between particle-associated and free water phases may promote niche partitioning between various microbial groups with restricted tolerance to even extremely low levels of dissolved oxygen, metal tolerance or specialized electron acceptors (59).

Although we have begun to identify the ‘usual suspects’, little is know about the diversity and cellular abundance of identified microbial groups, their spatial and temporal dynamics and the ecological interplay between physical, chemical and biological processes within OMZ systems. It is important to realize that microbial community diversity studies based on SSU rDNA sequence identity alone can only describe the general taxonomic composition of the stratified OMZ system. Although taxonomic identity hints at metabolic potential based on affiliation with known physiological groups, true insight into metabolic capacity can only come through a combination of functional gene screening, cultivation or enrichment studies and the application of environmental genomic techniques. In order to achieve a systems level understanding of OMZ’s it is essential to integrate across multiple scientific disciplines including
physical oceanography and environmental microbiology, to perform regular and standardized sample collections, and to ask fundamental biogeochemical questions. In this light, the OMZ itself must take on the properties of a model system similar to well characterized organisms in modern cellular, developmental and molecular biology. For most OMZ systems this is a difficult proposition given the logistics and planning required for even a single sampling trip. Therefore, it is essential that to identify locations where regular access and dedicated infrastructure are available for long term and repetitive observations. Saanich Inlet, located on the coast of Vancouver Island, British Columbia, fulfills many of the requirements for a model OMZ.

**Saanich Inlet is a Model OMZ**

Previous studies have confirmed the biological and chemical relevance of OMZ’s, and recent microbial community profiles have determined that the microbial nutrient and gas cycling occurring in these environments is of immediate and considerable concern to atmospheric gas regulation. Recent insights into global biogeochemical cycles (e.g. annamox and anaerobic methane oxidation) have modified climate change and nutrient cycling models and we now have the opportunity to take our knowledge of these environments one-step further; to glimpse the impact future changes in marine oxygen content will have on microbial communities and their regulation of other climate-associated gases. Of the three most potent greenhouse gases (nitrous oxide, carbon dioxide, and methane), the Black Sea is the most frequently studied OMZ for the microbial regulation of methane production and oxidation (19, 64). Yet, this OMZ lacks the changes in anoxia that would make it a suitable site to study the role oxygen flux in an environment can have on the microbial community and methane cycling. Saanich Inlet (Fig. 2) is
a seasonally anoxic OMZ recognized for microbial controls on methane cycling (75) and thus is
an excellent model OMZ.

Saanich Inlet is a glacially derived marine fjord, approximately 225 m in depth, located at
the southeastern tip of Vancouver Island, British Columbia, Canada (23). An 80-m sill is located
at the mouth of the inlet (70) and affects the biological and chemical conditions of the inlet by
restricting the circulation of deep basin waters that would typically replenish water column
oxygen levels. Thus, this sill is partly responsible for the seasonal anoxia that defines Saanich
Inlet (23). The bottom waters of Saanich Inlet generally become anoxic during summer months,
but are renewed with oxygen and nutrient rich waters through a deep-water turnover event (1).
Although the exact timing of the renewal can be variable (and, rarely, not occur at all) it is
seasonally associated with late summer or fall (1). One of the primary sources of freshwater to
Saanich Inlet is the Fraser River, which discharges into the Strait of Georgia 50 km from the
mouth of Saanich Inlet (23). The Frasier River is replenished by melting snows in the spring, and
swells to its highest levels in June (23). This summer freshet eventually makes its way through a
series of tidal channels to the Pacific Ocean, and contributes to the renewal of Saanich Inlet (23).

Seasonal renewal of Saanich Inlet brings both oxygen and nutrients (primarily in the form
of nitrate) to the bottom waters (1). The additional nutrients contribute to the abnormally high
primary productivity of Saanich Inlet which, in turn, contributes to the seasonal anoxia (70).
Daily levels of primary production peak in the spring (May) and again in late summer (August)
with grazing, nutrient-depletion, and photo-inhibition contributing to a decrease between June
and July (70). Levels of primary production in Saanich Inlet are considered high, not only
compared to similar fjords in British Columbia, but also to Saanich Inlet’s source water, the
Strait of Georgia (70). This productivity, combined with weak vertical circulation, can
contribute to the oxygen demand on deep waters and lead to anoxia (70). With the variety of factors affecting the oxycline in Saanich Inlet, it is understandable that Saanich Inlet is largely defined by its relatively unstable oxic/anoxic interface (35).

The relationship between the physical and chemical properties of Saanich Inlet have intrigued oceanographers for decades, and have made the fjord an excellent field site for biogeochemical process measurements, including methane cycling (1, 14, 15, 35, 47, 75). The studies of methane oxidation in Saanich Inlet spanned both the water column (47) and sediments (14, 15) before Ward et al. (75) conducted the first high-resolution study of methane flux and oxidation in the water column of Saanich Inlet.

Saanich Inlet, like all aquatic environments, cannot exist in isolation from the atmosphere; and although gas flux from surface waters to the atmosphere is typically recognized for regulating global oxygen and carbon dioxide levels (66), the flux of methane from these environments is now a concern given its potency as a greenhouse gas (6, 33). Thus, in an effort to better resolve how methane flux from Saanich Inlet to the atmosphere is controlled Ward et al. (75) studied methane oxidation levels in the water column, especially at the oxic/anoxic interface, by measuring methane and oxygen concentrations, methane oxidation rates, and bacterial abundance over the course of the summer (July to September), with an intensive focus on August. Their intriguing results suggest that, although methane levels are high in the anoxic waters of Saanich Inlet, methane flux to the atmosphere is almost completely negated due to biological oxidation of methane (75). With the oxic/anoxic interface defined as the point where oxygen disappeared and hydrogen sulfide was detected, Ward et al. (75) determined that the interface ranged from 128 m to 140 m, and was the location within which bacterial abundance and methane oxidation rates were highest. Methane concentrations increased from the interface
to depth and from July to September. A peak in methane concentration was measured in September at 160 m and was the result of a suspected mixing event whereby methane-rich deep waters were brought to shallower depths. Methane oxidation was highest at the interface, with rates in the deeper waters nearly 100-fold less. Seasonal measurements of methane oxidation were not made, although Ward et al. (75) suggested that Saanich Inlet would be similar to other water bodies that undergo seasonal stratification and have highest levels of methane oxidation in autumn. Ward et al. (75) concluded that a more extensive time series is required to fully resolve the factors that remove methane from the water.

Evidence of methane cycling in the sediments (14, 15), methane oxidation in the water column, and biological control of atmospheric methane flux (75) in Saanich Inlet confirm its distinctive nature and strongly recommend it as an ideal OMZ laboratory. The relative ease of collecting water samples from Saanich Inlet allows and in-depth analysis of the relationship between the microbial community and oxygen and methane dynamics over space and time. However, a complete lack of knowledge of the members of the microbial community in Saanich Inlet, and a lack of seasonal methane profiles, made this relationship impossible to assess. Thus, we conducted a study in which the microbial community of Saanich Inlet was examined spatially through a high-resolution community profile taken at four depths spanning the oxycline in February 2006 and temporally at eight sampling points throughout the course of 2006 (February to November). At all microbial sampling times and depths, gas data for oxygen and methane was also collected. Nitrate data also collected at these times and depths indicated that a turnover event occurred between July and September, making this study a truly comprehensive profile of the chemical and microbial population of Saanich Inlet.
MATERIALS AND METHODS

Sample Collection

Sampling was done aboard either the CCGS John P. Tully (T) or the R/V Strickland (S) during the 2006 calendar year (Table 1). Sixteen intervals traversing the Saanich Inlet depth-continuum were chosen for high-resolution gas analysis (10, 20, 40, 60, 75, 85, 90, 97, 100, 110, 120, 135, 150, 165, 185 and 200 m). In addition, 20-L samples were collected for four distinct depth intervals spanning the oxycline for microbial community analysis (Table 1). All water sampling was conducted using standard Niskin or GOFLO bottles. In order to concentrate microbial biomass for downstream genomic DNA extraction, the 20-L samples were filtered through 47-mm GF/D prefilters, 2.7 μm nominal cut-off (Whatman, NJ) in-line with 0.22-μm Sterivex-GV filters (Millipore, MA) using a Masterflex L/S 7553-70 peristaltic pumping mechanism (Cole-Parmer, IL). After filtration, 1.8 ml of storage/lysis buffer (40mM EDTA pH 8.0, 50mM Tris pH 8.3, 0.75M Sucrose) was added to each filter prior to storage at -80°C.

