GEOCHEMICAL REQUIREMENTS OF THE ANAEROBIC OXIDATION OF

METHANE IN THE EEL RIVER BASIN

A Dissertation in

Geosciences

by

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ABSTRACT

Although the anaerobic oxidation of methane (AOM) is a widely studied process, many of the geochemical requirements for it remain a mystery, in part because the responsible organisms are not in pure culture. It has been shown that freshwater AOM proceeds with nitrite and nitrate. However, before this study the only known electron acceptor in marine AOM was sulfate. The work of this study helps to illuminate some of the requirements of marine AOM in the Eel River Basin (ERB), CA, focusing on the methane source and electron acceptors which allow for this globally significant process to proceed.

In Chapter 2, I use a finite difference thermal history model to indicate areas within the ERB that are capable of thermogenic methane production. Using the model results, I propose a correlation between areas with high rates of hydrocarbon production, methane seep location, and thus the areas within the ERB where high rates of AOM occur. The results of this study not only provide a potential link between geophysics/tectonics and microbiology, but also provide target areas within the ERB that could be used for microbiologic studies. Chapters 3 and 4 are incubation studies, targeted at understanding the role of electron acceptors, using sediment from methane seeps in the ERB. Methane oxidation is monitored by measuring the incorporation of $^{13}$C, from $^{13}$CH$_4$, into the carbon dioxide in the headspace. In Chapter 3, I examine how the rate of AOM changes at varying sulfate concentrations, with a focus on concentrations lower than 1 mM. Although it is often stated that methane oxidation occurs in a 1:1 ratio with sulfate reduction, I find that at these low sulfate concentrations, methane oxidation and
sulfate reduction are uncoupled, with methane oxidation rates sometimes an order of magnitude higher than sulfate reduction rates. Our experimentally determined rates of AOM are then put into an early Earth atmospheric photochemical model where it is shown that AOM causes a faster rise of oxygen and faster re-rise of methane than models that do not contain AOM. In Chapter 4, I test whether electron acceptors other than sulfate can be used in marine AOM. My results show the first direct evidence that both manganese (in the form of birnessite) and iron (in the form of ferrihydrite) can be used in marine AOM. Although the rates of manganese- and iron-dependent AOM are slower than sulfate-dependent AOM, these processes have the potential to gain more energy from methane oxidation. In addition, manganese- and iron-dependent AOM have the potential to be significant processes on early Earth when sulfate levels were extremely low.

Chapter 5 continues the study of manganese- and iron-dependent AOM using phylogenetics and fluorescence *in situ* hybridization (FISH). In addition we incubated the experiments demonstrating manganese-dependent AOM with $^{15}$NH$_4$Cl, during which active cells incorporate the $^{15}$N, and measured target aggregates from the incubation using FISH coupled to secondary ion mass spectrometry (FISH-SIMS) to determine the active cells in our incubation. Based on phylogenetic analysis, we find that both manganese- and iron-dependent AOM appear to be performed by distinct microbial assemblages and/or mechanism as compared to sulfate dependent AOM. SIMS analysis of aggregates in the manganese incubation indicate that mixed and mixed-cluster aggregates (of archaea and bacteria) and archaea of sarcina morphology are active and thus are likely responsible for manganese-dependent AOM.
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Chapter 1

Introduction

In this dissertation, I explore some of the geochemical requirements of the anaerobic oxidation of methane (AOM). My research explores the link between geophysics/tectonics and methane seep location (and as a result locations where high rates of AOM proceed), indicating that tectonics has the potential to influence where AOM occurs. In addition, my research investigates a variety of electron acceptors that have the potential to be coupled to AOM.

Anaerobic Oxidation of Methane (AOM)

Estimates of AOM in marine sediments indicate that this process can consume up to 90% of the methane released in these settings (15), substantially limiting the release of methane to the atmosphere and making AOM a significant part of the global methane cycle today. AOM was likely a major part of methane cycling during the Archean as well. A new model suggests that AOM played an important role in the rise of oxygen and the subsequent re-rise in methane during the Archean (4). In addition, the extremophilic organisms involved in AOM are of great interest to astrobiologists, not only for their potential to be a form of early life on Earth, but also because they are good Earth-analogue microbes for potential life on places such as Mars and Titan.

The net reaction for the AOM in marine systems is often framed as
with the oxidation of methane coupled to the reduction of sulfate. Much geochemical and experimental evidence suggests that AOM is mainly coupled to sulfate reduction (1, 2, 9, 10, 14-18, 21, 25-27, 29, 31-36, 38, 41-45), despite the fact that other electron acceptors such as nitrate, manganese, and iron are energetically more favorable. AOM proceeds in freshwater samples in the absence of sulfate provided nitrate or nitrite is present (37). This work contrasts with the work of Nauhaus et al. (2005) (27), who found no evidence for marine AOM in the absence of sulfate. Although several electron acceptors were reduced during incubations, Nauhaus et al. (2005) (27) report no transfer of the $^{14}$C label from methane to carbon dioxide in the absence of sulfate.

However, there is evidence that sulfate reduction and AOM can be uncoupled in marine settings. Specifically, the highest rates of methane oxidation in marine sediment do not always correlate with the highest sulfate reduction rates (19). In addition, there is evidence for the reduction of other electron acceptors, such as manganese and iron, in areas where the AOM occurs (D’Hondt et al. 2004 (5)). Furthermore, some sulfate reducing bacteria can facultatively use electron acceptors other than sulfate (28, 40). It is therefore not conclusive that marine AOM requires sulfate under all circumstances.

There are currently three known archaeal groups of anaerobic methanotrophs responsible for anaerobic methane consumption: ANME-1 and ANME-2 (33) and ANME-3 (20). Despite the fact that these organism are not in pure culture, it has been demonstrated that both ANME-1 and ANME-2 are directly responsible for AOM as shown by measuring $^{13}$C depleted biomass for both groups using fluorescence *in situ* hybridization (FISH) coupled to secondary ion mass spectrometry (SIMS) (33). ANME commonly have
sulfate reducing bacteria as syntrophic partners, in which methane is oxidized to an unidentified intermediate, and then further oxidized by the sulfate reducer using sulfate (2, 15, 31, 33). However, ANME-1 and some ANME-2 have been found to live independently, suggesting that they do not require a physically associated sulfate reducing bacteria to perform AOM, and thus might also be capable of sulfate reduction (32, 33).

ANME-2 are closely related to Methanosarcinales, an archaeanal order of methanogens. Because of their close relationship to methanogens, it is often hypothesized that anaerobic methanotrophy is simply reverse methanogenesis. Hallam et al. 2003 (13) performed genomic analysis on archaeanal methanotrophs from marine sediment to test this hypothesis. The results of these studies show that ANME-1 and ANME-2 (but to a lesser extent) have most of the genes needed to perform reverse methanogenesis (13). Specifically, it is thought that the first step in AOM is catalyzed by methyl coenzyme reductase A (mcrA), which is the terminal step in methanogenesis (12, 13). Due to these similarities between methanogens and anaerobic methanotrophs, trace methane oxidation (TMO) in pure cultures of methanogens has been used to infer chemical properties of AOM (23, 24).

The most common sulfate reducers found with ANME-1 and ANME-2 are closely related to Desulfosarcinales (31, 32). The sulfate reducers most commonly associated with ANME-3 are related to Desulfobulbus (29). In addition to Desulfosarcinales and Desulfobulbus, a variety of additional sulfate reducers have been found in methane seep environments including Desulfovibrio, Myxobacteria (31, 32, 39), and Desulfuromonas acetoxidans (39). Sulfate reducers make the net AOM reaction thermodynamically favorable, presumably by consuming the product of AOM and oxidizing it with sulfate. It
has been hypothesized that hydrogen, acetate, formate, methanol, carbon monoxide, methylamines, or direct transfer of electrons are intermediates for the AOM, but Nauhaus et al. 2005 (27) concluded that none of these compounds are intermediates. Moran et al. 2007 (24) also demonstrated that acetate is not a likely intermediate for AOM using TMO studies in *Methanosarcina acetivorans*. A relatively new hypothesis, proposed by Moran et al. (2007) (24), is that the intermediate of AOM is a methyl sulfide, most likely methyl mercaptan (MeSH) or dimethyl sulfide (DMS). The evidence for methyl sulfides as an intermediate in the AOM comes from studies of TMO by the methanogen *Methanosarcina acetivorans*. Moran et al. 2007 (24) found that the most abundant products in TMO by this species of methanogens are methyl sulfides (specifically MeSH and DMS).

**Eel River Basin**

The Eel River Basin (ERB), located on the North American Plate off of the northern coast of California, is in a highly active tectonic area. The Mendocino Triple Junction (MTJ), the location where the North American plate, the Gorda plate, and the Pacific Plate meet, is located slightly south of the ERB. The MTJ is migrating northward towards the ERB and has altered both the heat flow and the thickness of the crust in the ERB (6-8, 11). The crustal thickening results in uplift of the continental portion of the ERB (7, 8). Because of this uplift, and thus increased amounts of erosion, the Eel River has one of the largest normalized sedimentation rates (average annual suspended-sediment yield/square mile of drainage area) in the United States (3).

During Fall 2006, we collected methane seep sediments from the ERB using the submersible Alvin. These samples, as well as samples collected by the R/V Western Flyer in Fall 2005, were used throughout my dissertation to study the geochemical requirements
of AOM. The locations of the methane seeps throughout the ERB are not well confined, although attempts have been made using bathymetry and backscattering (30). The methane in the ERB has a large component of thermogenic methane, although biogenic methane is present as well (22, 35). Although much research has been done in the ERB regarding methanotrophs as well as tectonics, the source of thermogenic methane has not been defined.

Summary of dissertation

This study helps to constrain the geochemical requirements of AOM. Chapter 2 focuses on determining the source of thermogenic methane in the ERB and whether there is first order correlation between areas of thermogenic methane production and known methane seeps throughout the basin, and thus areas where high rates of AOM occur. Potential sources of thermogenic hydrocarbons are assessed based on the thermal history of the ERB, determined using a finite difference thermal model. This chapter is in preparation for publication in Geo-Marine Letters. The authors of this manuscript are Emily J. Beal and Kevin P. Furlong. Chapters 3-5 use incubations to study the requirements for AOM. Chapter 3 examines the effect of sulfate concentrations on the rate of AOM. Specifically, we determine how much AOM occurs at low sulfate concentration (comparable to levels during the Archean) and relate these results to the potential role AOM played in the rise of oxygen. This chapter is in review at Geobiology. The authors listed on this manuscript are Emily J. Beal, Mark W. Claire, and Christopher H. House. Chapters 4-5 test the theory that sulfate is the sole electron acceptor used in AOM in marine systems. In these chapters, it is shown that both manganese (in the form of birnessite) and iron (in the form of ferrihydrite) can be used to oxidize methane. The organisms responsible for these
newly demonstrated metabolisms are then characterized using phylogenetic analysis of the 16s rRNA and mcrA genes, FISH, and FISH-SIMS. Chapter 4 is published in the July 10, 2009 issue of Science with authors Emily J. Beal, Christopher H. House, and Victoria J. Orphan. Chapter 5 is in preparation for Applied and Environmental Microbiology and will be submitted with the following authors: Emily J. Beal, Christopher H. House, and Victoria J. Orphan.

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Chapter 2

Thermogenic hydrocarbon potential of the Eel River Basin and its potential link to microbiota

Abstract

The numerous methane seeps in the off-shore Eel River Basin (ERB), California (CA) harbor microorganisms that consume methane in a process termed the anaerobic oxidation of methane (AOM). Although much has been learned about the requirements for AOM, studies have not focused on the source of methane in these ERB methane seeps where these microorganisms thrive. Here, we examine a possible link between tectonics, methane production, and methane seep location in the ERB. We first use a thermal history model which indicates that the Franciscan Complex is the likely source of thermogenic methane within the ERB. We then show that there is a potential correlation between the highest rates of recent and current hydrocarbon production in the Franciscan Complex and the known locations of thermogenic methane and methane seeps in the ERB. Our thermal history model results are then used to predict additional areas within the ERB which may contain dense areas of methane seeps, and could thus also be areas with large amounts of AOM occurring.
Background

The Eel River Basin (ERB), located on the southern Cascadia accretionary margin, contains abundant methane seeps. These seeps are the site of numerous studies investigating the anaerobic oxidation of methane (AOM), which consumes up to 90% of methane released in marine settings (31). Carbon isotopes of methane, as well as \( C_1/(C_2+C_3) \) (where, \( C_1 = \text{methane}, C_2 = \text{ethane}, C_3 = \text{propane} \) suggests that these methane seeps are, at least partially, thermogenic in origin (21, 30), although the source rock remains unclear (33). The ERB had a very high sedimentation rate in the Pliocene (5) from onshore erosion of the rapidly uplifting Coastal Range driven by the northward migrating Mendocino triple junction (MTJ) (7-9). It is possible that this high sedimentation rate caused by the relative young sedimentary basin (~5.3 m.y.) to produce thermogenic hydrocarbons basin (19, 25). Conversely, the basement Franciscan Complex could be the source of thermogenic hydrocarbons in the ERB, with the recent ERB sedimentation driving enhanced production. In addition to the uncertainty in the source of thermogenic hydrocarbons in the ERB (24, 33), it is also not clear if there is a link between tectonics, thermogenic hydrocarbon production, and methane seep location (and thus the creation of habitats for the organisms responsible for AOM). Although many studies have focused on the characterization and requirements of AOM (1, 3, 4, 11, 14-18, 20, 26-30, 38), there have been no studies that attempt to correlate the tectonics of an area with methane seep formation.

Carbon isotopes of methane with values averaging \( \delta^{13}C \sim -43 \% \) (PDB) and \( C_1/(C_2+C_3) \) ratios between 70 and 350 imply that there is abundant thermogenic methane
throughout the ERB (21, 30). Although minor amounts of oil have been observed in the 
off-shore ERB (21, 30), which could be residual oil produced before the onset of ERB 
sedimentation, an additional possibility that should be considered is that much of the 
methane is biogenic. Organisms involved in AOM can consume enough methane to 
increase the C1/(C2+C3) ratio to thermogenic methane levels (37). In addition, as 
microorganisms anaerobically oxidize methane, they preferentially consume 12C and 1H 
leaving the residual methane enriched in 13C and 2H (Figure 1). In this manner, AOM 
can fractionate biogenic methane in a manner that the carbon isotopic composition of 
biogenic methane appears to be thermogenic in origin (2, 37). However, organisms 
involved in AOM fractionate hydrogen isotopes to a much larger extent than carbon 
isotopes. Because of this, the isotopic composition between biologically fractionated 
biogenic methane and thermogenic methane is distinguishable (37) (Figure 1). 
Therefore, measuring the hydrogen isotopic composition of the methane in the ERB 
would provide an additional means to test its origin. Since current data suggests that 
thermogenic methane is present in the ERB, for the purpose of this study, we assume that 
observed thermogenic methane is not derived from biogenic methane.

Four major stratigraphic units (comprised of shale, siltstone, and sandstone), 
which overlie the Franciscan Complex basement, are found in the offshore ERB. These 
sequences have a maximum thickness of more than 2.5 km and a minimum thickness of 
~ 1 km (12). The oldest of these units, ranging in age from Miocene to middle Pliocene 
is a shale (12), and thus is a potential source rock for the observed thermogenic 
hydrocarbons in the offshore ERB. The onshore analogue of these shales contain an 
average of 0.5 % TOC (total organic carbon) (35). Due to the proximity of the ERB to
Figure 2-1. (from Coleman et al. 1981). The effect of microbial oxidation of methane on the carbon and hydrogen isotopic composition of biogenic methane. As shown, methane oxidation can enrich biogenic methane to the point it appears thermogenic. However, the hydrogen isotopes of microbial altered biogenic methane and thermogenic methane are substantially different, allowing for the differentiation between these methane types.
the northward migrating Mendocino Triple Junction (MTJ), both the heat flow and the thickness of the crust beneath the basin have been recently altered (7-9, 13). The crustal thickening drives the uplift of the continental portion of the ERB (8, 9), increasing the rate of sedimentation, causing the ERB to have one of the highest normalized sedimentation rates (average annual suspended-sediment yield/square mile of drainage area) in the United States (5). Although high sedimentation rates, such as observed in the ERB, can potentially decrease heat flow by as much as a factor of 2 (19, 25), the associated heating with burial will increase temperature of the deepest units. It is, therefore, possible that the elevated temperature caused by the MTJ migration and high sedimentation rates has altered the ERB in a manner to allow for thermogenic methane to be produced from the recently buried shale. Alternatively, it has been argued that the Franciscan Complex, which has TOC usually within the range of 1-2 % (22, 35), is potentially the source rock (24, 33).

Here we show how changes in sedimentation rate have affected the potential for hydrocarbon production throughout the ERB region, both within the shale as well as within the underlying Franciscan Complex. We used a finite difference thermal history model to calculate temperature histories as well as amounts and timing of potential hydrocarbon production at various depths (6). A time-temperature index (TTI) index was not used as a proxy for hydrocarbon production since it has been found to substantially under predict hydrocarbon production in area with high sedimentation rates (34), and does not differentiate between hydrocarbon production from different kerogen types. Instead we used a ‘kinetic’ model of hydrocarbon production \( \text{mg}_\text{hydrocarbon}/\text{g} \text{TOC} \) that predicts production from Type I, II, and III kerogens (34). These model results of potential
hydrocarbon production are assessed for consistency with the geologic development of the region and compared with known methane seep location to determine if the highest hydrocarbon production potentials are associated with methane seep location. The ability to explore links between geophysics/tectonics and microbiological processes has the potential to help define target areas for future microbiologic studies.

Methods

We divided the off-shore ERB into 23 sections (shown in Figure 2) and estimated stratigraphic thicknesses (total thicknesses shown in Figure 3), as well as timing of depositional and erosional events for each section. Estimates were based on the isopach maps and published data of Gulick et al. (12). We utilized this deposition history in our finite difference thermal model (6) to determine the thermal history and hydrocarbon production for each section. Model results are total hydrocarbon production, not only methane, and therefore are a maximum when considering methane production. Hydrocarbon production was calculated using activation energies for each kerogen type based on the ‘kinetic’ model of Tissot et al. (34), which accounts for the breaking of different bonds (and thus activation energies) between the different kerogens. Activation energies of kerogen types were from Ungerer and Pelet (36). The assumed total mass fraction of type II and II kerogens in our model runs are 630 mg_{hydrocarbon}/g_{TOC} and 250 mg_{hydrocarbon}/g_{TOC} respectively (34). Thermal conductivities used in the model runs for the stratigraphic units are as follows: 2.5 W/mK for the Franciscan Complex (23), 1.7 W/mK for the shale (10), 2.9 W/mK for the siltstones (10), and 3.0 W/mK for the sandstone (10).
Figure 2-2. Location of the Eel River Basin (ERB) and the Mendocino Triple Junction (MTJ). The overlying grid represents the study area, which is divided into 23 section, used in the thermal models. Filled stars indicate known locations of thermogenic methane and methane seeps within the ERB. The outlined stars show areas we predict to have thermogenic methane (from the Franciscan Complex) and/or methane seeps based on our model results. Outlined sections indicate the areas, with varying depositional histories, where models runs are shown in Figures 2-3 and 2-4.
Figure 2- 3. Total estimated depths of the sedimentary sequences within the ERB used in thermal modeling. Estimates are based on isopach maps create by Gulick et al. (11).
Heat flow was 80 mW/m² (8), consistent with the subduction of very young oceanic lithosphere. Surface temperature was 4 °C. Specified points within the stratigraphic column were monitored throughout the model, the location of the top of the shale and bottom of shale, as well as points corresponding to the present day depths of 3, 4, 5, 6, 7, 8, 9, and 10 km. Erosional events were included in several model runs, to assess the impact of missing sections. These events were modeled to occur between 2.5 – 1.5 m.y. (12) and assumed to erode either 150 or 300 m of sediment in sections along the western margin of the ERB (Figure 2, sections 4, 7, 10, 143, 16, 19). Because we know current stratigraphic thicknesses, erosion implies that there was an increase in deposition (by the amount we eroded) before the erosional period. Therefore, once assumed erosion occurs, we return to the observed thicknesses for all sequences. All models were run for 10 m.y. before onset of ERB sedimentation with no burial to determine the approximate maturity of units at the start of the recent ERB history. Each model was also run into the future for 20 m.y. with varying deposition rates (0 km/m.y., 0.4 km/m.y, or 0.64 km/m.y). Current methane seep location and thermogenic methane locations are based on Orphan et al. (30) and Kvenvolden and Field (21).

**Results**

Various deposition histories were examined for each section shown on Figure 2, including varying amounts of erosion since the onset of ERB sedimentation and varying current depositional rates, that satisfy data from Gulick et al. (12). Here we describe a representative set of results, focusing mainly on those model runs without erosion and
with a current depositional rate of 0.64 km/m.y (12). Figure 4 shows an example of one of our model runs (section 23 in the southern part of the ERB shown on Figure 2). Figure 4a shows an example burial history of those we used in our model runs. Using this burial history, we calculate the temperature (Figure 4b), the total amount of hydrocarbon production (oil and gas) from type II (Figure 4c) and type III (Figure 4d) kerogens, as well as the rates of hydrocarbon production from these kerogen types (Figures 3e-f) through the history of the ERB.

Figure 5 shows potential hydrocarbon production at a suite of ERB locations, with varying depositional histories, over time. Here, we show results for positions that currently correspond to depth intervals of 3 km, 4 km, 5 km, 6 km, 7 km, 8 km, 9 km, and 10 km, as well as the top and bottom of the shale sequence. The depth for each point at the beginning of the model runs is defined by the current depth of the point (3 km, 4 km, etc.) minus the estimated amount of assumed net deposition for the given section of the ERB. As our model continues into the future, each point is buried deeper due to continuing sedimentation (at the current sedimentation rate). Significant hydrocarbon production at current depths below ~ 6 km appears to have occurred before the onset of sedimentation for the ERB; units that are currently shallower than ~ 6 km began to produce hydrocarbons (from type II and III kerogens) concurrent with the onset of recent sedimentation (starting ~5 m.y.) (Figure 5). The peak rates of hydrocarbon production occur in the 3 - 5 km depth range for type II hydrocarbons, and 4 - 6 km depth range for type III hydrocarbons. Currently, hydrocarbons are beginning to be produced as shallow as 3 km depth for type II kerogens and at 4 km depth for type III kerogens (as seen by the increase in hydrocarbon production rate at these depths (Figure 5).
Figure 2-4. An example of model results for depth/burial history (a), temperature (b), hydrocarbon production from type II and III kerogen (c and d), rates of hydrocarbon production from types II and III kerogens (e and f) for section 23 (shown in Figure 2-2). Figure (a) shows burial history for the current depths beginning at the top of figure in the following order: top of shale, bottom of shale, 3 km, 4 km, 5 km, 6 km, 7 km, 8 km, 9 km, 10 km. Figure (b) shows temperature history for the same depth (in the same order). The data labels indicate the current depth, shown in (a), of all monitored points. Dashed lines indicate onset of burial (5.3 m.y.) and present day (0 m.y.). Some depths show (>5 km figure c and >6 km figure d) production of hydrocarbons before onset of ERB sedimentation (5.3 m.y.). Figure e and f, show the rates of hydrocarbon production from type II (figure e) and type III (figure f) based on the model results shown in figures c and d.

Figure 2-5 (next 3 pages). Hydrocarbon production and production rates for a variety of sections (labeled with section number). Unidentified lines for production type II figures correspond to the top and bottom of the shale. Unidentified lines for production type III figures correspond to the top and bottom of the shale and 3 km. All depth correspond to the current sediment depth for the given point. Dashed lines represent the beginning of ERB sedimentation (5.3 m.y.) and the present day (0 m.y.)
Our model results for the total amounts of hydrocarbons produced from type II kerogens (mg(hydrocarbon)/g(TOC)) at bottom and top of the shale sequence for all of the examined sections of the ERB are shown in Figure 6. Our results indicate that the shale, which overlies the Franciscan Complex, is currently producing only minor amounts of hydrocarbons from type II kerogens (Figure 6a-b) and no hydrocarbons from type III kerogens (data not shown). In the unrealistic event that sedimentation in the ERB stops today, our model runs indicate that the shale would still not be producing hydrocarbons within 30 m.y. However, if the present day sedimentation rate estimated at 0.64 km/m.y by Gulick et al. (12) continues, the shale could be producing hydrocarbons from both type II and III hydrocarbons within approximately 5 m.y. Even if the lower estimate of current sedimentation of 0.4 km/m.y. (32) is used in the thermal model, the shale would be producing hydrocarbons within ~ 6-7 m.y..

In contrast to the shale, the Franciscan Complex (the underlying basement) is capable of producing substantial hydrocarbons. Type II kerogens are according to our model results currently maturing at the depth of 4 km, and have generated hydrocarbon at ranges between 100 – 350 mg(hydrocarbon)/g(TOC) (Figures 4 and 6a). By 5 km depth, within the Franciscan Complex, significantly more hydrocarbons are being generated (in the range of 500 – 650 mg(hydrocarbon)/g(TOC)) (Figure 7b). The highest total amounts of hydrocarbons produced from type II kerogens are, for the most part, within sections along the margins of the model grid (Figure 7). Although there has likely been significant hydrocarbon production at depths greater than 5 km (Figure 5), most of that hydrocarbon was generated before the onset of ERB sedimentation. For this reason, it is likely that it migrated or was lost from the ERB before 5 Ma. The averaged rates of
Figure 2-6. Current potential for hydrocarbons (mg\(_{\text{hydrocarbon}}\)/g\(_{\text{TOC}}\)) from type II kerogens at the bottom of the shale sequence (a) and at the top of the shale sequence (b). The grid represents the area modeling within the ERB as indicated in Figure 2-2. Note that these values include amounts of hydrocarbons (including oil and gas) produced before the onset of sedimentation of the ERB which likely are gone from the basin.
Figure 2-7. Current potential for hydrocarbons (mg(hydrocarbon)/g(TOC)) from type II kerogens at 4 km (a) and at 5 km (b). The grid represents the area modeling within the ERB as indicated in Figure 2-2. Note that these values include amounts of hydrocarbons (including oil and gas) produced before the onset of sedimentation of the ERB which likely are gone from the basin.
hydrocarbon production, since the onset of ERB sedimentation, from type II kerogens was calculated at 4 km and 5 km (Figure 8). At 4 km, the highest average hydrocarbon production rates are along the western margin of our study grid. However, the peaks rates increase and shift to the middle of our study grid at 5 km.

It is estimated that towards the end of the late Pliocene, small amounts of erosion (~150 m) occurred along the western margin of the ERB (12). We tested the possible effect on hydrocarbon generation of these amounts of erosion in our model by increasing sedimentation before the start of the erosional period. This increase in sedimentation, for each section, deposits a sediment layer equal to the known thickness of stratigraphic unit in which the sedimentation occurs plus the estimated amount of erosion. Adding erosion to our model runs in this manner allows us to maintain current known sediment thicknesses. Although it might be expected that erosion would decrease hydrocarbon potential, it in fact increases it due to the increased rate of deposition before the erosional period (and thus transient deeper burial) as compared to models without erosion. When the above amounts of erosion are included in the thermal history model of the section along the western margin, the potential production of hydrocarbons from type II increases by ~ 8 % at 3 km and by ~ 5 % at 4 km (data not shown). When the amount of erosion is doubled to 300 m, we see an increase in production from type II kerogens at 3 km and 4 km to ~ 12 % and ~ 9 % respectively.