Gas Collection and Analysis

Water for major and trace gas analysis was collected into glass serum bottles from the Niskin or GOFLO bottles described above. Great care was taken to avoid the introduction of bubbles. Each bottle was allowed to overflow two to three times, before being topped off and poisoned with 100 μl of saturated HgCl₂. Serum bottles were subsequently stoppered, crimp-sealed and stored at 4°C. Dissolved gases (oxygen, argon, methane) were measured using Membrane Inlet Mass Spectrometry (MIMS) (71) and standards for calibration were generated by equilibrating water samples at constant temperature with standard gas mixtures. The ratio of oxygen to argon was used to calculate biological oxygen saturation, which is a measure of net
oxygen in the water column. This measure removes physical influences from oxygen measures (e.g. temperature and salinity) and reflects changes in oxygen present due to either respiration or photosynthesis. It is analogous to a biological oxygen concentration, where a positive value indicates a net production of oxygen in the water column and a negative value indicates a net loss of oxygen.

DNA Extraction

Sterivex filters were allowed to thaw on ice prior to the addition of 50 μl of lysozyme (0.125 mg/μl). Filters were then incubated for 1 hour at 37°C with intermittent mixing. After this 50 μl of Proteinase K (Qiagen, CA) and 100 μl of 20% SDS were added and incubation continued at 55°C for 1 hour with intermittent mixing. The lysate was then removed using a 3-cc syringe. Filters were rinsed with 1 ml of lysis buffer that was then added to the original lysate. An equal volume of phenol:chloroform:IAA (25:24:1, pH 8.0) was added to the lysate and gently mixed. The aqueous layer was collected and an equal volume of chloroform:IAA (24:1) added. After gentle mixing the aqueous layer was collected and loaded onto Microcon Centrifugal filter devices (0.5ml) (Millipore). Washing with TE (pH 8.0) and DNA collection procedures were in accordance with the Microcon Centrifugal filter device protocol. DNA was suspended in 180 μl of TE (pH 8.0). DNA was quantified on a NanoDrop Spectrophotometer (NanoDrop, DE) using ~2 μl of sample. DNA quality was assessed by running 5 μl of each sample along with 100, 250 and 500 ng of HindIII ladder (New England Biolabs, MA) on 1% agarose gels in 1X TBE overnight at 15 volts.
PCR Amplification of Functional and Taxonomic Markers

DNA extracts from the February 19, 2006 depth series (10 m, 100 m, 125 m, and 215 m) were used to assess overall microbial community diversity and function with respect to dominant markers for methane oxidation pathways. Bacterial and archaenal small subunit (16S) ribosomal DNA (rDNA) sequences were amplified using the archaeon-specific primers A20F (5'-- TTCCGGTTGATCCYGCCRG) and A958R (5'--YCCGGCGTTGAMTCCAATT) and the bacterial-specific primers B27F (5'--AGAGTTTTGATCCTGGCTCAG) and U1492R (5'-- GGTTACCTTAGTTACGACTT) using the PCR profile: 3 min at 94°C followed by 35 cycles of 94°C for 40s, 55°C for 1.5 min, 72°C for 2 min and a final extension of 10 min at 72°C. Each 50µl reaction contained 1µl of template DNA, 1µl each 10 µM forward and reverse primer, 2.5U Taq (Qiagen), 5 µl 10mM deoxynucleotides, and 41.5 µl 1x Buffer (Qiagen Taq Polymerase Kit). Bacterial particulate methane monooxygenase subunit A (pmoA) sequences were amplified using primers A189F (5'--GGNGACTGGGACTTCTGG) (29) and mb661R (5'--CCGGMGCAACGTCYTTACC)(9) using the PCR profile: 30 cycles of 96°C for 25s, 54°C for 45s, and 72°C for 50s. Each 50µl reaction contained 1µl of template DNA, 2 µl each 10µM forward and reverse primer, 2.5U Taq (Qiagen), 4 µl 10mM deoxynucleotides, and 40.5 µl 1x Buffer (Qiagen Taq Polymerase Kit). Attempts were made to amplify archaenal methyl coenzyme M reductase subunit A (mcrA) using primers ME1 (5'--GCMATGCARATHGGWATGTC) and ME2 (5'--TCATKGCRTAGTDDGGRTAGT) (26) using the PCR profile: 3 min at 94°C followed by 35 cycles of 94°C for 40s, 52°C for 1.5 min, 72°C for 2 min and a final extension of 10 min at 72°C. Each 50-µl reaction contained 1µl of template DNA, 1 µl each 10 µM forward and reverse primer, 2.5U Taq (Qiagen), 5 µl 10 mM deoxynucleotides, and 41.5 µl 1x Buffer (Qiagen Taq Polymerase Kit). In order to optimize ME primer specificity, a range of MgCl2
concentrations was also tested (1 mM to 25 mM). However, no amplification was achieved from the Februauery DNA extracts.

**Clone Library Construction and Screening**

SSU (16S) rDNA and *pmoA* amplicons were visualized on 1% agarose gels in 1X TAE and purified using the MinElute PCR Purification Kit (Qiagen, CA) according to the manufacturer's instructions. Samples were recovered in 30 μl of elution buffer (10mM Tris-Cl pH 8.5) following a ten minute incubation at room temperature. Approximately 4 μl of each purified product was cloned into a pCR4-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad CA) and transformed by chemical transformation into Mach-1-T1R cells according to the manufacturer's instructions. Transformants were transferred to 96-well plates (4 for each amplicon per depth) containing 180 μl L*\textsubscript{b}kans\textsubscript{50} and 10% glycerol and grown overnight at 37°C prior to storage at -80°C. Cloned inserts were amplified directly from glycerol stocks with M13F (5'- GTAAAACGACGGCCAG) and M13R (5'- CAGGAAACAGCTATGAC) primers using the 16S rDNA PCR protocol with an additional 10 minutes at 94°C added to the very beginning of the PCR profile to ensure adequate cell lysis.

One 96-well plate per amplicon per depth was selected for DNA fingerprint screening using the common 4-base cutter Rsa I (Invitrogen, CA). Each digestion reaction consisted of 2 μl of 10x React 1 buffer, 5 μl of M13 amplified PCR product, 16 μl of sterile water and 10U of Rsa I. Reactions were incubated for 2 hours at 37°C followed by Rsa I inactivation at 65°C for 10 minutes. Restriction patterns were visualized by running 5 μl of each RSA I digestion mixture on a 2% agarose gel (20 cm in width) in 1X TAE for 90 minutes at 120 V. Restriction patterns were visually inspected and unique patterns selected and run against one another for confirmation.
Unique restriction patterns were selected for Sanger sequencing through the McGill University and Genome Quebec Innovation Centre (Montreal, Quebec, Canada). Sequence data was collected on an ABI Prism 3100 DNA sequencer (Applied Biosystems Inc, Foster, CA) using Big Dye™ chemistry (PE Biosystems, Foster, CA) according to manufacturer’s instructions. Plasmids were sequenced bidirectionally with M13F and M13R primers. Sequences were edited manually from traces using Sequencher software V4.1.2 (Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic Analysis

SSU (16S) rDNA Sequences were analyzed using the ARB program (50). Sequences were imported into the full-length 16S SILVA database (www.arb-silva.de) and aligned to the closest relative. Tree construction was performed for all bacteria SSU sequences from Saanich Inlet using the neighbour joining method implemented in ARB. Tree construction for alpha and gamma proteobacteria SSU rDNA was performed using PHYML with 1,000 bootstrap replicates. Deduced amino acid sequences for environmental pmoA clones were determined from the nucleotide sequence and aligned using the Clustal method implemented in MegAlign (DNA Star, Madison WI). A phylogenetic tree for unique pmoA sequences was generated using distance and parsimony methods implemented in PAUP version 4.0b10 (68). Bootstrapping for distance and parsimony was accomplished with 1,000 replicates per tree using heuristic search methods.

Denaturing Gradient Gel Electrophoresis

To target the variable regions 1 through 3 (V1 - V3) of the bacterial 16s rDNA, primers 357f (5'- TACGGGAGGCAGCAG) and 907r (5'- CCGTCAATTCCMTTTGAGTTT) (56) were
used. A GC clamp (5'-CGCCCGC CGCCCGC CGC CGCCCGCGCGTCCCGC GCCCCGGCCCGC) was added to the 5' end of 357f and, to aid in gel visualization, a Cy5 fluorophore (Integrated DNA Technologies, IA) was added to the 5' end of 907r. PCR amplification was with the following program: 5 minutes at 95°C to activate the HotStar Taq (Qiagen, CA) then 9 cycles of 94°C for 45 s, 65°C for 45 s with a touchdown of -1°C per cycle followed by 72°C for 1 min. This was followed by 24 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min. Lastly, there was a final extension at 72°C for 12 min. The PCR reaction consisted of 1 µl of each primer (10 µM), 5 µl of 10x Buffer (Qiagen, CA), 5 µl of 10 mM deoxynucleotides, 2.5 U Hotstar Taq (Qiagen, CA), 1 µl of 100x diluted DNA extract, and 36.5 µl of ddH2O.