A similar analysis of hydrocarbon production potential from type III kerogens was completed for the same scenarios as described for type II kerogens. Hydrocarbon production from type III kerogens, which began as a result of recent deposition, occurs within current unit depths ranging from ~ 5 – 7 km (Figure 8). At the current depth of 5
km, production from type III kerogen ranges from $30 - 100 \, \text{mg}_{\text{hydrocarbon}}/g_{\text{TOC}}$ (Figure 9a). By 6 km depth, production has increased to approximately $150 - 200 \, \text{mg}_{\text{hydrocarbon}}/g_{\text{TOC}}$ (Figure 9b). The highest hydrocarbon production potential from type III kerogens is mainly along sections at the margin of the grid. At depths below 6 km, the Franciscan has likely generated large amounts of hydrocarbons. However, these deeper units had already begun to mature before the onset of the ERB and thus most of the produced hydrocarbons at this depth were likely lost before sedimentation began. The highest averaged rates of hydrocarbon production (since the onset of ERB sedimentation) from type III kerogens also peaks along the western margin of our study grid at 5 km and in the center of our study grid at 6 km (Figure 10). When 150 m of erosion is included for the section along the western margin, potential production increases by $\sim 5\%$ at 5 km and $<1\%$ at 6 and 7 km. This amount slightly increases to $\sim 8\%$ at 5 km, $\sim 1\%$ at 6 km, and $< 1\%$ at 7 km.

Discussion

Carbon isotopes as well as $C_1/(C_2+C_3)$ ratios suggests that thermogenic hydrocarbons are prevalent in the ERB (21, 30). Although the possibility that thermogenic hydrocarbon migrated from outside of the ERB cannot be ruled out, we focus on sources within the ERB. Potential source rocks are either a shale sequence or the underlying Franciscan Complex. The shale sequence is not likely a significant source of the hydrocarbons observed in the ERB today, although this unit could likely begin to produce within the next $\sim 5$ m.y. The Franciscan Complex, in contrast to the shale, has
Figure 2-8. Current averaged rates of hydrocarbons (mg_{hydrocarbon}/g_{TOC}) from type II kerogens at 4 km (a) and at 5 km (b) since the onset of the ERB sedimentation (5.3 Ma). The grid represents the area modeling within the ERB as indicated in Figure 2-2. Note that these values include amounts of hydrocarbons produced (including oil and gas) before the onset of sedimentation of the ERB which likely are gone from the basin.
Figure 2-9. Current potential for hydrocarbons (mg_{hydrocarbon}/g_{TOC}) from type III kerogens at 5 km (a) and at 6 km (b). The grid represents the area modeling within the ERB as indicated in Figure 2-2. Note that these values include amounts of hydrocarbons produced (including oil and gas) before the onset of sedimentation of the ERB which likely are gone from the basin.
Figure 2-10. Current averaged rates of hydrocarbons (mg_{hydrocarbon}/g_{TOC}) from type III kerogens at 5 km (a) and at 6 km (b) since the onset of the ERB sedimentation (5.3 Ma). The grid represents the area modeling within the ERB as indicated in Figure 2-2. Note that these values include amounts of hydrocarbons produced (including oil and gas) before the onset of sedimentation of the ERB which likely are gone from the basin.
the potential to be currently producing significant amounts of hydrocarbons, from both type II and type III kerogens (Figures 4-5). Most of the kerogen in the Franciscan Complex are gas prone type III kerogens (35), indicating that if the Franciscan Complex is the source rock for thermogenic hydrocarbon in the ERB, only a small amount of oil will be produced. This expectation is congruent with observations made in the ERB, where little oil has been observed (12, 21, 30).

The onset of sedimentation in the ERB ~5.3 Ma dramatically altered the potential of hydrocarbon production from the basement Franciscan Complex, causing much of the shallower depths to move into conditions favoring hydrocarbon maturation (Figure 4). Type III kerogens began to mature at 4-5 km as recently as ~3 Ma while, type II kerogens began to produce hydrocarbons at 3-4 km during this time. Because the large differences in sedimentation rates throughout the basin we observe significant variability in hydrocarbon production potential throughout the basin (Figures 4-8). At 5 km depth, hydrocarbon production from type III kerogen is as much as a factor of three greater, such as seen in sections 13, 16 and 19 (Figure 9a) than at low producing areas, such as sections 14 and 15 (Figure 9a). Similarly, at 4 km depth, production of hydrocarbons from type II kerogens vary by a factor of three (such as sections 16 and 19 as compared to 14 and 15, Figure 7a). There is greater potential hydrocarbon production deeper than the depths discussed above. However, at these depths, hydrocarbon production was high before the onset of the ERB sedimentation (Figure 5) and thus there is a likelihood that the hydrocarbons produced at these depths were lost. In addition, the highest rates of hydrocarbon production are observed at 4-5 km (Figure 5). Therefore, the shallower,
slightly less mature depths, have the greater potential to be modern sources of hydrocarbons, including methane.

Most faults throughout the ERB are high-angled thrust faults associated with plate convergence (12). The dips of these faults are typically greater than 60 °, and thus hydrocarbons migrating from ~6 km should be transported almost directly upward, with the maximum distance of offset from their origin ~ 4 km. We therefore might expect a correlation between surficial hydrocarbon concentrations and areas with either the highest hydrocarbon production rate or total amount of hydrocarbon production. In the case of the ERB, the highest rates of hydrocarbon production appear to be more important as an indicator of possible methane seep locations as compared to the total amount of produced hydrocarbons. The total amount of hydrocarbon production includes any hydrocarbons produced before the onset of ERB sedimentation (Figures 3 and 4). Therefore, the total amount of hydrocarbon production for each section is an overestimate of the amount of hydrocarbons currently available in the area. In contrast, the calculated rates of hydrocarbon production (Figures 3, 4, 7 and 9) only include hydrocarbons produced since the onset of ERB sedimentation, and thus identify areas producing hydrocarbons currently and in the recent past.

The black stars on Figure 2 represent the location where studies have indicated the presence of thermogenic hydrocarbons (21, 30). Most studies have focused on the southern section of the ERB, and thus only the locations of methane seeps in this part of the basin are well known. It is interesting to note that the two areas with known abundant (thermogenic) methane seeps (stars on Figure 2) also have high rates of hydrocarbon production in our model (Figures 7 and 9). Although there is not sufficient data to show
a definite correlation between high rates of hydrocarbon production in the ERB and methane seep location, we can use our model data (in conjunction with known methane seeps) to predict other areas of the ERB which may contain high densities of methane seeps.

Our model results indicate that the likely source of thermogenic hydrocarbons in the ERB is the Franciscan Complex in which the organic matter is mainly comprised of type III kerogens. We can therefore focus on production rates of type III kerogens when predicting methane seep locations (Figure 10). Both of the known locations of thermogenic methane within the ERB have an averaged hydrocarbon production rate greater than 22 mg$_{\text{hydrocarbon}}$/g$_{\text{TOC}}$/m.y. for type III kerogens. Using the results, such as shown in Figure 10, for all modeled section with hydrocarbon production rate greater to 22 mg$_{\text{hydrocarbon}}$/g$_{\text{TOC}}$/m.y., we can predict which areas of the ERB may have the highest potential for methane seeps. Using these methods, we expect that much of the central part of our modeled grid (shown in Figure 2) likely contain abundant methane seeps.

Conclusions

The results of our thermal history model show that the sedimentary sequences overlying the Franciscan Complex throughout the offshore ERB are not currently capable of producing thermogenic hydrocarbons. However, specific depth intervals in the Franciscan Complex (~ 5 km), have likely been producing hydrocarbons for the last 3 m.y as a result of the recent sedimentation of the ERB. The produced methane may then be transported to the surface using high-angle thrust faults as conduits, and thus surface
methane seep density may be correlated with the rate of hydrocarbon production within the 
underlying Franciscan complex. In the two areas where methane seeps and thermogenic 
methane have been studied, they correlate with the areas we expect to currently be 
generating hydrocarbons at a high rate. Building on the expected relationship between 
seep location and areas with high rates of hydrocarbon production, we predict that the 
central part of our study grid will likely also contain dense methane seeps.

The idea that tectonic history can dictate the location of methane seeps, and thus the 
location where the highest rates of marine AOM occur, is exciting to explore because it 
provides a direct link between Earth processes and life. In addition, it provides a 
mechanism to potentially predict the locations where both highest and lowest rates of AOM 
may be occurring within the ERB. This, in turn, provides an additional estimate on the 
total amount of AOM within the ERB, and thus the impact from the ERB on global 
methane cycling. If similar thermal tectonic modeling is performed for a variety of diverse 
location along continental margins (where most AOM occurs), an additional constraint on 
global rates of AOM could be added.

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Chapter 3

High rates of anaerobic methanotrophy at low sulfate concentrations with implications for the past and present methane cycle

The anaerobic oxidation of methane (AOM) is a key factor in regulating atmospheric methane concentrations, and hence affects both global temperatures and the atmospheric redox state. Despite its importance to global biogeochemical cycles, the relationship between sulfate concentration and the rate of AOM has not been well constrained. Here, we present measurements showing substantial methane oxidation at lower sulfate concentrations than previously expected, with no significant decrease in the rate of AOM until sulfate levels are well below 1 mM. At sulfate levels below 1 mM, there appears to be a strong decoupling of AOM and sulfate reduction, with methane oxidation rates sometimes exceeding sulfate reduction by almost an order of magnitude. The fact that AOM can proceed rapidly at such low sulfate levels allows for the possibility that high rates of AOM occurs both in freshwater environments (lakes, rivers, etc.) as well as in deep ocean sediments. In addition to the implications for present day methane cycling, it has been suggested that AOM is a significant factor in the rise of atmospheric oxygen 2.4 billion years ago. When our experimental data is included in a biogeochemical model of Earth’s redox evolution, the rise of oxygen occurs more abruptly compared to previous models using semi-analytic estimates of AOM rates. In addition, both oxygen and methane reach higher concentrations after the rise of oxygen then in the previous modeling.
Introduction

The first evidence that AOM occurs in the modern ocean comes from geochemical porewater profiles of marine sediment (3, 41, 57). Porewater profiles show that methane is being anaerobically oxidized in marine sediments, and that peak methane oxidation often corresponds to the peak sulfate reduction rate for a given sample. These geochemical profiles, as well as molecular studies, indicate that sulfate is the primary electron acceptor in AOM today (2, 3, 7, 28, 49, 57). In addition, methane oxidation can be coupled to iron and manganese oxide reduction in marine environments (5), and nitrate and nitrite reduction in freshwater environments (56).

Since the original detection of AOM, much has been learned about the process, although the responsible organisms are not in pure culture. Fluorescence in situ hybridization (FISH) coupled to secondary ion mass spectrometry (SIMS) has shown that archaea, often in a syntrophic association with bacteria, are responsible for sulfate-dependent AOM (51). In addition, many molecular biology techniques using DNA and lipids, as well as incubations studies using both labeled and unlabeled substrates have given much insight into how this process occurs (7, 18, 19, 21, 22, 26-29, 35, 39, 45, 46, 49-52, 55, 60, 61).

Today, marine sulfate-dependent AOM is performed by three groups of archaea, ANME-1, ANME-2, which are distantly related to cultured Methanosarcinales, and ANME-3 (7, 39, 49, 51, 59). Organisms involved in nitrate-, manganese-, and iron-dependent AOM are yet to be characterized. ANME-2 and ANME-3 are typically found in a syntrophic relationship with sulfate reducing bacteria suggesting that methane oxidation
occurs in the archaeal partner and sulfate reduction in the bacterial partner (39, 50, 51). In this case, the ANME oxidize methane to an unidentified intermediate, which is passed to the bacterial partner, where it is used to reduce sulfate. The consumption of the AOM intermediate by sulfate-reducing bacteria maintains the intermediate at a very low concentration, keeping AOM energetically favorable.

The ANME groups are found in all methane seep environments, with either ANME-1 or ANME-2 dominant in the environment (39). Nauhaus et al. (46) found that differences in temperature, pH, salinity, and methane and sulfate concentrations cannot completely explain the dominance of ANME-1 or ANME-2 in different settings, although it was found that ANME-2 are slightly more adapted to cold temperatures than ANME-1. Knittel et al. (39) also found that sulfate and methane concentrations are not correlated with a specific ANME group. This indicates that both ANME-1 and ANME-2 are capable of adapting to a wide variety of environmental conditions, including sulfate concentrations.

The anaerobic oxidation of methane (AOM) is traditionally thought of as a sulfate-dependent process described by the following net reaction:

\[
\text{SO}_4^{2-} + \text{CH}_4 \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O} \tag{1}
\]

The result of this reaction is a localized increase in alkalinity, leading to precipitation of AOM associated carbonates. Provided there is sufficient reduced iron present, the produced sulfide is precipitated as iron sulfide. Consequently, the process of AOM consumes methane without releasing a reduced species back into the atmosphere. In addition, sulfate-dependent AOM does not alter the biospheric atmospheric oxygen flux, and hence increases the oxidation state of Earth’s atmosphere by decreasing the net methane flux relative to the net oxygen flux. By contrast, the aerobic oxidation of methane
does not change the atmospheric redox state, as the net stoichiometry is equivalent to that of respiration (11).

Recently, Catling et al. (11) have suggested that anaerobic oxidizers of methane might play a significant role in the largest biogeochemical change in Earth’s history - the ‘Great Oxidation Event’ or GOE. Prior to 2.4 billion years ago (Ga), Earth’s atmosphere did not contain O$_2$, as evidenced by the mass independent fractionation of sulfur isotopes (MIF-S) in sedimentary rocks (17, 53). The MIF-S signal disappeared between 2.45 and 2.33 Ga (6) signifying the end of reducing atmospheric conditions (63), and is closely followed temporally by evidence of widespread oxidation in surficial environments (9, 31). Among the many consequences of increasing free oxygen in the atmosphere was the onset of oxidative weathering (30), which flooded the ocean with sulfate as sedimentary continental sulfides (predominantly pyrites) are oxidized to soluble sulfates. An upper limit on sulfate concentrations in Archean (< 2.5 Ga) oceans has been placed at 0.2 mM, with concentrations increasing to ~2 mM by the mid-Proterozoic (20).

The late Archean biosphere was quite different than our own. After the evolution of oxygenic photosynthesis, which may have occurred by 2.9 Ga (47), organisms ancestral to modern cyanobacteria were likely the dominant primary producers (14). These organisms would have produced local free oxygen, but the ocean/atmosphere system around them contained sufficient reducing power to neutralize the oxygen (32), limiting the atmospheric concentrations to trace levels (36). Methanogens have been postulated to be a major player in late Archean ecosystems (37), and in collaboration with fermenters, would have processed a large fraction of the primary organic carbon into methane. The net effect of these two metabolisms working in concert is the effective
conversion of two neutral species into the strong redox couple of CH$_4$ and O$_2$, with the
net stoichiometry CO$_2$ + 2H$_2$O $\rightarrow$ CH$_4$ + 2O$_2$ producing fluxes of CH$_4$ and O$_2$ in a 1:2
ratio (12, 38). In the modern Earth system the net ratio is less than 1:2, as a significant
portion the CH$_4$ generated by methanogens is consumed by AOM before reaching the
atmosphere. Thus a strong positive feedback between increasing oxygen levels, oceanic
sulfate levels, and AOM throttling of the methane flux could have driven drastic change
in the redox state of Earth’s atmosphere.

Methods

Sampling/Incubations

Sediment samples were taken in September 2006 from the Eel River Basin (off
the coast of California, USA) during a cruise of the R/V $\text{Atlantis}$ with the submersible
ALVIN (Woods Hole Oceanographic Institution). Sediment was removed from cores (in
3 cm sections) and stored in sterile whirl-pack bags, which were sealed in thick mylar
bags containing argon, soon after coring (aboard the ship). The push core used was PC12
(0-19 cm) from dive A4265. This sediment was stored at 10$^\circ$ C for approximately 14
months in these sealed anaerobic mylar bags before incubation. The entire 19 cm
sediment core was homogenized in an anaerobic chamber and combined with artificial
sulfate-free seawater in a 1:3 ratio. The seawater was made following the recipe of
Lyman and Fleming (40), excluding the sulfate salts. It was degassed using a mixture of
N$_2$ and CO$_2$ (in the ratio of 80:20), reduced by adding 0.5 g L$^{-1}$ Na$_2$S·9H$_2$O, and then
autoclaved. This slurry was stored for 6 months in a 1 L glass bottle sealed with a headspace of CH₄ and CO₂. When no sulfate was detectable in the slurry (after 6 months), it was evenly distributed to 19 120 mL bottles. These bottles contain approximately 10 mL of sediment and 30 mL of seawater. We created a concentrated solution of degassed sterile Na₂SO₄ and injected the appropriate amount into the bottles to get the following approximate starting sulfate concentrations: 4 bottles at 30 mM, 3 bottles at 15 mM, 3 bottles at 1 mM, 3 bottles at 0.5 mM, 3 bottles at 0.1 mM, and 3 with no injected sulfate. Bottles were shaken twice a week (after isotopic measurements). The gases from the anaerobic chamber were vacuumed from each bottle and a headspace of 2.5 bars CH₄, 35 mL CO₂, and 6 mL ^13^CH₄ was added. One bottle containing 30 mM sulfate was then autoclaved to confirm that no abiotic isotope exchange occurs between methane and carbon dioxide. These bottles were set up using ice packs so that they never warmed and were stored at 10º C for the duration of the experiment.

**Carbon Isotope Measurements/Methane Oxidation Rates**

Carbon isotopic measurements of CO₂ were made on a Finnigan MAT 252 dual-inlet mass spectrometer (Thermo Finnigan, Bremen, Germany), with precision better than 1 ‰, as described in Moran et al. (42). Isotope measurements were taken twice a week for the duration of the experiments. For each measurement, 5.0 mL of headspace was taken from each incubation. This amount was not replaced over time. δ^{13}C_{CO₂} values were converted to the amount of methane oxidized using equations published in Moran et al. (43). We first considered the mass balance of CO₂ in our incubations:
\[ F_T n_T = F_i n_i + F_{\text{CH}_4} n_{\text{ox}} \quad (2) \]

where \( F_T \), \( F_i \), and \( F_{\text{CH}_4} \) are the fractional abundance of carbon isotopes \( (^{13}C/^{13}C+^{12}C) \) in the total amount of CO2 \( (F_T) \), in the initial CO2 (before any methane has been oxidized) \( (F_i) \), and in CO2 derived from oxidized methane \( (F_{\text{CH}_4}) \). The CO2 injected into our incubations had a value of \( F_T = 0.0111 \) \( (\delta^{13}C = -37.8 \, \text{‰}) \). \( n_T \), \( n_i \), and \( n_{\text{ox}} \) represent the moles of CO2 in the total amount of CO2, the initial amount of CO2, and the moles of methane oxidized, where

\[ n_T = n_i + n_{\text{ox}} \quad (3) \]

\( F_{\text{CH}_4} \) is then defined by using mass balance:

\[ F_{\text{CH}_4} n_{\text{CH}_4} = F_{\text{stock}} n_{\text{stock}} + F_{\text{label}} n_{\text{label}} \quad (4) \]

where \( F_{\text{stock}} \) and \( F_{\text{label}} \) are the fractional abundance of carbon isotopes in the unlabeled methane and labeled methane added to the incubations. \( n_{\text{stock}} \) and \( n_{\text{label}} \) are the moles of unlabeled \( (n_{\text{stock}}) \) labeled methane in our incubation \( (n_{\text{label}}) \), and total methane \( (n_{\text{CH}_4}) \). \( F_{\text{stock}} = 0.0105 \) \( (\delta^{13}C = -60 \, \text{‰}) \), \( F_{\text{label}} = 0.99 \), and \( F_{\text{CH}_4} = 0.039 \) in our incubations. Substituting equation (4) into equation (2) and solving for \( n_{\text{ox}} \) we obtain:

\[ n_{\text{ox}} = \frac{(F_T n_T - F_i n_i) / (F_{\text{stock}} n_{\text{stock}} + F_{\text{label}} n_{\text{label}})}{F_{\text{CH}_4}} \quad (5) \]

We then solve for \( n_{\text{ox}} \) iteratively by first assuming that \( n_T = n_i \). On subsequent iterations it is assumed that \( n_T = n_i + n_{\text{ox},i} \), where \( n_{\text{ox},i} \) the value \( n_{\text{ox}} \) of calculated from the previous iteration. Within 3-4 iterations the value of \( n_{\text{ox}} \) stabilizes and this value is used as the amount of methane oxidized between two time points. We included the decrease in the amount of gases in our headspace overtime (due to sampling) when calculating the amount of methane oxidized in each incubation. We exclude possible CO2 produced by remineralization of organic matter and therefore our estimates of the amount of methane...
oxidized are a minimum. In addition, we do not include possible methane produced by methanogens. It was found that methanogenesis occurs at ~ 10 % the rate of AOM in methane seep sediments (48), corresponding to a maximum contribution of ~12 μmoles of methane (Table 3-1). This potential contribution of methane is within the uncertainty of the calculated concentration of methane in our incubation and thus we believe it is reasonable to neglect it.

Methane oxidation rates were calculated by taking the difference in the amount of methane oxidized between time points and dividing it by the number of days between them. The error associated with methane oxidation rates comes from uncertainty in values needed to convert δ values to the amount of methane oxidized. These include the amount of CO₂ (+/- 0.5 mL) and ¹³CH₄ (+/- 0.5 mL) injected into the headspace, uncertainty in the pressure of methane in the headspace (+/- 0.1 bar), and uncertainty in headspace volume (+/- 5 mL). We estimate the error in converting δ values to amounts of methane oxidized to be ~ 7 %. This error was propagated to methane oxidation rates using the root mean square method and estimated to be 10 %.

**Sulfate Measurements/Sulfate Reduction Rates**

Sulfate was measured using the SulfaVer4© method from Hach, where barium in the reagent combines with the sulfate to precipitate barium sulfate. The amount of turbidity (measured on a spectrometer at λ = 450 nm) is proportional to the amount of barium sulfate formed. The amount of turbidity relative to the amount of sulfate was calibrated using sulfate standards (ranging in concentration from 0 -30 mM sulfate).
Although there were no known interferences for turbidity measurements in our samples, we created our standards using artificial seawater with varying amounts of sulfate to be consistent with the samples. All samples from incubations were centrifuged to remove sediment from the seawater before sulfate measurements. In addition, we measured the turbidity of the seawater before the sulfate measurements to confirm that there were no interferences in our samples. Sulfate measurements were made immediately after sampling to minimize potential sulfide oxidation to sulfate. Using this method we reliably detected no sulfate (< 30 μM) in various sulfate free high sulfide samples, indicating that the procedure resulted in little, to no, sulfide oxidation.

For incubations starting with > 5 mM sulfate, we made a solution of 0.517 g SulfaVer4© in 50 mL of DI water and pipetted 1.45 mL into cuvettes. We sampled 0.5 mL of slurry (1:3 ratio of sediment to seawater) for each of these sulfate measurements, centrifuged slurry to remove sediment, and pipeted 0.05μl of sample into the cuvettes containing the SulfaVer4© solution. We allowed the reaction to proceed for exactly 5 min before turbidity was spectrographically measured. Incubations with a starting sulfate concentration < 5 mM were measured similarly. 1.5 mL of slurry (1:3 ratio of sediment to seawater) was removed from these incubations. After centrifugation, 0.5 mL were injected into a cuvette containing 0.1 mL SulfaVer4© solution (0.3 g SulfaVer4© in 5 mL water). The turbidity in each cuvette containing samples was measured in triplicate. In addition, sulfate concentration was determined in duplicate at each time point for each subsample. Our lowest detection limit for sulfate was 30 μM.

Sulfate measurements were taken weekly, not twice a week, so as not to oversample the bottles. Because two methane oxidation rates were taken each week, and
only one sulfate reduction rate, the same sulfate reduction rate is plotted with two methane oxidation rates. Sulfate reduction rates were determined by taking the difference between two sulfate measurements and dividing this number by the time between them. The removal of seawater was taken into account when determining moles of sulfate as well as sulfate reduction rates in the incubations. The time points with reported sulfate concentrations that are lower than 30 μM are extrapolated using the last data point with measurable sulfate (> 30 μM) and the subsequent data point, where there was no measurable sulfate (assumed to be 0 mM). Error for sulfate concentrations was determined experimentally using standards and determined to be 10%. This error was propagated to sulfate reduction rates using the root mean square method and estimated at 15%.

**Free energy calculations**

The free energy of sulfate-dependent AOM (eq. 1) at varying sulfate concentrations was calculated using the follow equation:

\[
\Delta G = \Delta G^0 + RT \ln \left( \frac{[\text{HS}^-][\text{HCO}_3^-]}{[\text{CH}_4][\text{SO}_4^{2-}]} \right)
\]  

(6)

where \( R = 0.008314 \text{ kJ/mol}^{-1} \text{ K}^{-1} \), \( T = 283 \text{ K} \), \([\text{CH}_4] = 1.5 \text{ mM} \) (as determined using Henry’s law), \([\text{HCO}_3^-] = 11 \text{ mM} \) (as determined using Henry’s law), \([\text{HS}^-] = 2 \text{ mM} \) (based on added amount of sulfide), \([\text{SO}_4^{2-}] \) varies based on incubation. \( \Delta G^0 \) of sulfate-dependent AOM (eq. 1) is -16.6 kJ/mol.
Results/Discussion

Incubation Experiments

The maximum methane oxidation rate observed for all incubations occurred between days 3 and 6, not between days 0 and 3 (Figure 3-1). However, the lag in sulfate reduction seems to be minimal, as compared with the observed lag in methane oxidation, with most incubations experiencing their peak sulfate reduction rates within the first week of incubation (Figure 3-2). This is perhaps due to the fact that the sediment was sulfate free for 6 months before the start of the experiment, and therefore the AOM intermediate could have built up in the sediment making AOM much less energetically favorable. When sulfate was added to the incubations the sulfate reducers could immediately begin to oxidize organic matter, including the AOM intermediate. However, the AOM organisms needed their waste product, the AOM intermediate, to be drawn down before they could reach their peak methane oxidation rate. Because the peak methane oxidation rates do not occur at the beginning of the incubations, the data from 0-3 days are plotted separately than the other data. These data are labeled as “lag phase” in the figures. By examining the data from the incubations that start at 15 mM and 30 mM sulfate, where methane oxidation rates stay almost constant throughout the experiment, one can estimate that the lag phase data points underestimate actual methane oxidation rates by 2-4 μmoles/day (Figure 3-3).

Rates and amounts of methane oxidation vary with respect to sulfate concentration, with the bottles containing the most sulfate having both the most methane oxidation as well as the highest rates of AOM (Figure 3-3, Table 3-1). There does not appear to be a
Figure 3-1. $^{13}$C enrichment of CO$_2$, reported in $^{13}$F$_{CO2}$ ($^{13}$C/$(^{13}$C+$^{12}$C)) values, through time. The data labels indicate the approximate starting sulfate concentration for the triplicate incubations. These values were then converted to moles methane oxidized, shown on the right hand axis. The data plotted are the average of the three incubations for a given sulfate concentration. The killed control is the autoclaved incubation, which shows that no abiotic isotope exchange occurs between the $^{13}$CH$_4$ and the $^{13}$CO$_2$. Days 0-3 are labeled as lag because peak methane oxidation occurred for all incubations after day 3. The insert contains the rates of methane oxidation for the triplicate incubations throughout the experiments. The error bars are the range of the triplicate incubations (some ranges are within the data point).
Figure 3-2. Sulfate reduction rates as a function of sulfate concentration. The labels for the data indicate the approximate starting sulfate concentration for each of the incubations. Data were fit to the form of the Michaelis-Menten equation. The insert shows sulfate reduction rates as a function of time.
Figure 3-3. Methane oxidation rates plotted against sulfate concentrations. Methane oxidation rates are calculated for each of the data points shown in Figure 3-1 (each point in Figure 3-1 is the average of the triplicate incubations). Because each methane oxidation rate is associated with a range of sulfate concentrations (ex: the sulfate concentration in an incubation from days 0 to 3), the average sulfate concentration for the given time interval is plotted. Methane oxidation rates do not decrease until sulfate levels fall below 1 mM. The shown trendline was used in the redox model. The data from days 0-3 are plotted separately due to an observed lag phase. The insert is an enlargement showing methane oxidation rates at low sulfate concentrations. The data were fit to the form of the Michaelis-Menten equation. Lag phase points (open diamonds) were excluded from the fit.
Table 3-1. Averaged measured parameters for sulfate-dependent AOM incubations.