PCR products were purified using the MinElute PCR Purification Kit (Qiagen, CA) and eluted with 30 µl of elution buffer (10mM Tris-Cl pH 8.5). 200 ng of each sample was loaded on a 6% polyacrylamide gel (37:5:1) containing a 30% to 70% gradient of denaturant (100% denaturant consists of 40% [vol/vol] formamide and 7 M urea) and run in 1xTAE at 85 V for 20 minutes with no samples loaded. Then, after samples were loaded, it was run for 15 minutes at 20 V and then at 85 V for 14 hours. Gels were visualized with a Typhoon Imager using a 670 BP/30 Cy5 (Red 633 nm) laser, a 480 photo multiplier tube, normal sensitivity and a pixel size of 50 µm.

Nested PCR was used to amplify the third variable region (V3) of archaeal 16S rDNA. Primers A20F and A958R were used (under the same conditions described previously) to provide a 930 bp product which was cleaned using the MinElute PCR purification Kit (Qiagen). This product was then amplified using the primers PARCH340F (5' CCCTACGGGG(C/T)GCA(G/C)CAG) and PARCH519R (5'-TTACCGCGGC(G/T)GCTG). A GC clamp (56) was added to the 5' end of PARCH340F and, to aid in visualization, a Cy5
fluorophore (Integrated DNA Technologies, IA) was added to the 5' end of PARCH519R. The PCR reaction consisted of 0.25 µl of each primer (100 µM), 5µl of 10x Buffer (Qiagen, CA), 4 µl of 10 mM deoxynucleotides, 2.5 U Hotstar Taq (Qiagen, CA), 2 µl of PCR product (from A20F and A958R), and 38 µl of ddH₂O. The PCR cycle consisted of 5 min at 95°C to activate the Hotstar Taq followed by 2 min at 92°C and then 30 cycles of 92°C for 1 min, 53.5°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 6 min. The PCR product was then cleaned and 400 ng of DNA was loaded onto an 8% acrylamide gel with a 15% to 50% denaturing gradient which was run in 0.5X TAE buffer for 10 min at 20 V, and then for 3 h at 200 V. Gels were visualized as described previously.

DGGE Gel Analysis

Fingerprints were analyzed using GelCompar II™ (Applied Maths, Belgium) software. Banding patterns across gels were normalized using a previously constructed ladder tagged with a FAM fluorophore (Integrated DNA Technologies, IA), 2 µl of which was added to each lane along with the samples and visualized on a Typhoon Imager using a 520 BP/ 40 Cy2, BlueFAM (Blue2 488 nm) laser, 480 photo multiplier tube, normal sensitivity and a pixel size of 50 µm. After normalization, bands were selected by the program using a minimum profiling level of 5% relative to maximum (to ensure that differences in total lane intensity did not affect band selection) and compared with 1% position tolerance and 1% optimization to allow for tolerance of band shifts within and amongst lanes. Band intensity was assessed using relative band surface measures of densiometric curves extracted from the fingerprints by the program (to allow for comparison across lanes). Tables of band presence or absence, or relative band intensity were exported from the program and modified to form appropriate matrices for the PC-ORD Software
For the purpose of further analysis bands were considered operational taxonomic unites (OTU's). Clustering analysis was also performed on the fingerprints using a Pearson correlation with an unweighted pair group method with arithmetic mean (UPGMA) algorithm. Densiometric curve data were used to produce the dendograms.

Richness and Diversity Estimation

Richness is a measure of total species (or OTU's) in a sample. To calculate richness fingerprint bands and RFLP patterns were used as functional OTU's. The Shannon-Wiener diversity index \( H' = - \sum_{i=1}^{S} p_i \log p_i \) is a measure of diversity of a sample, taking into consideration both the total number of species \( S \) and the relative abundance (or 'evenness') of those species \( p_i \) (39) and was calculated using PC-ORD software (53).

Statistical Analysis

Ordination of fingerprint data (band intensity) and environmental data (methane and oxygen concentrations) was conducted using nonmetric multidimensional scaling (NMS) (40, 41, 52). This method allows for relationships between variables to be analyzed without making any underlying assumptions about the distribution of the data. The Bray-Curtis distance measure is appropriate for community ecology, and was used in the ordination analysis. For both bacterial and archaeal data, 40 runs with real data and 50 runs with randomized data (for the Monte Carlo test) were conducted. A random starting figure was used to begin the ordination, and final assessment of dimensionality performed using the NMS scree plot. The relationship between fingerprint patterns was best represented for both bacteria and archaea in 3 dimensions. Environmental data could then be overlain on the NMS ordination, with the direction and length
of the vector indicating its relative strength. R-squared values were calculated to measure the
strength of correlation between the distance matrix and the axis, while r-squared values were
measured to indicate the correlation of the environmental variable vector with the axis.

Multi-response permutation procedures (MRPP) were conducted using PC-ORD (53) on
both bacterial and archaeal data to test the null hypothesis of no difference in ranked distances
amongst members of the same group. Fingerprint data was grouped according to depth, and a
Ranked Sørensen non-metric distance measure used to conduct the test. Two important values,
the test statistic T (describing the separation amongst groups) and the agreement statistic A
(within group similarity) were determined by MRPP. The more negative the T value, the stronger
the separation, while A values range from −1 to 1, where A=1 indicates all items within a group
are identical, A=0 indicates heterogeneity within a group is the same as would be expected by
chance and A<0 when there is less agreement between groups. A p-value was determined, and
results were considered significant if p<0.05.
RESULTS

Environmental Data

The chemical profile of Saanich Inlet was distinguished by the changes in oxygen and methane with depth, and by the changes in this depth profile over the course of a year (14, 15, 23, 47, 75). This chemical variability made it essential to measure oxygen and methane concentrations for Saanich Inlet over a wide range of depths and times. A high-resolution depth profile was determined in February and, based on the data collected, a series of depths were chosen to represent the water column chemistry throughout the year.

February gas data (Fig. 3) show the depth stratification of methane and oxygen in Saanich Inlet. The oxygen concentration was high at the surface and decreased steadily until a depth of 125 m. After this point oxygen levels remained fairly stable, with the lowest concentration occurring at 215 m. The methane concentration was below 200 nM from the surface to approximately 125 m, where it drastically increased to approximately 400 nM. This gradient is quite steep compared to the slow increase in methane concentration that occurred from 135 m (400 nM) to 215 m (800 nM). This chemical profile confirmed previous evidence of an oxic/anoxic interface, and a possible methane-oxidizing zone at approximately 125 m (75). This interface spans several meters, and is a unique location in the water column where oxygen levels decrease to near zero, while methane levels are rapidly increasing. Thus, to monitor this zone and the oxic and anoxic environments above and below it, gas measurements were taken at 10 m, 100/105 m, 120/125 m, and 200/215 m seven more times throughout the following year (Table 1).

There were changes in both oxygen and methane concentrations in Saanich Inlet from February to July 22 (Fig. 4 and 5). Samples taken from 10 m exhibited not only much higher
oxygen concentrations than those at 100 m, 125 m, and 200 m, but also far greater variation (Fig. 4). At 10 m depth the oxygen concentration peaked in June and decreased sharply over the course of July. February and July 22 samples from 10 m had very similar concentrations, with July 22 being slightly less. In comparison, samples from 125 m and 200 m exhibited a relatively stable pattern, with highest levels seen in February and lowest levels seen in May. Although 200 m depth had greater oxygen saturation than 125 m in February, it decreased from February to April and from that point on remained relatively stable. At 100 m oxygen concentrations decreased between April and May and were relatively stable until July 22.

As was expected from the February profile, and previous measurements made in Saanich Inlet (75), methane concentrations were highest at 200 m for all sampling times (Fig. 5). Methane samples at 200 m were also the most dynamic, ranging from 700 nM, in February, to 1500 nM on July 22. Methane concentrations at 200 m increased from February to May, decreased in June by nearly 200 nM, and increased again in July. Other than a minor peak in the methane concentration on July 6 at 125 m, the three shallower depths had relatively constant and low (below 200 nM) levels of methane.

16S rDNA Sequence Analysis

Because of the unique nature of Saanich Inlet’s chemical profile (high levels of methane in anoxic bottom waters and fully oxic shallow waters with low methane levels) a profile of the microbial community was conducted to determine whether the bacteria and archaea present in Saanich Inlet are similar to those found in other oxygen minimum zones (OMZ’s). This was achieved by phylogenetic analysis of 16S rDNA clone libraries using an ARB (50) database that contained relevant OMZ sequences (22, 43, 51, 73, 81). This analysis revealed that the majority
of bacterial sequences found at all depths were Proteobacteria (Fig. 6), which is similar to sequence data collected from the Black Sea, Baltic Sea, Cariaco Basin, Arabian Sea, and Namibian Upwelling (22, 43, 51, 73, 81). Saanich Inlet sequences also belonged to the OD1, Cyanobacteria, Actinobacteria, Nitrospina, Gemmatimonadetes, Chalditrhix, Plactomyces, Verrucomicrobia, Bacteroidetes, and Spirochaetes groups. With the exception of the Gemmatimonadetes (minorly represented at 100 m), members of these groups are also found in the other OMZ’s mentioned above (22, 43, 51, 73, 81).

The clone libraries indicated community depth trends specific to Saanich Inlet. The most noticeable trend was the increasing proportion of proteobacteria with depth (Fig. 6). Although alpha proteobacteria decreased with depth, to the point of being completely absent from the 215-m library, gamma proteobacteria increased with depth and were the only proteobacteria represented at 215 m. Beta proteobacteria, delta proteobacteria, and epsilon proteobacteria represent minor members of the community, and were totally absent at 215 m. The very minor representation of epsilon proteobacteria in the library sets Saanich Inlet apart from the other OMZ’s used for comparison (22, 43, 73, 81). The other bacterial divisions are present in Saanich Inlet at relatively low abundance, with the greatest representation and variety found at 10 m and 100 m (Fig. 6).

The dominance of the gamma proteobacteria at depth in Saanich Inlet warranted a more detailed resolution of this group. Upon closer examination, it was found that relatives of sulfur-oxidizing bacterial symbionts (Oceanspiralles_5 Oleiphilaceae members) were by far the most prevalent members of the gamma/beta proteobacteria detected, and they dominated the clone library from 215 m (Fig. 7). Also, groups with possible functional significance in methane cycling, members of the Methylophilaceae (found at 10 m and 100 m) and members of the
Methylococcaceae (found at 100 m and 125 m), comprised a very small part of the gamma proteobacteria community.

Keeping the methane profile chemical profile of Saanich Inlet in mind, I further analyzed the two groups in which methane-oxidizing bacteria are known, the alpha proteobacteria (amongst which the Type-II methanotrophs cluster) and gamma proteobacteria (amongst which Type-I methanotrophs cluster). Sequences from these groups in Saanich Inlet were found to cluster with sequences from known oxygen minimum zones, methanotrophs, and several known symbiont relatives (Fig. 8). Alpha proteobacterial sequences from Saanich at 10 m and 100 m were related to those of sulfite oxidizers, Baltic Sea, Black Sea, and Arabian Sea species (22, 43, 73). However, a much wider range of Saanich Inlet sequences were found in the gamma proteobacteria, particularly sequences from 10 m and 100 m, which are related to those of methylotrophic species and a sequence from the Namibian upwelling. As well, Saanich Inlet sequences from 100 m and 125 m clustered with those of cultured methanotrophs, a Black Sea sequence, and a sequence isolated from an anaerobic methane oxidizing community in sediments. Several Saanich Inlet sequences from 10 m are related to Namibian upwelling, Arabian Sea, and Black Sea sequences and are also closely related to a large group of Saanich Inlet sequences from 100 m, 125 m, and 215 m that grouped very closely to sulfur-oxidizing symbionts isolated from several eukaryotic organisms.

Analysis of the archaeal 16S rDNA gene clone libraries indicated that the archaea detected belonged almost exclusively to the crenarchaeota. Euryarchaeaa represented less than 20% of the libraries from 10 m, 100 m, and 125 m and were completely absent from 215 m. Archaeal sequences from Saanich Inlet were also related to sequences from other OMZs, although the other OMZs exhibited the reverse distribution of archaea, with euryarchaeaa more
abundant than crenarchaea. As well, no members of the archaeal 16S rDNA clone libraries were related to known methanogens or anaerobic methanotrophs (ANME groups). This is unlike the Black Sea, a methane rich OMZ (19).

**pmoA**

Taking into consideration the methane concentrations found in Saanich Inlet, the inability to amplify the marker gene for methanogens (methyl coenzyme-M reductase; *mcrA*) (27), and the presence of 16S rDNA genes associated with methanotrophs in the Inlet (reported above), an effort was made to better understand the methane oxidizing community of Saanich Inlet. This was accomplished by analysis of clone libraries containing a fragment of a gene encoding a key enzyme component involved in methane oxidation, the alpha subunit of particulate methane monooxygenase (*pmoA*) (76). The phylogeny of the genes detected was subsequently analyzed by comparison to existing classification of the gene. This analysis established that *pmoA* sequences detected in Saanich Inlet were related primarily to Type-I methanotrophs (Fig. 9). Although one phylotype was found to belong to the Type-II methanotrophs, the majority of *pmoA* phylotypes belonged to Type-I, and displayed a distinct depth distribution (Fig. 10). Phylotype A was found at all depths, although its relative abundance decreased with depth, whereas the relative abundance of phylotype B, also found at all depths, increased with depth. Other *pmoA* phylotypes (J and K) were far less abundant than A and B and were absent from the 215-m library. The relationship of the *pmoA* genes found in Saanich Inlet to other OMZ’s and methane-oxidizing communities was determined through phylogenetic analysis of the gene as well. This analysis demonstrated that the phylotypes found in Saanich Inlet represented novel *pmoA* sequences. Phylotype A was most closely related to *pmoA* from the eastern Pacific ocean.
OMZ and distantly related to \textit{pmoA} from cultured methanotrophs. Phylotype B was most closely related to \textit{pmoA} sequences from deep sea hydrothermal vents and methanotrophic symbionts of the mussel \textit{Bathymodiolus}, and was also related to the cultured methanotroph \textit{Methylomonas sp. LW15}. (Fig. 8)

**Denaturing Gradient Gel Electrophoresis**

Saanich Inlet is recognized for its seasonal anoxia (23, 47, 75) and the chemical profile of Saanich Inlet obtained over the course of the year indicated that, along with great spatial variability, there were temporal changes in oxygen and methane concentrations in the water column that culminated in a deep-water turnover event between July 22 and September. To study whether the bacterial and archaeal community also demonstrated spatial and/or temporal change, denaturing gradient gel electrophoresis was used to obtain bacterial fingerprints for each of the four depths at all eight sampling times.

On the basis of Pearson correlation, fingerprints from February at 10 m and 100 m cluster more closely than those from 125 m and 200 m (Fig. 11). Archaeal fingerprints from February at 10 m and 100 m also cluster together, although the fingerprint from 200 m groups more closely to this cluster than that from 125 m, unlike for the corresponding bacterial fingerprint (Fig. 11). Thus, bacterial and archaeal communities at 10 and 100 m are relatively similar and distinct from those of greater depth.

When all the sampling times were analyzed, the primary trend evident from Pearson correlation of bacterial and archaeal DGGE fingerprint data was a clustering of fingerprints by depth, with a few notable outliers (Fig. 12 and 13) This suggests that community composition was generally stable at each depth over most of the year. For bacteria (Fig. 12), the 10-m and
100-m fingerprints cluster together and are grouped apart from those of 125 m and 200 m, which form their own cluster. All 10-m samples cluster together save for April, which was dissimilar to all other fingerprints. The 100-m samples from February clustered with the 10-m samples. The 200-m cluster displayed the tightest clustering, with September and November grouping most closely with each other. The samples from 125 m formed two clusters; a cluster that included May, June, and July samples, and a cluster that included February, April, September, and November samples. September and November samples at 100 m clustered with September and November at 125 m. Further confirming the interpretation of Pearson correlation data that suggested the samples are primarily clustered by depth, MRPP analysis indicates that there is a significant separation of fingerprints based on depth and that fingerprints at the same depth are more similar than dissimilar (T=-8.24; A=0.16; p<0.01).

Pearson correlation analysis of archaeal DGGE fingerprints (Fig. 13) indicated that they also cluster primarily by depth. The July 6 fingerprint at 10 m was unlike others from that depth as it grouped with fingerprints from 100 m and 200 m. As well, the fingerprint from May at 100 m is grouped more closely with the May 10-m sample than other 100-m samples, as was seen with the bacterial DGGE fingerprints. Save for May, the 100-m samples cluster tightly, as do the fingerprints from February to July 6 at 125 m. The remaining samples from 125 m (September, November and July 22) cluster with September and November at 200 m. The MRPP analysis of archaeal fingerprints indicates a conclusion similar to that for bacterial DGGE fingerprints; there is a separation of fingerprints based on depth and similarity of samples within a depth (T=-12.8; A=0.3; p<0.01).

For all samples, visual inspection of the DGGE gels confirmed the similarity analyses. The variable presence and intensity of visible bands corresponded with calculated relative
differences in similarity. It should be noted as well that for the depth series with the most visually evident fingerprint variability, archaea at 200 m, the bands that appeared in September and November were, according to visual inspection, not novel to 200 m as they were also seen in the 125-m fingerprints.

**Diversity and Richness**

Diversity, as calculated by the Shannon-Wiener index, and richness values were also utilized as indicators of spatial variability and temporal change. The bacterial 16S rDNA clone libraries showed a depth-dependent diversity trend, with overall richness and diversity decreasing substantially below 100 m (Table 2). In comparison, diversity of the archaeal 16S rDNA clone-libraries (Table 3) remained relatively constant from 10 m to 125 m, with a noticeable decrease in diversity and richness at 200 m. When richness values for archaea were taken into consideration, archaea and bacteria differed drastically once again. Archaeal richness values were similar to bacterial values found at 200 m, and were much less than bacterial values at 10 m, 100 m, and 125 m.