<table>
<thead>
<tr>
<th>Initial/ Ending Sulfate Concentration (mM)</th>
<th>Total Methane Oxidized (μmole)</th>
<th>Maximum/Minimum AOM Rate (μmole/day)*</th>
<th>Maximum/Minimum Sulfate Reduction Rate (μmole/day)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/0</td>
<td>4</td>
<td>0.3/0.17</td>
<td>0/0</td>
</tr>
<tr>
<td>0.08/0</td>
<td>12</td>
<td>1.5/0.19</td>
<td>0.3/0</td>
</tr>
<tr>
<td>0.6/0</td>
<td>33</td>
<td>4.9/0.24</td>
<td>3.0/0</td>
</tr>
<tr>
<td>0.9/0.001</td>
<td>52</td>
<td>6.6/0.44</td>
<td>4.5/0.2</td>
</tr>
<tr>
<td>15.1/10.4</td>
<td>116</td>
<td>7.9/4.8</td>
<td>10.5/6.2</td>
</tr>
<tr>
<td>30.8/25.8</td>
<td>120</td>
<td>7.9/5.3</td>
<td>9.9/8.5</td>
</tr>
</tbody>
</table>

* Data from insert of Figure 3-1.
** Data from insert of Figure 3-2.
significant decrease in the rate of methane oxidation until sulfate concentrations are well below 1 mM (Figure 3-3), in agreement with results from Iversen and Jørgensen (34). Although methane oxidation rates and sulfate concentrations do not linearly correspond over the range of sulfate concentrations tested, they do appear to follow Michaelis-Menten kinetics with a half saturation constant of ~0.1 mM (Figure 3-3, Table 3-1).

The incubations starting with approximately 1 mM sulfate have a peak AOM rate of 85 % - 100 % of the rate from the incubations with the approximate starting sulfate concentrations of 15 mM and 30 mM. Due to the lag in methane oxidation at the start of the experiment, this peak value does not occur at values near 1 mM but instead occurs when sulfate concentrations in the incubations are around 0.35 mM. The maximum rate of methane oxidation in the incubations with starting sulfate concentrations ~0.5 mM is between 60% and 75% of the rate seen in the incubations starting with 15 mM and 30 mM sulfate. The peak rate in the incubations starting with approximately 0.5 mM sulfate occurs when they have an average sulfate concentration of 0.25 mM. However, in the incubations that started at ~0.1 mM sulfate, methane is oxidized at maximum of 20 % of the rate as the high sulfate level bottles. The maximum AOM rate in these incubations occurred when the average sulfate concentration had dropped to 0.045 mM.

The large range of sulfate concentrations (1-30 mM) at which methane oxidation stays almost constant (Figures 3-1 & 3-2) cannot be explained by phylogenetic differences between the incubations. Our sediment was homogenized before incubation and thus each bottle received approximately the same proportion of each of the ANME groups. The maximum methane oxidation and sulfate reduction rates occurred in all incubations within a week of when they received sulfate. Girguis et al. (19) and Nauhaus (44) approximates
the doubling time of anaerobic methanotrophs to be greater than 3 months. In addition, Orcutt et al. (48) report no significant change in microbial community over a 2 month incubation. Therefore, the proportions of anaerobic methanotrophs in our incubations likely did not change.

The sulfate free incubations all show minimal methane oxidation (3 % of the maximum AOM rate of the 30 mM incubations) (Figure 3-1). These incubations have no free energy available to them through sulfate-dependent AOM, and thus one would expect to see no signal. Previous experiments, that were each monitored, for about one year, without the addition of any new substrates, also show this trend(4). If AOM is responsible for this signal, the mechanism is not yet known, as there is no measureable sulfate present in these incubations. Alternately, as methanogens produce methane, they also anaerobically oxidize small amounts of methane in process termed trace methane oxidation (TMO) (23, 43, 64). In incubation experiments, Moran et al. (43) found that methanogens perform TMO at rates ranging from ~0.01 – 3 μmoles/day. Rates of TMO varied due to substrate growth, methanogen strain, and temperature. The AOM rates seen in our sulfate free incubations are around 0.2 μmole/day, and therefore fall in the known range of rates for TMO. If methanogens are responsible for the signal seen in these incubations through TMO, their growth substrates are yet to be determined.

The highest rates of sulfate reduction occur at the highest sulfate concentrations (15 and 30 mM) (Figure 3-2, Table 3-1). The rate of sulfate reduction, as compared to sulfate concentration, follows Michaelis-Menten kinetics, although there is much scatter in the data (Figure 3-2). Methane oxidation rates and sulfate reduction rates rarely correlated in a
Figure 3-4. Methane oxidation rates (calculated from the data in Figure 3-1) plotted with sulfate reduction rates. The labels for the data indicate the approximate starting sulfate concentration for each of the incubations. High sulfate reduction rates are often associated with high methane oxidation rates. The insert is an enlargement of the figure focusing on the incubations with low sulfate reduction rates, where methane oxidation rates can be as much as an order of magnitude higher than them (x-axis ranges from 0 – 0.6 mM, and y-axis ranges from 0 – 8 μmole/day).
1:1 ratio (Figure 3-4). We found that at high sulfate concentrations (> 10 mM) methane oxidation and sulfate reduction rates are loosely coupled with the ratio of methane oxidation to sulfate reduction ranging from 1:1 to 1:2. However, at low sulfate concentration, methane oxidation rates can greatly exceed sulfate reduction rates (Figure 3-4). In the cases where sulfate reduction is greater than methane oxidation, sulfate is likely being used to oxidize organic matter, which produces isotopically light CO₂. Because of this, our calculations of the amounts of methane oxidation are a minimum value.

At sulfate concentrations lower than 1 mM, methane oxidation greatly exceeds sulfate reduction, often by an order of magnitude (Figure 3-4) showing a strong decoupling between methane oxidation and sulfate reduction. It is not apparent what electron acceptor is responsible for AOM at low sulfate concentrations. In parallel research, Orphan et al., (33) report evidence that ANME-2 methanotrophs can be substantially more active than their sulfate-reducer partners. In addition, environmental geochemical studies indicate that sulfate reduction and methane oxidation are not always coupled (35). Our data further support this idea, and shows that, in particular, low sulfate concentrations seem to enhance this decoupling.

The implications of our experimental results for the stoichiometry of AOM at low sulfate concentrations are not clear. Conceptually, rates of AOM exceeding that of sulfate reduction in our bottles would imply another oxidant other than sulfate. However, it is doubtful that oxidized Mn or Fe would have persisted during the pre-incubation, especially considering the high concentration of sulfide, which would abiotically reduced any oxidized Mn an Fe (1, 8), in the experiments. Perhaps, at high methane to sulfate ratios, AOM loses some of its efficiency resulting in the loss of electrons, hydrogen, or other
reduced metabolites to other microbial processes (besides sulfate reduction) in the sediment. These other microbial processes could include methanogenesis, which would reduce the amount of net methane destroyed by these high AOM rates. While the high rates of AOM at low sulfate concentrations (< 1 mM) may not fully translate into net methane oxidation in marine sediment, the observed kinetics for AOM at low sulfate still demonstrates that sulfate-dependent methanotrophs can utilize remarkably low sulfate concentrations (~0.2 mM). This observation has implication for the late Archean water column when sulfate was first becoming appreciable and atmospheric methane was high (discussed below).

**Energetics**

The incubations with the most sulfate also have the most energy available to them through sulfate-dependent AOM (Figure 3-5). Although sulfate reduction rates do not correlate linearly with sulfate concentration, they do show a strong linear correlation with their available free energy (Figure 3-5). This suggests that organisms involved in sulfate reduction are driven by the amount of energy available to them. However, methane oxidation rates only correspond linearly with available energy up to around 0.5 mM sulfate (Figure 3-5). Above this level, bottles with increased free energy (due to an increase in sulfate) do not show greater methane oxidation rates. Although it appears that there is a strong decoupling between methane oxidation rates, sulfate concentrations, and the available free energy, the rate of AOM is strongly influenced by sulfate concentration at low sulfate levels (Figures 3-1 – 3-4).
Figure 3-5. Averaged maximum sulfate reduction and methane oxidation rates from triplicate incubations plotted with the corresponding free energy from sulfate-dependent AOM and sulfate concentration. Open circles are sulfate reduction rates. Closed squares are methane oxidation rates. Maximum sulfate reduction rates for the incubations of approximate starting sulfate concentrations of 0.1, 0.5, and 1 mM occurred at their initial sulfate concentrations. Maximum sulfate reduction rates for the incubations starting at 15 mM sulfate occurred at the averaged sulfate concentration of 11.8 mM, and at 27 mM for the incubations starting at 30 mM sulfate. The peak methane oxidation rates for these incubation occurred between days 3 and 6 of the experiments. All free energies are calculated for sulfate-dependent AOM. The sulfate concentration used in the calculations corresponds with the peak methane oxidation/sulfate reduction rate occurred.
Model

Claire et al. (63) describe a biogeochemical box model for the evolution of the redox state of Earth’s atmosphere over geologic time. The model includes parameterized atmospheric chemistry, hydrogen escape, crust/mantle evolution and degassing, oxidative weathering, and simple biologic parameterizations. The purpose of the model was to elucidate the major controls on the evolution of atmospheric oxygen, and to provide a self-consistent quantitative framework to explore theories for the GOE. Here, we follow the approach of Catling et al. (11) who use the framework of the Claire et al. (63) model, but only examine the atmospheric redox transition itself. In this manner, our results are independent of assumptions about volcanic/metamorphic outgassing and hydrogen escape, which primarily affect the “timing” of the anoxic/oxic transition in the numerical model (11). At the time of the oxic transition, important variables in the redox balance of the atmosphere are the net flux of free oxygen due to organic carbon burial, $F_B$, the reducing fluxes from volcanism and metamorphism, $F_V$ and $F_M$, oxidative weathering, $F_W$, and the biological fluxes of oxidizing and reducing gases. Hydrogen escape is negligible at this time, and for the transition to occur, the numerical values of $F_B$ and $F_V+F_M$ sum to zero, an assumption which is independent of any specific conceptual model for why the oxic transition occurred. As $O_2$ accumulated during the GOE, oxidative weathering became a quantitatively significant portion of the redox balance. Therefore, in our simple model, the most important redox fluxes at the time of the oxic transition are biology and oxidative weathering.

The model solves the following equations:
\[
\frac{d}{dt} [\text{O}_2] = 2\phi - 2k_{\text{eff}} [\text{O}_2] [\text{CH}_4] - F_W + F_{\text{M}} - (F_{\text{V}} + F_{\text{M}})^0 \text{ at time of oxic transition}
\]

\[
\frac{d}{dt} [\text{CH}_4] = (\phi - F_{\text{AOM}}) - k_{\text{eff}} [\text{O}_2] [\text{CH}_4]
\]

where \(\phi\) is the biospheric \(\text{CH}_4\) flux, \(k_{\text{eff}}\) captures the net photochemical interaction of \(\text{CH}_4\) and \(\text{O}_2\) (63), and \(F_{\text{AOM}}\) is the flux of methane consumed by AOM. Oceanic sulfate concentrations are estimated as \([\text{SO}_4^{2-}] = 0.03[\text{O}_2]^{0.4}\) (11) and oxidative weathering as \(F_W = 0.0065[\text{O}_2]^{0.4}\) (63), with \([\text{O}_2]\) in Tmol \((10^{12}\) moles). Two semi-analytical parameterizations for \(F_{\text{AOM}}\) as a function of oceanic sulfate concentrations were described in Catling et al. (11). The first method used the simple estimate that 45\% of the modern oxidative weathering flux is used to oxidized sulfides to sulfates, and assumed that all sulfate was “available” for AOM. The second method was drawn from the sedimentology literature which shows that, in many situations, the AOM rate is limited by the diffusion of sulfate into sediments. Both of these parameterizations yielded faster timescales for the oxic transition and featured both oxygen and methane rising to higher values, when compared to \(F_{\text{AOM}} = 0\).

Here, we replace these semi-analytic approximations with a fit to the data in Figure 3-3:

\[
F_{\text{AOM}} = \alpha \times \left( \frac{7.5 \times [\text{SO}_4^{2-}]}{0.093 + [\text{SO}_4^{2-}]} \right)
\]
The conversion factor, $\alpha$, between the measurement units [$\mu$mol day$^{-1}$] and the flux unit of the global biogeochemical model [Tmol ($10^{12}$ moles) yr$^{-1}$] is $365/10^{18}$ before accounting for the difference in surface area between the measurement bottle and the global extent of sediments. Assuming that 10% of the global ocean area ($1.8 \times 10^{18}$ cm$^2$) has sediments which produce/consume methane, and given our experimental sediment surface area of $\sim$20 cm$^2$, we obtain a conversion factor of order unity. Given the significant uncertainties in these numbers, we adopt a proportionality constant of 1, and drive equation 2 with the AOM parameterization in equation 3.

The model results in Figure 3-6 further strengthen the contention of Catling et al. (11) that anaerobic oxidizers of methane can play a significant role in the evolution of redox gases in the atmosphere in general, and in the structure of the GOE in particular. The considerably higher AOM rates at lower sulfate concentrations than expected produce lower steady-state methane levels in the Archean, and drive a strong positive feedback on atmospheric oxygen concentrations, once methane levels begin to collapse. Using the empirical AOM data, oxygen levels rise more abruptly than previously estimated, allowing methane to quickly re-rise to $\sim$ 20 ppm, which might aid in recovery from the postulated “snowball Earth” episode at this time period. At the time of the oxic transition, the direct effect of biology on the bulk composition of the atmosphere was perhaps at its maximum in Earth’s history (13), and the positive feedback provided by methane oxidizers was likely a key factor in establishing the dominance (and irreversibility) of the oxic biosphere.

An additional factor that might affect the redox balance in the Archean is that both manganese- and iron-dependent AOM were likely a large part of Archean methane cycling (5). Studies found that the rates of manganese- and iron-dependent AOM are 27% and 12
Figure 3-6: The evolution of atmospheric CH$_4$ (solid lines) and O$_2$ (dashed lines), varying the parameterization for the anaerobic oxidation of methane. The thin black lines show the evolution of the atmosphere when AOM is parameterized via sulfate’s diffusion properties (“method 2” of Catling et al. 2007), while the thick black lines show the same model run but using equation 3 for AOM. Both models feature a collapse in CH$_4$ concentrations, a dramatic rise in O$_2$, followed by a re-rise in CH$_4$ enabled by the photochemical shielding by O$_3$, as captured by our atmospheric chemistry parameterization.
% respectively of the rate of sulfate-dependent AOM (at 5 mM sulfate) (5). Since the data presented here indicated that there would be little increase in the rate of AOM between 5 mM sulfate and present day oceanic sulfate levels (28 mM), we estimated that together, manganese- and iron-dependent AOM, consume methane at 39 % of the rate of the modern sulfate-dependent rate. Estimates of atmospheric methane during both the Archean and the Proterozoic are high (38, 54), and thus methane was likely not a limiting factor in AOM during these time periods. In addition, large amounts of manganese and iron oxides were provided to the oceans from photooxidation(5), suggesting that manganese- and iron-dependent AOM were likely not limited by the availability of methane, manganese, or iron during the Archean and was only limited by the kinetics of the reaction. When 39% of the modern sulfate-dependent AOM rate is added to equation (3), the length and structure of the transition event is unchanged from that shown on Figure 3-6, although steady state Archean CH₄ values are up to 50 % lower. If large AOM rates are a feature of the Archean, quantitatively more reducing gases than proposed in Claire et al. (63) would be needed to delay the oxic transition to 2.4 Ga, although the amount required falls within the large uncertainties inherent in creating a redox budget for the Archean.

**Further implications**

The earliest viable evidence for the anaerobic oxidation of methane (AOM) comes from a large negative carbon excursion in sedimentary rocks ~2.7 Ga, which is normally attributed to aerobic methanotrophy (24). Based on current growth rates of anaerobic methanotrophs, Hinrichs (25) finds that these organisms produce enough
biomass to account for the 2.7 Ga carbon isotope excursion, without aerobic methanotrophy playing a role. However, an estimate of the average sulfate concentration in oceans 2.2 Ga is ~1 mM (10, 16), implying that global sulfate levels would have been even lower ~2.7 Ga. Conventionally, it is thought that only small amounts of methane are consumed by AOM at these sulfate levels, due to the belief that methane oxidation and sulfate reduction always occur in a 1:1 ratio (45, 46). Therefore, assuming that sulfate is the dominant electron acceptor in marine anaerobic methanotrophy, AOM would have been sulfate-limited during this time interval. Our results, presented here, suggest that significant amounts of AOM can occur at sulfate levels well below 1 mM, indicating that sulfate-dependent AOM would not have been as limited in the Archean as previously thought. Even if the sulfate concentrations at 2.7 Ga had not increased from the level of the early Archean of 0.2 mM (20), we estimate that AOM organisms would have been able to oxidize methane at 68 % of the rate as organisms today, and thus could have contributed significantly to this carbon isotope excursion.

Our results, which show that large amounts of methane oxidation occurs at significantly lower sulfate levels than previously assumed, not only influence our understand of early Earth processes, but also influence our thinking of present day processes. Freshwater environments, such as lakes and rivers, have average sulfate levels around 0.1 mM (62), and are supplied with methane through methanogens living in the anoxic sediments. Our results suggest that at these sulfate levels, methanotrophic archaea are capable of oxidizing methane at ~50 % of the rate of organisms living in marine settings, and thus implicating non-marine environments as an important methane sink. Indeed, it has been recently shown that AOM is occurring in freshwater environments
with low sulfate levels (15, 58), and thus an important factor when exploring current methane cycling.

In addition, our results indicate that organisms involved in AOM can survive on very little energy availability (in sulfate concentrations ranging from 0 mM – 1 mM). In fact, it appears that AOM organisms, or methanogens involved with TMO, can survive and metabolize within a closed system for at least one year. This allows for the possibility that AOM or TMO is occurring in the deep biosphere where there are significant amounts of methane, but little or no measurable sulfate.

References


Chapter 4

Manganese- and Iron- Dependent Marine methane Oxidation

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\textbf{Abstract}

Anaerobic methanotrophs help regulate Earth’s climate and may have been an important part of the microbial ecosystem on the early Earth. The anaerobic oxidation of methane (AOM) is often thought of as a sulfate-dependent process despite the fact that other electron acceptors are more energetically favorable. Here, we show that microorganisms from marine methane seep sediment in the Eel River Basin (ERB), CA are capable of using manganese (birnessite) and iron (ferrihydrite) to oxidize methane, revealing that marine AOM is coupled, either directly or indirectly, to a larger variety of oxidants than previously thought. Large amounts of manganese and iron are provided to oceans from rivers, indicating that manganese and iron dependent AOM have the potential to be globally significant.
**Background/Results/Discussion**

AOM occurs in freshwater samples in the absence of sulfate provided nitrite or nitrate is present (11, 32). Incubation studies show manganese (MnO$_2$) or iron (FeCl$_2$ and FeCl$_3$) addition to anoxic sediments and digested sewage increases the ratio of methane oxidized to methane produced (39). However, there has been no direct evidence for AOM in the absence of sulfate in marine samples (23). Studies of pore-water geochemistry show manganese and iron reduction in areas where AOM occurs (9), and the highest AOM rates in marine sediment do not always correlate with the highest sulfate reduction rates (17). Furthermore, sediments of the uplifted Franciscan Complex, a paleo-analogue of the ERB, show methane-derived $^{13}$C-depleted carbonate associated with rhodocrosite (MnCO$_3$) (15). In addition, there is enrichment of manganese and other metals in methane seep associated carbonates from the Black Sea (34).

Here, we show that birnessite (Figure 4-1), and ferrihydrite (Figure 4-2), can be used as electron acceptors in marine AOM. Large amounts of manganese (~19 Tg/year (31)) and iron (~730 Tg/year (20)) are provided to continental margins from rivers (3). Iron and manganese are provided to the ERB, in this manner, by high sediment discharge from the Eel River, which drains the northern California Coast Range (38). If the entire global flux of manganese and iron is used to oxidize methane, it could account for about one fourth of present day AOM consumption. Even if only a small percentage of the influx of manganese and iron is used for AOM, it still has the potential to be a large methane sink since both manganese and iron can be oxidized and reduced 100 – 300 times before burial (7).
Figure 4-1. $^{13}$C enrichment of CO$_2$ reported in $^{13}$F$_{CO2}$($^{13}$C/$^{13}$C+12C) values and converted to moles methane oxidized. The incubations with manganese oxidize (birnessite) oxidize about 3.5 times more methane than the live control (sulfate free, no provided electron acceptor), indicating that manganese can be used as an electron acceptor in AOM. Error bars represent the range of the triplicate incubations. The standard deviations of the triplicate incubations for the birnessite and live controls are within the symbol for each data point. In addition, when more birnessite is injected into the cultures, the rate of AOM increases ~30%, from ~11 μmole/year/cm$^3$sed (days 23 –43) to ~14 μmole/year/cm$^3$sed (days 43 – 57).
Figure 4-2. $^{13}$C enrichment of CO$_2$ reported in $^{13}$F$_{CO2}$ ($^{13}$C/$^{13}$C+$^{12}$C) values and converted moles methane oxidized. The incubations with iron (ferrihydrite) oxidize about 5 times more methane than the live control (sulfate free, no provided electron acceptor), indicating that iron can be used as an electron acceptor in AOM. Error bars represent the range of data from the triplicate incubations. The standard deviations of the triplicate incubations for the ferrihydrite and live controls are within the symbol for each data point.
Methane seep sediment from the ERB was incubated with methane, $^{13}$C-labeled methane, CO$_2$, and artificial sulfate-free seawater. Triplicate incubations were either given sulfate, birnessite, ferric oxyhydroxide, ferrihydrite, nitrate, nitrate and sulfate, or no electron acceptor (live control). The birnessite and ferrihydrite experiments were pre-incubated (1) to insure that they were sulfate free. As methane is oxidized, the $^{13}$C-label is transferred from methane to CO$_2$, and thus we can monitor AOM by measuring the $^{13}$C enrichment in the CO$_2$ throughout the experiment. $\delta^{13}$CO$_2$ values are then converted into the amount of methane oxidized (21, 22).

We measured $^{13}$C-enrichment of CO$_2$ in cultures supplied with sulfate, birnessite, and ferrihydrite indicating that AOM can proceed in the absence of sulfate if birnessite (Figure 4-1) or ferrihydrite (Figure 4-2) is present (2). Sulfate was measured (SulfaVer4© method from Hach) periodically in all live control, birnessite, and ferrihydrite incubations to show that they remained sulfate free (< 30 μM sulfate) for the duration of the experiment. An autoclaved bottle (Figures 4-1 and 4-2), containing either birnessite or ferrihydrite, shows that no abiotic isotopic exchange between methane and CO$_2$ and no abiotic production of sulfate occurs. Incubations with just nitrate, as well as with nitrate and sulfate, appear to inhibit AOM [see APPENDIX A (Figure A-1)]. We also see no evidence for AOM in the presence of ferric oxyhydroxide [see APPENDIX A (Figure A-1)].

The net reaction for the AOM is often framed as:

$$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}$$  \hspace{1cm} (equation 1)

AOM with sulfate (equation 1) provides organisms with a potential Gibbs free energy of $\Delta G = -14$ kJ/mole for our in situ conditions. All of the sulfate incubations
reported here oxidize methane at an approximate rate of 52 μmole/year/cm$^3_{sed}$ (Figure 4-1), corresponding to a potential energy gain of 0.7 J/year/cm$^3_{sed}$ (Table 1). However, the oxidation of methane with birnessite (simplified to MnO$_2$) yields $\Delta G = -556$ kJ/mole at our in situ conditions (equation 2).

$$\text{CH}_4 + 4\text{MnO}_2 + 7\text{H}^+ \rightarrow \text{HCO}_3^- + 4\text{Mn}^{2+} + 5\text{H}_2\text{O} \text{(equation 2)}$$

The observed rate of birnessite dependent AOM is 14 μmole/year/cm$^3_{sed}$ (Figure 4-1), which equals a potential energy gain of 7.8 J/year/cm$^3_{sed}$ (Table 1). Therefore, although the rate of sulfate dependent AOM is about four times faster than birnessite dependent AOM, the birnessite incubation have the potential to gain 10 times the amount of energy than the sulfate incubations.

AOM coupled to ferrihydrite (simplified as Fe(OH)$_3$) reduction (equation 3) yields a potential free energy of $\Delta G = -270.3$ kJ/mol at our in situ conditions.

$$\text{CH}_4 + 8\text{Fe(OH)}_3 + 15\text{H}^+ \rightarrow \text{HCO}_3^- + 8\text{Fe}^{2+} + 21\text{H}_2\text{O} \text{(equation 3)}$$

The incubations with ferrihydrite oxidize methane at an average rate of 6 μmole/year/cm$^3_{sed}$ (Fig 2), corresponding to a potential energy gain of 1.6 J/year/cm$^3_{sed}$ (Table 1). This shows that the microorganisms responsible for ferrihydrite dependent AOM have the potential to receive energy at approximately twice the rate of sulfate dependent AOM, despite the fact that they are oxidizing methane at approximately one tenth the rate.

Previous culture studies have found that microorganisms from the Back Sea can reduce manganese oxides more efficiently than ferrihydrite (36). This result is consistent with our experiment, where we see that manganese dependent AOM occurs at a faster rate
<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate (μmole/year/cm³_sed)</th>
<th>Potential Energy Gain (J/year/cm³_sed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₄²⁻ + CH₄ → HCO₃⁻ + HS⁻ + H₂O</td>
<td>52</td>
<td>0.7</td>
</tr>
<tr>
<td>CH₄ + 4MnO₂ + 7H⁺ → HCO₃⁻ + 4Mn²⁺ + 5H₂O</td>
<td>14</td>
<td>7.8</td>
</tr>
<tr>
<td>CH₄ + 8 Fe(OH)₃ +15H⁺ → HCO₃⁻ + 8Fe²⁺ +21H₂O</td>
<td>6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 4-1. Rates and potential energy gain from AOM with different electron acceptors
than iron dependent AOM. Both manganese and iron dependent AOM occur at much slower rates than sulfate dependent AOM, although they are significantly more energetically favorable. This can be explained by considering that manganese and iron oxides are both solids, and thus less accessible than sulfate. Despite the slower methane oxidation rates of manganese and iron dependent AOM, it is likely that they are a significant part of biogeochemical methane cycling.

There are three known archaeal groups responsible for AOM: ANME-1 and ANME-2 (28) and ANME-3 (18). ANME commonly have sulfate reducing bacterial partners, often related to Desulfosarcinales and Desulfobulbus (25-27, 30). However, ANME-1 and some ANME-2 have been found to live independently, suggesting that they may not need a physically associated sulfate reducing bacteria to perform AOM (27, 28). The fact that ANME are often found with sulfate reducers does not necessitate that sulfate is needed for AOM to proceed. Some sulfate reducing bacteria can facultatively use electron acceptors other than sulfate (8, 24, 35). In fact, one species of Desulfobulbus is capable of iron reduction (16). The presence of greigite magnetosomes in sulfate reducing bacteria associated with ANME-2 from the Black Sea further suggests a role in iron cycling (33, 34).