The diversity indices for the February 16S rDNA clone libraries and DGGE fingerprints had similar trends (Tables 2 and 3). Although not as evident as in the bacterial clone library, the DGGE diversity was generally highest at 10 m and 100 m and lowest at 200 m. There was not an obvious decrease between 100-m and 125-m fingerprints as was seen in the clone libraries, but most samples, with the exception of low values for May at 10 m and September at 125 m, do show higher diversity at shallow depths. Archaeal fingerprint diversity also exhibited trends similar to the archaeal clone libraries, with very little change seen amongst 10-m, 100-m, and 125-m samples. From February to July 6 there was a decrease in fingerprint diversity at 200 m,
much as was seen in the clone library, yet July 22, September, and November fingerprints at 200 m show no such decrease. Richness values for bacterial fingerprints are generally higher than those for the archaeal fingerprints (as was seen in the clone libraries), although the sudden decrease at 200 m is not as evident in the fingerprint data as it was in both the bacterial and archaeal clone libraries.

**Nonmetric Multidimensional Scaling**

Nonmetric multidimensional scaling (NMS) is an excellent method to compare community change to environmental data that, for Saanich Inlet, consisted of oxygen and methane concentrations at specific depths from February to July 22. The ordination of bacterial and archaeal DGGE fingerprints resulted in a general clustering of fingerprints according to depth (Fig. 14 and 15), much as was seen in the Pearson correlation of DGGE fingerprints (Fig. 12 and 13). NMS ordination of bacterial DGGE fingerprints showed that axis 3 represented 32.9% of the variance, whereas axis 2 represented 23.9% of the variance in the samples. Oxygen and methane are correlated with these two axes, with oxygen correlating more strongly with axis 3 than axis 2 ($r^2=0.305$ and $r^2=0.164$, respectively) and methane correlating more strongly with axis 2 than axis 3 ($r^2=0.096$ and $r^2=0.200$, respectively). The ordination of bacterial fingerprints was by depth, with 200-m samples clustering quite tightly and fingerprints from 10 m grouping together loosely. Samples from 10 m are far more distantly placed from other fingerprints, including those from 100 m, 125 m, and 200 m. Notable exceptions to the general depth clusters include the fingerprint from May at 100 m, which grouped with 10-m samples, and those samples from May, September and November at 200 m, which group with fingerprints from 100 m and 125 m. These fingerprints were also outside their depth clusters in the Pearson correlation.
analysis. The correlation of methane and oxygen vectors with the ordination axes, axes that resulted in a grouping of fingerprints primarily by depth, is the first indication that these chemical variables are related to the depth distribution of microbial community fingerprints.

NMS ordination of archaeal DGGE fingerprints resulted in axis 2 representing 39.4% and axis 3 representing 39.4% of the variance in the samples. Oxygen is correlated to both axis 2 and axis 3, although more strongly to axis 3 ($r^2 = 0.116$ and $r^2 = 0.440$, respectively). Methane is correlated almost solely to axis 3 ($r^2 = 0.028$ and $r^2 = 0.240$, respectively). As was seen in the bacterial NMS ordination, 10-m samples clustered relatively loosely and included the fingerprint from May at 100 m. Fingerprints from 200 m cluster tightly with the exception of September and November 200-m fingerprints, which grouped more closely to samples from 100 m and 125 m. The overall trends in ordination of archaeal fingerprints are similar to those of the bacterial fingerprints, with fingerprints clustering primarily according to depth. As well, the correlation of methane and oxygen vectors with the ordination axes, as was seen in the bacterial fingerprint ordination, indicates that methane and oxygen are related to the depth distribution of archaeal community fingerprints.
DISCUSSION

As the impact of microbial metabolism in marine environments on both biogeochemical cycling (31) and atmospheric conditions (37) is slowly elucidated, there is a compelling requirement to identify the organisms that comprise these communities. My analysis of Saanich Inlet provided a comprehensive profile of the microbial community in February 2006, and my study of the temporal dynamics of the microbial community gave some insight into the environmental characteristics that may impact its composition.

Microbial Community Profile: February 2006

A microbial community profile is an extremely valuable tool in understanding how one study site relates to another and, to a very limited extent, is capable of giving an idea of the functional potential of some microbial groups present. Our analysis of Saanich Inlet indicated that its microbial community was quite similar to other OMZ's used for comparison. Of course, caution must be taken when assessing the degree of similarity or difference amongst communities; site to site variations in nutrient levels, light levels, and dissolved gases (such as hydrogen sulfide) are just a few of the factors that can affect microbial community composition (36), and they were not considered here. However, keeping in mind the dominant and well-recognized affect anoxia has on communities (28, 45, 46, 80), it is useful to compare a seasonally anoxic environment with established biological controls on methane flux to OMZ’s which are stably stratified and, save for the Black Sea, are not recognized for methane cycling.

Although the generally low representation of groups outside of the proteobacteria makes assessing the consequences of their presence/absence difficult (especially as experimental confounders such as 16S rDNA clone library bias may affect relative abundance assessments
(74)), these groups give some indication of the general carbon and nitrogen cycling processes occurring in Saanich Inlet. Members of the *Bacteroidetes* are recognized for their ability to degrade a wide variety of high-molecular-weight compounds, including proteins and carbon sources such as chitin (10). Members of this group normally associate with particles (12, 81), and their absence in Saanich Inlet below 100 m may be due to a lack of oxygen, which would be required to degrade organic matter. Their general under representation in the clone library is slightly puzzling, as the high productivity of Saanich Inlet would seem to make it an ideal environment for these exoenzyme-producing heterotrophic bacteria. However, this group is known to be difficult to study using conventional clone library techniques, and studies to assess their diversity in marine environments have used more specific 16S rDNA primers for clone library analysis of *Bacteroidetes* (3). Future studies using different sets of universal 16S primers may indicate that the *Bacteroidetes* is better represented in Saanich Inlet. It is interesting to note, however, that according to the study by Vetriani et al (73), the *Bacteroidetes* were not represented in the Black Sea, although this may be due to a lack of resolution in the oxic zone, where the highest levels of primary productivity would take place.

*Actinobacteria* are well represented in both coastal and open ocean waters (60), and in most of the OMZ’s studied save for the Black Sea and Cariaco Basin. Once again, their niche as heterotrophic organisms capable of aerobically degrading a wide variety of compounds is likely responsible for their representation at 10 m and 100 m in Saanich Inlet, and for their absence in OMZ’s where the anoxic chemocline was the focus of community profiling (51, 73). A relationship previously noted between beta proteobacteria and *Actinobacteria* (25, 60) may also be occurring in Saanich Inlet, where members of the beta proteobacteria are represented at the same depths and with similar relative abundance as the *Actinobacteria*. In fact, the beta
proteobacteria represented in Saanich Inlet belong to similar groups, especially *Methylophilus*, as those found by Rappé et al. (60) in coastal ocean waters. The data on the detection, abundance and depth distribution of taxa containing nitrite-oxidizing bacteria in Saanich Inlet may help elucidate the nitrogen cycling taking place in the water column. The distribution of *Nitrospina* (detected at 100 m and, to a lesser extent, 10 m) was similar to the nitrococcus group of the gamma proteobacteria (found at 100 m) and may indicate that nitrite oxidation takes place close to the oxic/anoxic interface. Members of this group were in other OMZs, except the Black Sea and Cariaco Basin, likely for reasons related to sample resolution at the oxycline (as described above). Notably, ammonia-oxidizing bacteria were not detected in Saanich Inlet. This may be a result of crenarchaeal dominance of this nutrient-cycling niche (54). In fact, the alpha-subunit of the ammonia monoxygenase (*amoA*) gene found in crenarchaeota was detected in Saanich Inlet (unpublished data).

The *Planctomyces* have been recently recognized as the group containing the so-called ‘annamox bacteria’ (42). Although members of this group were not abundant in Saanich Inlet (and no sequences related to candidate annamox bacteria were detected), caution should be taken in concluding this group is not functional relevant. In the study conducted by Woebken et al. (81) specific *Planctomyces* 16S rDNA primers had to be developed to study this group, and whereas *Planctomyces* were poorly represented in the microbial community survey conducted by Vetriani et al. (73) in the Black Sea, Kuyper et al. (42) went on to detect and describe the annamox bacteria in a more focused study of the same environment.

Markedly absent from Saanich Inlet were any sequences similar to *Pseudomonas*-like species found in abundance in the Black Sea and Baltic Sea as well as in the Cariaco Basin and Namibian Upwelling. Remarkable for their degradation of chitin and secretion of extracellular
enzymes (2, 30), their lack of representation in a highly productive environment like Saanich Inlet is puzzling, although not completely unusual, as this group was not detected in the Arabian Sea (22) either. Environmental conditions, including a lack of marine eukaryotes with which Pseudoalteromonas species normally associate (30) may be responsible for the absence of this group in Saanich Inlet. It is also possible that the group was not abundant at the time of sampling and may increase throughout the year, although this is unlikely given the stability of the bacterial fingerprints over time.

Proteobacterial sequences were abundant in Saanich Inlet, as they were in most OMZ’s studied. Within this group, however, there are several thought-provoking similarities and differences between Saanich Inlet and the other OMZ’s studied. Whereas alpha proteobacteria, beta proteobacteria, and gamma proteobacteria sequences detected in Saanich Inlet appear quite similar to those from other OMZ’s, the lack of representation of the delta proteobacteria and epsilon proteobacteria sets Saanich Inlet apart.

The alpha proteobacteria represented in Saanich Inlet belong to well-studied groups. These included sequences related to SAR11 and Type-I methanotrophs, which were detected in other OMZ’s (save for the Cariaco Basin, in which there is a conspicuous absence of alpha proteobacteria). Sequences related to Roseobacter species (data not shown) and Sulfitobacter species were also present in Saanich Inlet, and these groups are also found in the coastal waters studied by Rappé et al. (60).

Relatives of sulfur-oxidizing bacterial endosymbionts within the gamma proteobacteria were detected in all four depths in Saanich Inlet, and in all the OMZ’s studied save for the Cariaco Basin. Although variations in experimental methods make comparing the relative abundance of this group over different OMZ’s difficult, their nearly complete dominance of all
phytotypes found at 215 m is unique to Saanich Inlet. Making the presence of these organisms all the more fascinating, Saanich Inlet is the shallowest of the OMZ's used for comparison and is not known to host the deep-sea mussels from which the endosymbionts were originally isolated (18). The functional role of these organisms is perhaps one related to sulfur cycling, yet 16S rDNA sequence data is far from adequate evidence to make a confident assessment. As well, sulfur species were not analyzed in this study, making interpretations about the sulfur cycle difficult. And although these sequences were found primarily below the oxic/anoxic interface, where hydrogen sulfide may be available for oxidation (hydrogen sulfide was detected at 215 m in February by its distinctive odor), the lack of oxygen at 215 m makes drawing conclusions about their metabolic activity extremely difficult. Given the wide geographic distribution of these gamma proteobacteria in oceanic systems, further study into their functional niche would be worthwhile. Their abundance in Saanich Inlet makes it an ideal environment to employ metagenomic techniques to further resolve this group’s metabolic pathways.

The sequences related to Type-I methanotrophs detected in Saanich Inlet are likely of functional significance, and are similar to sequences found in the Black Sea. Detected where they were expected, at the oxic/anoxic interface in Saanich Inlet, these groups seem less abundant than may have been expected given the levels of methane oxidation measured by Ward et al. (75). However, 16S rDNA clone library analysis is subject to DNA extraction, PCR amplification, and DNA cloning biases (74), all of which make abundance measures difficult. Yet, the phylogenetic relationship between these sequences and those found in the Black Sea does indicate that Saanich Inlet is similar to an OMZ where methane-cycling takes place.

The few delta proteobacteria sequences found in Saanich Inlet were not like those found in other OMZ’s. Poorly represented in the 16S rDNA clone library, there were no sequences
related to delta proteobacteria known to cycle sulfur species. As mentioned previously, sulfur cycling in Saanich Inlet is not well understood, and there may be a seasonal sulfur phenomenon responsible for the abundance of this group that caused it to be poorly represented in February.

Of the groups not detected in Saanich Inlet in February, the relatives of the epsilon proteobacteria *Thiomicrospira denitrificans* (capable of fixing carbon dioxide using sulfide and nitrate) is most remarkable. The abundance of this group in other OMZ’s, save for the Arabian Sea, is notable and perhaps indicative of a functional niche for these organisms. Previous studies have suggested that the accumulation of hydrogen sulfide is required (48) and that the epsilon proteobacteria are best represented in deep basins where hydrothermal vents are present (48, 49). Thus, the seasonal nature of the anoxia, combined with the relatively shallow depth of Saanich Inlet may give some insight as to why the group is not represented. Nevertheless, it is important to remember that any microbial profile is a snapshot of a certain time and space. Oxygen concentrations at depth in Saanich Inlet are highest in February, and decrease throughout the year, making it possible that the epsilon proteobacteria will become more abundant in later months.

The role archaea play in the oceanic environment is being elucidated at a rapid pace, especially their role in anaerobic methane oxidation (4) and nitrogen cycling (44). In Saanich Inlet the abundance of crenarchaeal groups compared to euryarchaeal ones sets it apart from the only other OMZ recognized for methane cycling, the Black Sea. Within the archaeal populations in the Black Sea sequences related to both methanogens and ANME-1 (methane producers and consumers) were detected, whereas in Saanich Inlet the few euryarchaeal sequences recovered were not related to either of these groups and were not present at all at 215 m. However, both of these groups are strict anaerobes, and oxygen levels in February may have been too high to allow
the growth of these species. Saanich Inlet is more similar to the Namibian Upwelling system, where crenarchaea dominated the 16S rDNA clone library in the anoxic zone (130 m). Both Saanich Inlet and the Namibian Upwelling are rich in nutrients, and thus the crenarchaeal distribution may be a reflection of the role these organisms play in nitrogen cycling.

**Methane Cycling in Saanich Inlet**

The microbial community profile suggested methane-oxidizing bacteria were present in the water column of Saanich Inlet, and the clone library profile of the gene particulate methane monooxygenase (pmoA) confirmed that genetic evidence of bacterial methane oxidization was present at all depths. Interestingly, amongst the novel phylotypes detected, there is a distinct depth distribution, with one phylotype dominant at 10 m and one at 215 m. Although these data were initially surprising, given methane oxidation can only occur in aerobic environments, evidence of aerobic methanotrophs in anaerobic waters was also found in the Black Sea (19, 64). Because a measure of the gene’s presence in an environment is not an accurate indicator of the associated enzyme’s activity, Schubert et al. (64) postulated that the detection of pmo in anaerobic waters was as a result of ‘sinking’ methanotrophs from the oxic to anoxic waters. This is unlikely in Saanich Inlet, as phylogenetic assessment of the gene indicates that the copies detected in abundance in shallower waters are different from those abundant at depth. In fact, the study by Schubert et al. (64), which employed DGGE to detect different 16S rDNA phylotypes associated with Type-I methanotrophs, also indicated there was a chemocline-dependent distribution of phylotypes. Thus, their hypothesis that these groups are as a result of methanotrophic communities falling through the water column may be incorrect. They also postulated that these groups might not be active at this depth and at such low levels of oxygen.
This possibility raises the question as to why a specific phylotype of inactive organisms would be found below the chemocline. One possibility is that these Type-I methanotrophs are capable of utilizing very low levels of oxygen that may be transiently present in the anaerobic zone of the water column (oxygen concentrations below 1 μM cannot be measured). Although these questions cannot be answered with current data from Saanich Inlet, the data collected do suggest that some of the pmoA phylotypes isolated (J and K) may be restricted to certain oxygen concentrations. Saanich Inlet provides an excellent site to study this further; as the increased anoxia over time at depth may allow a study of what methanotrophic communities are more or less abundant at certain oxygen concentrations. Sequence data obtained from the February clone libraries will allow Saanich-specific primers to be developed for a technique such as quantitative PCR (Q-PCR). Utilizing Q-PCR would allow for abundance of the phylotypes present in Saanich Inlet to be resolved with far greater accuracy, and would be appropriate to study spatial differences and temporal changes.

The presence of pmoA at depth in Saanich Inlet, combined with the absence of both mcrA and groups related to either methanogens or ANME in the archaean 16S rDNA clone libraries suggests that aerobic methane oxidation is the only part of the methane cycle occurring in the water column of Saanich Inlet. As discussed previously, the relative levels of oxygen at depth in Saanich Inlet, compared to the Black Sea where these archaean groups were detected (19, 64, 73), may explain the absence of these strict anaerobes. However, the absence of these groups from Saanich Inlet’s water column by no means precludes the possibility that they will be found in the sediments. In fact, the levels of anaerobic methane oxidation measured in the sediments were four times greater (14, 15) than the highest rates of methane oxidation observed by Ward et al. (75). Investigation, using 16S rDNA and mcrA clone libraries, into what archaean groups are
present in the sediments in Saanich Inlet may serve to greatly enhance our knowledge of methane cycling in this system. The data collected to date suggest that seasonal anoxia does not allow the organisms involved in the anaerobic components of the methane cycle to flourish.