To study the microbial communities responsible for manganese dependent AOM, we sampled one incubation from each set of conditions at the end of the experiment and determined changes in the microbial assemblage based on 16S rRNA and methyl coenzyme M reductase (mcrA) gene diversity. Over the course of the 10-month incubation (1) a shift was observed in the archaeal diversity relative to the starting sediment. The proportion of phylotypes associated with the crenarchaeota increased in both the manganese incubation
and the live control (sulfate-free, no added electron acceptor), while the sulfate incubation supported an increase in euryarchaeota, in particular phylotypes belonging to ANME 2b and 2c [see APPENDIX A (Figure A-2)]. Uncultured phylotypes belonging to Marine Benthic Group D (MBGD) were the most abundant in the starting sediment and remained a significant component of the archaeal diversity in all treatments, representing 35% or more of the total clones [see Appendix A (Figure A-2)]. The metabolic potential of MBGD is not currently known, however it is interesting to note that the closest cultured relative of many of the recovered phylotypes are methanogens (80% identity) and their potential role in methane cycling warrants further investigation. An increase in phylotypes associated with the Crenarchaeota Marine Benthic Group C (MBGC), which is absent in the sulfate incubations, was observed in both the manganese and live control incubations [see Appendix A (Figure A-2)].

16S rRNA phylotypes belonging to the known methanotrophic ANME groups comprised a relatively small proportion of all sediment incubations, with ANME-1 representing no more than 5% of the total archaeal diversity. However, analysis of the \( mcrA \) gene (specific for methanogens and methanotrophic archaea), indicated a greater diversity of the methanotrophic ANME than was recovered by the initial 16S rRNA screen. Specifically, with the exception of the sulfate incubations, the most common \( mcrA \) gene came from the ANME-1 (approximately 85%) [see Appendix A (Figure A-2)]. In the sulfate incubations, ANME-1 represented 42% of the recovered \( mcrA \) genes, with ANME-2 representing 46%. The manganese and sulfate incubations revealed an increase in diversity supporting a small percentage of ANME-3, not observed in the original sediment or live control [see Appendix A (Figure A-2)].
Approximately 40% of the bacteria found in the birnessite incubation are possible manganese reducers (Figure 4-3, [see Appendix A (Figure A-3)]). These include clones related to microorganisms found in heavy metal contaminated sites or from hydrothermal systems. Specifically, the groups Bacteriodes, Proteobacteria (including Geobacter), Acidobacteria, and Verrucomicrobia contain representatives likely capable of metal reduction (Figure 4-3) (10, 12-14). Bacteriodes are only present in the manganese and control incubations, while Acidobacteria are only present in the manganese incubations (Figure 4-3). The clones related to sulfur cycling in the birnessite incubations are almost all sulfur oxidizers, such as the ε-Proteobacteria Sulfurovumales. The bacteria in the sulfate incubations are dominated by sulfate reducers, mainly Desulfobulbus.

The large change towards manganese reducers observed in the bacterial community from the birnessite incubation suggests that bacteria are playing a vital role in manganese dependent AOM and that archaea are not solely responsible (Figure 4-3). In the birnessite incubation, the relative proportion of ANME-2 decreases, while Methanococccoides/ANME-3 increases and ANME-1 stays relatively constant (see Appendix A (Figure A-2). Overall, our data implies manganese dependent AOM is either carried out by ANME-1 and/or Methanococccoides/ANME-3 with a bacterial partner or that manganese dependent AOM in this case is not performed by archaea, but rather solely by bacteria. If bacteria are indeed solely responsible for manganese dependent AOM, it is likely that they do not contain the mcrA gene, as recently observed for nitrite dependent AOM (11).

Abiotic and biotic processes can oxidize sulfide to sulfur in the presence of metal oxides (4, 6). In principle, sulfur disproportionation producing transient sulfate, mediated
Figure 4-3. Percent distribution of recovered bacterial clones based on 16S rRNA genes in the starting sediment (Other includes clades OP3 and Marine Group A), live control, manganese (Other includes clades Elusimicrobia and KSB3), and sulfate incubations (Other includes clades Marine Group A, KSB3, GN02, and TM6). Sulfur-metabolism indicates phylotypes putatively involved in sulfur cycling. Metal associated represent phylotypes that are possible manganese reducers. Metal/Sulfur are the phylotypes that have the potential to partake in sulfur and/or metal cycling. The starting sediment was stored anaerobically for approximately a year before use, and therefore does not reflect the proportions of bacteria when it was sampled.
perhaps by *Desulfobulbus* (19) or ε-Proteobacteria, could be the underlying process observed, indirectly linking AOM to metal reduction. While the shift in the bacterial community from known sulfate-reducing bacteria to putative metal-reducing microorganisms in the birnessite incubations supports the idea that the AOM is directly linked to metal reduction, the observed shift in microbial community could also be a result of the stimulation of heterotrophic metal-reduction. If metal-reduction is indirectly linked to AOM in marine sediments, then the realized energy gain for the microorganisms directly catalyzing AOM would be much lower than that suggested in Table 1. Regardless of mechanism, the stimulation of AOM with Mn and Fe has important implications for capacity of CH₄ oxidation.

It is estimated that AOM consumes most methane released in marine settings, equaling 5-20% of today’s total global methane flux (37), making this process an important part of the global carbon cycle today. However, before Earth became oxygenated, growth of methanotrophs was limited by their ability to find electron acceptors. Based on the column integrated photooxidation rates of 5 mg/cm²/year of manganese and 200 mg/cm²/year of iron (5), on the order of 10,000 Tg/year of methane could be oxidized during this time period by manganese and iron dependent AOM, irrespective of whether the processes directly link metal reduction to methane oxidation. Estimates of the methane flux to the atmosphere during the Proterozoic are on the order of 1,000-10,000 Tg/year (29), meaning that manganese and iron dependent AOM had the oxidative potential to oxidize the entire early Earth methane flux. Thus, manganese and iron dependent AOM could have been extremely important methane sink, as well as energy sources, for the early biosphere.
References

1. A full description of the methods can be found in the Materials and Methods section in Appendix A.
2. See relevant Appendix A text for discussion regarding dissolved manganese and iron concentrations.
3. There are minor contributions of manganese and iron from hydrothermal systems and aeolian input. See Appendix A text for further discussion.
13. Gremion, F., A. Chatzinotas, and H. Harms. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a
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35. Tebo, B. M., and A. Y. Obraztsova. 1998. Sulfate-reducing bacterium grows with Cr (VI), U (VI), Mn (IV), and Fe (III) as electron acceptors. FEMS microbiology letters 162:193.


Chapter 5

Molecular Characterization of Metal Dependent Marine Methane Oxidation

Recently we reported that both manganese and iron can be used as electron acceptors in methane oxidation in marine sediments, which previously was thought to be exclusively a sulfate-dependent process. However, the mechanism and the organisms responsible for manganese- and iron-dependent AOM remained elusive. Here, we focus mainly on characterizing organisms involved in manganese-dependent AOM.

Comparison of 16S rRNA phylotypes from our various incubations indicates that manganese-dependent AOM is likely occurring by a different mechanism and/or different microbial assemblage from that of both iron- and sulfate-dependent AOM. Through FISH and FISH-SIMS analyses using $^{13}$C and $^{15}$N labeled substrates, we find that the most active cells during manganese dependent AOM are primarily mixed and mixed-cluster aggregates of archaea and bacteria that are distinct from the ANME-2/Desulfosarcina-Desulfococcus (DSS) shell aggregates that are catalyzing sulfate-dependent AOM in the control incubation. Based on the results from phylogenetic analysis, FISH and FISH-SIMS analysis, we suggest that Mn-driven AOM is fueled by a direct coupling of metal-reduction to methane oxidation, without transient sulfate production.
Introduction

By following a $^{13}$C-label during the anaerobic oxidation of methane (AOM), Beal et al. (3) found that manganese, in the form of birnessite, and iron, in the form of ferrihydrite, can be used in marine AOM in the absence of sulfate (3). Manganese- and iron-dependent marine methane oxidation occur at one fourth and one tenth the rate, respectively of sulfate-dependent AOM. Despite the slower rates, which are likely due to inaccessibility of the solid electron acceptors, both manganese- and iron-dependent AOM have the potential to significantly contribute to methane cycling throughout Earth history (3). Although nitrite and nitrate have been shown to be possible electron acceptors in freshwater environments (11, 33), there is no evidence that organisms in marine sediments are capable of metabolizing methane using nitrate (3, 27).

There is geologic and geochemical evidence for metal-dependent marine AOM. The carbonate in rhodocrosite (MnCO$_3$) deposits, throughout the California Coast Range, have isotopically light carbon indicating that the carbon source is methane (13). Both manganese and iron enrichment in methane associated carbonates have been observed in the Black Sea, as well (35). In addition, bacterial sulfate reducers, associated with anaerobic methanotrophs, sometimes contain greigite magnetosomes (34, 35). Porewater profiles from the Gulf of Mexico show strong decoupling between methane oxidation and sulfate reduction (18). Moreover, geochemical porewater profiles in the equatorial pacific near Peru show manganese and iron reduction occurring in the same sediment intervals as methane oxidation (9).
Today, marine sulfate-dependent AOM is performed by at least three groups of archaea: ANME-1, ANME-2, and ANME-3 (5, 15, 19, 26-30). ANME-1 are often found living independently suggesting that both methane oxidation and sulfate reduction can occur within one organism (29, 30). However, both ANME-2 and ANME-3 are usually found in organized aggregates with sulfate reducing bacteria (mainly Desulfosarcinales and Desulfobulbus) indicating that in some cases methane oxidation occurs in the ANME archaea but requires a syntrophic association with a sulfate-reducing bacterial partner (19, 29, 30). Interestingly, it has been found that one species of Desulfobulbus is capable of metal reduction (16).

Upon the original finding of AOM coupled to denitrification, data suggested that this process is performed by a consortia of archaea, which are distantly related to ANME-2, and denitrifying bacteria (33). However, with subsequent enrichment cultures, it was found that AOM coupled to denitrification appears to be carried out solely by bacteria with no involvement by any archaea (11). In addition, it was found that methyl-coenzyme reductase A (mcrA), the enzyme believed to activate methane in methanotrophic archaea, is likely not involved in bacterially mediated nitrite-dependent AOM (11).

Analysis of microbial communities from manganese-dependent AOM incubations show a significant enrichment of putative bacterial manganese reducers, suggesting their involvement in this process (2). In addition, the relative proportion of ANME-1 stays constant, ANME-2 decreases and ANME-3 increases relative to the controls (2), suggesting manganese-dependent marine methane oxidation likely occurs either by a consortia of ANME-1 or ANME-3 and manganese reducers, or solely by bacteria (2).
However, it remains possible that a transient, undetectable amount of sulfate was cycled in the incubations. The data presented here further characterize the communities responsible for manganese dependent AOM using FISH, and FISH coupled to secondary ion mass spectrometry (FISH-SIMS) in an attempt to determine the mechanism, as well as the microorganisms responsible for metal dependent AOM.

The SIMS study uses the novel technique of incubation with a $^{15}$N-labeled substrate (in our case $^{15}$NH$_4$Cl) to track *de novo* protein synthesis within either single cells or cell aggregates and thus has the capability to determine which cells have an active metabolism (31). $^{15}$N-tracers have been successfully used in studies with environmental samples to study metabolic activity, including those regarding AOM (7, 20, 31, 39). It has been demonstrated that using SIMS in conjunction with a $^{15}$N-tracer can accurately indicate active cells and cell aggregates within AOM incubations (31). However, there are potential drawbacks to this method, such as abiotic adherence of $^{15}$N to cells. In addition, different types of microorganism likely uptake $^{15}$NH$_4$Cl at different rates and therefore this method can only be used to positively identify active cells, and not relative activity between numerous targets.

**Methods**

**Incubations/Phylogenetic Analysis**

All analyzed samples came from the two incubation studies described by Beal et al. (2). The methane seep sediment samples used in the incubations came from the Eel
River Basin, CA (ERB) collected in either in August 2005 using the R/V *Western Flyer* (manganese experiments) or in September 2006 using Alvin and the R/V *Atlantis* (iron experiments). All incubations contained methane seep sediment (location described above), artificial seawater (24), and an added electron acceptor (sulfate, birnessite, ferrihydrite), or no added electron acceptor. Both the manganese and the iron incubations had separate controls containing sulfate, controls with no added electron acceptor, and an autoclaved control (to test for abiotic exchange between $^{13}\text{CH}_4$ and $^{13}\text{CO}_2$). All incubations were conducted in triplicate. A full description of the phylogenetic analysis for the manganese experiments can be found in Beal et al. (3), and for the iron experiments in Appendix B. At the end of the 10-month experiments, one of each of the triplicate incubations (for the manganese experiments and the ferrihydrite experiments) was amended with $^{15}\text{NH}_4\text{Cl}$ at a final concentration of 2 mM. The experiments were incubated at 10º C for an additional three weeks with the $^{15}\text{NH}_4\text{Cl}$ and then sampled (0.5 mL sediment/sea water slurry) for FISH- SIMS analysis to determine which target aggregates incorporated $^{15}$N during *de novo* protein synthesis (and thus are active in our incubations) (31). Beal et al. (2009) (3) extracted DNA from the manganese incubation, its associated controls, and the original sediment used for the incubations using an UltraClean Soil isolation Kit (Mo Bio Laboratories). The DNA was PCR amplified using three sets of primers: bacterial specific (27F and 1492R), archaeal specific (21F and 958R), and mcrA specific. Cloning was conducted using a TopoTA Cloning Kit (Invitrogen, USA). 46 archaeal, 47 bacterial, and 46 mcrA clones from the original sediment, 24 archaeal, 23 bacteria and 24 mcrA clones from the live control, 31 archaeal, 31 bacterial, and 32 mcrA clones from the manganese incubations, and 23 archaeal, 24
bacterial, and 24 mcrA clones from the sulfate incubations were screened (GENBANK accession numbers FJ264513 to FJ264602 and FJ264604 to FJ264884). Classification of archaea was based on the scheme presented in Knittel et al. (2004) (19). mcrA sequences were classified using close BLAST hits (36) and classified mcrA sequences from Hallam et al. (2003) (12). We used PAUP to create bacterial neighbor joining trees. Bacterial metabolisms were putatively identified using the phylogenetic association of clones (determined using the greengenes classify tool (10)) as well as using the closest BLAST hits (36). Sequences from the iron incubation, its associated controls (sulfate and no added electron acceptor), and the original sediment used in the iron incubations was analyzed in the same manner as described above for the manganese incubations (results in Appendix B). 32 archaeal, 27 bacterial, 32 mcrA and clones from the original sediment, 23 archaeal, 21 bacteria and 23 mcrA clones from the live control, 47 archaeal, 48 bacterial, and 47 mcrA clones from the iron incubation, and 24 archaeal, 23 bacterial, and 24 mcrA clones from the sulfate incubations were screened (GENBANK accession numbers GQ356797-GQ357040).