The relationship of the pmoA phylotype most abundant at depth to the pmoA associated with the Bathymodiolus symbiont in an intriguing one and raises questions about the community at depth in Saanich Inlet. Indeed, the relative abundance of the 16S rDNA clones related to the sulfur-oxidizing bacterial endosymbionts, and their abundance at depth, may indicate a relationship with the pmoA phylotypes detected at depth. Methanotrophic bacteria are known to be endosymbionts of mussels, and although no relatives to methanotrophic endosymbionts were detected in the 16S rDNA clone library, the abundance of relatives to sulfur-oxidizing bacteria raises interesting questions about the role of ‘endosymbiont relatives’ in Saanich Inlet. These questions further strengthen the case to resolve the metabolic nature of these organisms.

**Microbial Community dynamics: Spatial variability and temporal change**

The remarkable spatial and temporal chemical profile of Saanich Inlet is reflected by limited changes in the bacterial and archaeal communities with depth and time. The methods employed to analyze changes in the microbial community (Pearson correlation and NMS ordination) indicated that changes in oxygen and methane over depth appear to affect the distribution of the microbial community and that, save for the major water turnover event which occurred between July 22 and September, minor variations in chemistry from February to July 22 did not have a substantial effect. Of course, any assessment of the microbial ‘community’ dynamics demands a discussion of how the community was defined and represented in this analysis. Since profiling a microbial community over space and time demands a relatively high-
throughput technique that can compare all samples under set conditions, a 16S rDNA clone library (although capable of providing a high-resolution community profile) is an inefficient method to measure community change. Thus, 16S rDNA fingerprints derived from denaturing-gradient gel electrophoresis (DGGE) were used to represent the bacterial and archaeal communities found at each sample depth and time. Bands on these gels were considered operational taxonomic units (OTU’s), and were used as proxies to represent community diversity but not the individual bacterial or archaeal species themselves. Thus, variability in the number, placement, and relative size of these OTU’s is considered an appropriate measure of community, rather than species, change over time and space.

The spatial trends evident in the February DGGE dendograms are found throughout the temporal data as well. February dendograms suggested that microbial populations at 10 m and 100 m were more similar to those found at 125 m and 200 m, and this is reasonable to expect given the well-recognized effect of the oxic/anoxic interface.

The evidence that the temporal and spatial trends of the microbial community were relatively stable made the association of September and November fingerprints at 200 m with fingerprints from 100 m and 125 m of great interest. After taking into consideration that these were microbial fingerprints from after a deep-water renewal (indicated by a flow of nitrate into the bottom waters (1)) it is very likely that this is evidence that the community composition at 200 m has changed. It is unlikely, given the tight clustering of the September and November 200-m fingerprints with fingerprints from 100 m and 125 m, that novel bacterial or archaeal populations established themselves after the turnover. Rather, the NMS ordination and Pearson correlation suggest that the turnover event resulted in the mixing of populations from a shallower depth into deeper waters, although without sequence data this cannot be confirmed. The mixing
event may also have affected the relative abundance of groups already present at depth, but it is impossible to determine whether this is as a result of more of the same groups from shallower waters being introduced to the deep water, or a nutrient or chemical change which may affect the group’s abundance. Interestingly, the noticeably low diversity value of the 125-m fingerprint in September may also be an indication of a change in community composition.

The nutrient dynamics of Saanich Inlet may have influenced communities at shallower depths. The first instance of this is the bacterial fingerprint in April at 10 m, a sample that may have been taken when those waters were experiencing the first increase in light and temperature, resulting in the increased abundance of phytoplankton, and thus an associated increase in the availability of carbon and nutrients for bacterial groups that were minor community members in winter. It is possible these groups require the high nutrient levels present in spring, and are out-competed as the summer progresses. The clustering of both the bacterial and archaeal fingerprint from May at 100 m with with 10-m fingerprints, and the lower than typical bacterial diversity at 10 m in May, may be as a result of the spring bloom that typically occurs in Saanich Inlet (23). Light penetration to deeper waters and high nutrient concentrations in spring may have made it possible for groups that normally reside at the surface (where both light and nutrients are high) to grow at 100 m. When this bloom finished these groups would have been isolated at 10 m once again. Adding nutrient data (especially nitrogen species) to the NMS ordination would aid in assessing the impact of the spring bloom on microbial communities. It would also help resolve the factors affecting the sharp decrease in bacterial diversity between 100 m, and 125 m. As the data is now, it is difficult to determine whether this is a result of a sudden drop in oxygen concentration or a drop in available nutrients, both of which are plausible.
The NMS ordination of the microbial fingerprints is the first link between oxygen, methane, and the distribution of microbial populations throughout the year. The relatively strong correlation with the axes by both methane and oxygen indicates they are related to the ordination of the fingerprints. And since this ordination results primarily in clusters formed according to the depth of the fingerprint, a link between community composition and oxygen and methane can be made. The addition of other environmental factors such as temperature, salinity, and nitrogen species (as mentioned previously), would greatly enhance our ability to link community profiles with environmental data. It would also help to determine whether or not there are certain environmental factors that affect archaea to a greater or lesser extent than bacteria and help to explain why archaeal community diversity remains relatively stable above 200 m, whereas bacterial community diversity changes at the oxic/anoxic interface. As well, oxygen and methane data for September and November are currently unavailable, and are thus absent from the environmental vectors. This makes drawing conclusions about the affect changes in deep-water oxygen and methane levels have on the microbial community difficult. Along with the addition of environmental data, community assessment would greatly benefit from a study of what microbial species were most affected by the environmental variables. A first step in this study would be to sequence those bands that correspond to the operational taxonomic unites (OTU’s) found to be most strongly correlated to the final ordination of the fingerprints. Sequencing those bands will greatly increase our knowledge of the microbial species of Saanich Inlet that may be affected by the seasonal changes and/or environmental variables.
Conclusions and Future Research

This study has determined several important features of the microbial community in Saanich Inlet. As expected, the microbial community of Saanich Inlet was similar to other oxygen minimum zones, and some of its methanotrophic members are likely capable of carrying out methane oxidation in the water column. I found no evidence of methane production or anaerobic methane oxidation occurring in the water column, yet this is not entirely unexpected given the temporal oxygen profile of Saanich Inlet, and does not preclude these processes from occurring in the sediment. As well, the community appeared to be distributed primarily according to depth, and this distribution was related to methane and oxygen concentrations. Lastly, the microbial community at 200 m seemed to be affected by a deep-water renewal that occurred between July 22 and September of 2006.

There are many questions surrounding the microbial relationship to the chemical dynamics of Saanich Inlet that remain to be studied. A variety of environmental genomic techniques, such as metagenomic libraries and high-throughput 16S rDNA clone screening can better resolve the microbial community’s functional capacity in regards to nutrient cycling and gas-flux regulation. Questions related to the relatives of sulfur-oxidizing bacterial endosymbionts that dominate at depth, including those regarding their metabolic and symbiotic capacities, could be answered using these techniques. Further chemical profiling of sulfur species would be helpful in answering these questions, and would also help resolve if the relatives of the epsilon proteobacteria *Thiomicrospira denitrificans* are absent due to a lack of sulfide. Methane cycling can be further studied by assessing the temporal dynamics of *pmoA* using Q-PCR, and by measuring methane oxidation rates in Saanich Inlet to help determine whether or not these groups are metabolically active at depth. Additional environmental data, combined with
sequence data from the OTU's that were strongly correlated to the ordination, would make the NMS ordination a far stronger tool in assessing the impact of the chemical and nutrient profile of Saanich Inlet on the microbial community and how seasonal anoxia sets Saanich Inlet apart from other oxygen minimum zones. Indeed, the data collected and analyzed in this study only serves to emphasize that Saanich Inlet is an environment that will serve as a fascinating study system for many years to come.
Table 1 – List of sampling dates and depths from Saanich Inlet.

<table>
<thead>
<tr>
<th>Collection Date</th>
<th>Coordinates</th>
<th>Sample Depths (m)</th>
<th>Ship</th>
<th>Filter Volume (L)</th>
<th>DNA [μg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>02/19/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 100, 125, 215</td>
<td>Tully</td>
<td>15.5, 20.0, 17.5, 15.5</td>
<td>0.16, 0.18, 0.56, 0.13</td>
</tr>
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<td>04/13/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 100, 125, 205</td>
<td>Strickland</td>
<td>15.0, 15.0, 15.0, 10.0</td>
<td>0.93, 0.18, 0.51, 0.06</td>
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<tr>
<td>05/18/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 105, 125, 200</td>
<td>Strickland</td>
<td>15.2, 20.0, 20.0, 20.0</td>
<td>1.19, 0.80, 0.72, 0.11</td>
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<tr>
<td>06/07/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 105, 120, 200</td>
<td>Strickland</td>
<td>10.0, 20.0, 19.5, 20.0</td>
<td>1.89, 0.85, 0.95, 0.36</td>
</tr>
<tr>
<td>07/06/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 100, 120, 200</td>
<td>Strickland</td>
<td>13.0, 19.5, 18.5, 19.0</td>
<td>1.67, 0.69, 1.05, 0.19</td>
</tr>
<tr>
<td>07/22/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 100, 120, 200</td>
<td>Tully</td>
<td>15.0, 20.0, 20.0, 20.0</td>
<td>1.54, 0.67, 0.70, 0.22</td>
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<td>09/20/06</td>
<td>48°38'N 123°30'W</td>
<td>10, 105, 120, 200</td>
<td>Tully</td>
<td>16.0, 20.0, 20.0, 19.5</td>
<td>1.29, 1.50, 1.00, 0.01</td>
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<td>11/14/06</td>
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<td>Strickland</td>
<td>20.0, 20.0, 20.0, 20.0</td>
<td>0.52, 1.13, 0.70, 0.51</td>
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Table 2 – Measure of diversity (Shannon-Wiener index) of a bacterial 16S rDNA clone library and 8 bacterial DGGE fingerprints. Richness values are in parentheses. For the clone library n=92 at 10 m, n=91 at 100 m, n=94 at 120 m and n=96 at 200 m.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>10 m</th>
<th>100 m</th>
<th>120 m</th>
<th>200 m</th>
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<tbody>
<tr>
<td>Clone Library (February)</td>
<td>3.346 (39)</td>
<td>3.421 (43)</td>
<td>1.931 (19)</td>
<td>0.389 (7)</td>
</tr>
<tr>
<td>February 18</td>
<td>2.731 (21)</td>
<td>2.812 (21)</td>
<td>2.21 (17)</td>
<td>1.724 (13)</td>
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<tr>
<td>April 13</td>
<td>2.113 (10)</td>
<td>2.796 (22)</td>
<td>2.273 (18)</td>
<td>1.989 (15)</td>
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<tr>
<td>May 18</td>
<td>1.918 (12)</td>
<td>2.125 (11)</td>
<td>2.42 (19)</td>
<td>2.159 (14)</td>
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<td>June 7</td>
<td>2.184 (15)</td>
<td>2.585 (18)</td>
<td>2.33 (19)</td>
<td>1.989 (17)</td>
</tr>
<tr>
<td>July 6</td>
<td>2.806 (25)</td>
<td>2.48 (16)</td>
<td>2.479 (20)</td>
<td>2.065 (14)</td>
</tr>
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<td>July 22</td>
<td>2.517 (21)</td>
<td>2.587 (18)</td>
<td>2.378 (20)</td>
<td>2.137 (16)</td>
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<td>September 13</td>
<td>2.753 (26)</td>
<td>2.290 (15)</td>
<td>1.570 (13)</td>
<td>2.130 (16)</td>
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<tr>
<td>November 14</td>
<td>2.819 (24)</td>
<td>2.266 (13)</td>
<td>2.094 (18)</td>
<td>2.136 (19)</td>
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Table 3 - Measure of diversity (Shannon-Wiener index) of an archaeal 16S rDNA clone library and 8 archaeal DGGE fingerprints. Richness values are in parentheses. For the clone library n=91 at 10 m, n= 94 at 100 m, n=96 at 120 m and n= 93 at 200 m.

<table>
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<tr>
<th>Archaea</th>
<th>10 m</th>
<th>100 m</th>
<th>120 m</th>
<th>200 m</th>
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<tr>
<td>Clone Library (February)</td>
<td>1.154 (7)</td>
<td>1.35 (8)</td>
<td>1.38 (8)</td>
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<td>2.238 (12)</td>
<td>2.262 (11)</td>
<td>2.099 (11)</td>
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<tr>
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<td>2.292 (16)</td>
<td>2.217 (14)</td>
<td>2.134 (10)</td>
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<td>May 18</td>
<td>2.108 (12)</td>
<td>2.071 (11)</td>
<td>2.391 (15)</td>
<td>2.032 (11)</td>
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<td>June 7</td>
<td>2.141 (13)</td>
<td>2.228 (14)</td>
<td>2.388 (15)</td>
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<td>July 6</td>
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<td>2.202 (13)</td>
<td>2.225 (13)</td>
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<td>July 22</td>
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<td>2.269 (13)</td>
<td>2.198 (12)</td>
<td>2.201 (12)</td>
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<td>2.349 (15)</td>
<td>2.305 (16)</td>
<td>2.276 (13)</td>
<td>2.332 (14)</td>
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<td>2.205 (14)</td>
<td>2.107 (12)</td>
<td>2.206 (13)</td>
<td>2.289 (13)</td>
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Methanotrophy

Aerobic Water
• Methanotrophs

Oxic/Anoxic Interface
• Methanotrophs

Anaerobic Water
• ANME, SRB, Methanogens

Anaerobic Sediments
• ANME, SRB, Methanogens

Methanogenesis

Figure 1 - Model of methane cycling in an oxygen minimum zone. Arrows indicate flow of methane. ANME are anaerobic methane-oxidizing archaea. SRB are sulfate-reducing bacteria.
Figure 2 - Geographic location, depth profile, and decadel oxygen concentration measurements of Saanich Inlet. (Depth profile map courtesy Department of Fisheries and Oceans, Government of Canada. Oxygen profile courtesy P. Tortell and Institute of Ocean Sciences, Government of Canada)
Figure 3 - Methane concentration and biological oxygen saturation in Saanich Inlet on February 18, 2006.
Figure 4 - Biological oxygen saturation at sampling depths and times where DNA was also extracted from Saanich Inlet in 2006.
Figure 5 - Methane concentration at sampling depths and times where DNA was also extracted from Saanich Inlet in 2006.
Figure 6 - Depth distribution of bacterial and archael phyla in Saanich Inlet as determined by relative phylotype abundance in the 16S rDNA clone library for each depth. (For all 16S rDNA clone libraries 91 ≤ n ≥ 96)
Figure 7 - Depth distribution of beta proteobacteria (BP) and gamma proteobacteria (GP) in Saanich Inlet as determined by relative phylo­type abundance in the 16S rDNA clone library for that depth. (For all 16S rDNA clone libraries $91 \leq n \geq 96$)
Figure 8 - Unrooted tree of alpha and gamma proteobacteria from Saanich Inlet and reference sequences from oxygen minimum zones. Bootstraps over 70% shown. GenBank accession numbers given in brackets. Circles indicate percentage of each Saanich Inlet phylotype in the clone library for the depth indicated by colours.
Figure 9 - Unrooted tree of pmoA sequences from Saanich Inlet, cultured methanotrophs, and environmental samples. Bootstraps over 70% shown. GenBank accession numbers given in brackets. Circles indicate abundance of each Saanich Inlet phylotype in the total pmoA clone library while the name indicates the depth at which the phylotype was initially detected.
Figure 10 - Depth Distribution of *pmoA* phylotypes in Saanich Inlet as determined by relative phylotype abundance in the clone library for that depth. (For all libraries $91 \leq n \geq 96$).
Figure 11 - Pearson correlation of bacterial (A) and archaeal (B) DGGE fingerprints from February 2006. Scale indicates percent similarity.
Figure 12 - Pearson correlation of bacterial DGGE fingerprints. Scale indicates percent similarity.
Figure 13 - Pearson correlation of archaeal DGGE fingerprints. Scale indicates percent similarity.
Figure 14 - Bacterial DGGE fingerprint NMS ordination with oxygen and methane superimposed to represent the relative strength and direction of correlation with the ordination. Three axes produced the best ordination. Oxygen has an $r^2$ value of 0.305 for axis 2 and 0.164 for axis 3, while methane has an $r^2$ value of 0.096 for axis 2 and 0.200 for axis 3.
Figure 15 - Archaeal DGGE fingerprint NMS ordination with oxygen and methane superimposed to represent the relative strength and direction of correlation with the ordination. Three axes produced the best ordination. Oxygen has an $r^2$ value of 0.116 for axis 2 and 0.440 for axis 3, while methane has an $r^2$ value of 0.028 for axis 2 and 0.240 for axis 3.
REFERENCES:


ARB neighbour joining tree of bacterial 16S rDNA sequences isolated from Saanich Inlet with reference sequences

** Bacterial Diversity in the Oxygen Minimum Zone off Chile
† Known Methanotrophs
* Assorted Symbionts
** Isolated from 20 m in the Antarctic Ocean Water Column
†† Alpha-proteobacteria and dimethylsulphoniopropionate uptake after an algal bloom in the North Sea
APPENDIX B

Restriction Fragment Length Polymorphism (RFLP) Summaries for Bacteria

10 m RFLP’s A-T

10 m RFLP’s U-AM

100 m RFLP’s A-V

100 m RFLP’s W-AQ
APPENDIX C

Restriction Fragment Length Polymorphism (RFLP) Summaries for Archaea

Archaeal RFLP’s A-M
APPENDIX D

Restriction Fragment Length Polymorphism (RFLP) Summaries for *pmoA*

*pmoA* RFLP's A, B, J, K