We determine the relative amount of change based on 16S rRNA archaeal and bacterial sequences between the manganese incubation and its associated sulfate incubation as well as the iron incubation and the associated sulfate incubation. Bacterial 16S rRNA sequences are subdived into microorganisms involved in metal cycling, microorganisms involved in metal and sulfur cycling, microorganisms involved in sulfur cycling, and microorganisms with unknown/non-metal/sulfur metabolisms based on the classification described previously (3).
FISH

The sediment sampled (1 mL sediment/seawater slurry) after the period of $^{15}$NH$_4$Cl incubation was initially washed three times using 1.5 x phosphate-buffer saline (PBS) (PBS was added to sample, sample was centrifuged, supernatment was discarded). Samples were then fixed by adding 750 μL 4 % (wt/vol) paraformaldehyde and 250 mL 1.5 x PBS and incubating for 3 hours at 4º C. Samples were washed using 1.5 x PBS and then stored at -20º C in a 1:1 ethanol/PBS solution. Aggregates were separated from sediment on a Percoll density gradient at 4º C as outlined by Orphan et al. (2001) (30). The entire Percoll gradient (with the exception of the sediment pellet) was then filtered onto a 3 μm polycarbonate filter, 25 mm in diameter (Millipore, Billerica, MA, USA). Cells were transferred to a 1 inch glass round (29), followed by a ethanol dehydration series (50 %, 70 %, and 100 % ethanol in 1X PBS).

The protocol for FISH (32) was conducted on samples using oligonucleotide probes ARCH915 and EUB338_MIX (labeled with either CY3 or FITC, Sigma Oligo). Prior to viewing and mapping, hybridized slides were marked using a diamond pen to facilitate locating SIMS targets as described in Orphan et al (2002) (29). The slides were then counterstained with DAPI combined with a water-soluble glycerol and PBS mounting medium (at pH=8.0). Aggregates were identified using at 60x oil immersion objective (Olympus PlanApo). Both epifluorescent and transmitted light images, as well as epifluorescent “z-stacks” were collected using a DeltaVision RT deconvolution microscope system and the image analysis software Softworx version 3.5.1 (Applied Precision, Ithica, WA, USA). The locations of observed hybridized aggregates were
recorded for subsequent identification and analysis by SIMS. After identifying aggregates, the DAPI/ mounting medium was gently removed using DI water (which caused the loss of some of the aggregates). All aggregates were revisited and transmitted light images were collected at lower magnification (40x or 10x; Olympus objective UPlanApo). Low magnification (10x) paneled images were also collected for areas of the slide containing target aggregates and reference marks. These images were then used for locating target aggregates with the CCD camera system on the CAMECA 1270 ims ion microprobe.

15N and 13C analysis of cell aggregates using FISH-SIMS

We used the UCLA CAMECA 1270 IMS ion microprobe in a multi-collection mode to measure the carbon and nitrogen isotopic composition of positively identified archaenal/bacterial aggregates as described in Orphan et al. (31). The instrument was configured to use electron multipliers to simultaneously collect 12C13C− (on-axis) and 12C2− (off-axis). The magnet was cycled to use the on-axis detectors to collect counts for 12C14N− and 12C15N−. The primary Cs+ beam (typically 0.1 to 0.3 nA) had a spot size of approximately 15 μm. A correction for Instrumental fractionation was determined by measuring Escherichia coli cells, with known carbon and nitrogen isotopic compositions, on the SIMS (30, 31). Typical analytical precision on carbon and nitrogen isotopic measurements was less than 2 ‰ and 10 ‰ respectively. Although Orphan et al. (31) estimate that levels up to δ15N ≈ 650 ‰ can be measured due to abiotic adherence of
\(^{15}\text{N}\), we determine if our aggregates are active using the cutoff determined using our non-active sulfate control (discussed below) of \(\delta^{15}\text{N} \approx 150 \text{‰}\).

**Results**

**Phylogenetic comparison between manganese and iron incubations**

Comparison of phylogenetic changes of the manganese and the iron incubations relative to their associated sulfate incubations reveals only relatively minor changes in archaeal diversity based on 16S rRNA sequences (3). For example, the manganese and iron incubations are slightly less enriched in archaeal methanotrophs (ANME-1, ANME-2, and ANME-3) as compared to the corresponding sulfate incubations (3). Recovered 16S rRNA diversity from the manganese incubation had a decreased proportion of euryarchaeota group marine benthic group D (MBGD) and an increase in the crenarchaeota group marine benthic group C (MBGC) and the marine hydrothermal vent group (MHVG) relative to archaeal diversity in the control. Diversity surveys from the iron incubation, in contrast showed an increase in the MBGD and a slight decrease in MBGC (Appendix B).

In contrast to the analysis of the archaeal assemblage, analysis of the bacterial assemblage revealed a larger change in diversity within the manganese and iron incubations as compared to their associated sulfate incubations (Figure 5-1). Both the manganese and iron incubations have a relative decrease in bacterial sulfate reducers (with no known ability for metal reduction) relative to their respective sulfate controls.
Figure 5-1. Comparison of bacteria communities based on 16S rRNA from the manganese and iron incubations as compared to their associated sulfate incubation. Metal associated includes phylotypes putatively involved in manganese and iron cycling. Sulfur metabolism includes phylotypes involved only in sulfur cycling (i.e., sulfur oxidation, sulfur disproportionation, sulfate reduction). Metal/sulfur are those phylotypes that are capable of both metal (manganese and/or iron) and sulfur cycling. The delta Proteobacteria in the iron incubation show a large enrichment of organisms involved in both iron/manganese and sulfur cycling, many of which are also capable of sulfur disproportionation (Desulfobulbus, Desulfovibrio, and Desulfiromonas). In contrast, the manganese incubation shows enrichment of putative metal reducers, a slight decrease in organisms involved in metal/sulfur cycling, and a decrease in organism involved in sulfur cycling (mainly Desulfosarcina) as compared to its associated sulfate control.
In addition, both incubations are enriched with metal reducers as compared to their associated sulfate incubations (Figure 5-1). However, the manganese incubation shows notably more enrichment in putative metal reducers as compared to the iron incubations (Figure 5-1). Another major difference is the large increase in the iron incubation of bacteria which may be involved in both sulfur and metal cycling (Figure 5-1), including the δ-Proteobacterial groups *Desulfo bulbus*, *Desulfovibrio*, and *Desulfuromonas*. In contrast to this, the manganese incubation had a relatively large increase in putative metal reducers, belonging mainly to γ-Proteobacteria, δ-Proteobacteria, and Bacteriodetes, in combination with a slight decrease in the recovery of phylotypes affiliated with metal/sulfur cyclers (mainly *Desulfo bulbus* and *Geobacter* (3)).

**Fluorescence in situ Hybridization (Manganese and Iron incubation experiments)**

We observe that the sulfate incubation, run in parallel with the manganese incubation, contains shell aggregates (3 of 14 screened aggregates), such as Figure 5-2a, as well one mixed aggregate (Figure 5-2b). In addition, one large archaeal aggregate (20 μm by 35 μm) morphologically resembling ANME-1 (Figure 5-2c), 3 aggregates of archaea of sarcina morphology, and small clusters (5 - 10 μm) of bacteria (Figure 5-2d-e) were observed. Most aggregates (12 of 14 screened aggregates) were less than 15 μm in size. The variety and size of observed aggregates in our sulfate incubation are in good agreement with reports from other methane seep environments (5, 19, 26, 28-31).

Comparison of the aggregate morphology in the manganese incubation revealed notable differences in the archaeal/bacterial aggregate type relative to the associated
Figure 5-2. FISH images showing different aggregate types from the sulfate incubation. Cy3 (red) is bacteria and the FITC (green) is archaea. All images also contain DAPI (blue) (a) shell aggregate which is active in the incubation. (b) active mixed aggregate, (c) cluster of ANME-1 (archaea rods), (d) and (e) bacterial clusters.
sulfate incubation. Mixed (and mixed cluster) aggregates of bacteria and archaea appeared to be the most common aggregate type (excluding bacterial clusters) (8 of 33 screened aggregates), ranging in size from 10 to 40 μm (Figure 5-3a-d). A few shell aggregates (3 of 33 screened aggregates), typically observed in the sulfate incubation, were also present. Additionally, archaeal aggregates of sarcina morphology (3 of 33 screened aggregates), putatively identified as ANME-2 were also observed (Figure 5-3e-f). Archaea of cocci morphology (3 of 33 screened aggregates) were observed (such as Figure 5-3g), ranging in size from 10-30 μm. Many bacterial clusters (such as Figure 5-3h), ranging in size from a few μm up to 50 μm diameter, were also detected. In general, cell aggregates in the manganese incubation tended to be larger than those found in the associated sulfate incubation. 15 of the 33 screened aggregates were greater than 15 μm in diameter.

FISH analysis of the sulfate incubation, run in parallel with the iron incubation, showed a substantial number of the typical shell (9 of 17 screened aggregates) and mixed aggregates (5 of 17 screened aggregates) associated with sulfate-dependent AOM (similar to Figure 5-2a-b). These aggregates ranged in size from a few μm to ~20 μm in size. Additionally we found a few aggregates comprised solely of bacteria ranging in size from (5 - 15 μm) (such as Figure 5-2d-e). 4 of 17 screened aggregates are greater than 15 μm in diameter. The iron incubation did not show as large a change from its associated sulfate incubation as compared to the manganese incubation. FISH results from the iron incubation showed a substantial number of the typical sulfate-dependent AOM shell aggregates (5 of 18 screened aggregates), such as shown in Figure 5-2a.
Figure 5-3. FISH images showing different aggregate types from the manganese incubation. Cy3 (red) is bacteria and the FITC (green) is archaea. All images also contain DAPI (Blue) (a), (b), (c), (d) mixed and mixed-cluster aggregates, (e) and (f) archaeal aggregates of sarcina morphology, (g) archaea cocci, and (h) bacterial cluster. (f) shows slight bacterial associates, but we targeted only the archaea using SIMS. Small mixed and mixed-cluster aggregates (a and b) show more incorporation of $^{15}$N than the large mixed and mixed-cluster aggregates (b and c). In addition, archaeal groups (sarcina) show incorporation of $^{15}$N.
However mixed aggregates (4 of 18 screened aggregates, similar to Figure 5-2a-c) were also observed. In addition, bacterial clusters were recovered ranging in size from 10 - 20 μm. 7 of 18 screened aggregates are 15 μm or greater in diameter.

**13C/15N analysis of archaeal-bacterial aggregates by FISH-SIMS (Manganese incubation)**

The aggregates from the manganese incubation and its sulfate control, described in the previous section, were mapped (as described in methods) and the carbon and nitrogen isotopic composition measured by SIMS. A total of 10 aggregates from the sulfate incubation and 21 aggregates from the manganese incubation were measured for carbon and nitrogen isotopes. The difference in number of target aggregate as compared to those screened using FISH is due to the loss of aggregates in the wash step in preparation for SIMS (see Methods). Aggregate types were classified into 6 groups: shell aggregates (Figure 5-2a), mixed and mixed-cluster aggregates (Figures 5-2b, and 5-3a-c), archaea sarcina (such as Figures 5-2d-e), archaea rods (Figure 5-2c), archaea cocci (such as Figure 5-3g), and bacteria (such as Figures 5-2d-e and 5-3h).

At the conclusion of the manganese experiments, both a sulfate control and a manganese incubation were incubated with $^{15}$NH$_4$Cl, during which active cells incorporated this label during transcription and protein synthesis. SIMS results for the sulfate incubation indicated that shell and mixed aggregates, in most cases, have the largest amount of $^{15}$N incorporation (Figure 5-4a). However, we also observed a mono-specific bacterial cluster that was more active (i.e. enriched in $^{15}$N) than the shell
Figure 5-4. Carbon and nitrogen isotopic compositions of targeted aggregates. Aggregates classification scheme based on description in text as well as indicated in Figures 2 and 3. The top panel shows aggregates from the sulfate control. The bottom panel shows aggregates from the manganese incubation. Shell aggregates are active in the sulfate incubation, but not active in the manganese as indicated by incorporation of $^{15}N$. Mixed/mixed cluster and archaea sarcina are active in the manganese incubation (as shown by incorporation of $^{15}N$). Aggregates in the manganese (birnessite) incubation (bottom panel) are less active than those in the sulfate incubation (top panel) as expected due to the slower rate of manganese dependent AOM as compared to sulfate dependent AOM. Typical carbon isotopic composition of shell and mixed and mixed-cluster aggregates in the ERB range from -100 to -60 ‰ (although some have been found as enriched as -20 ‰). Archaea sarcina have typical $\delta^{13}C$ values ranging from -80 to -20 ‰. Bacteria are typically heavier, with $\delta^{13}C$ values typically between -45 ‰ and -20 ‰ (17, 29, 30). Because we do not know the $\delta^{13}C$ value of the target aggregates before incubation with $^{13}CH_4$, we cannot conclusively state which aggregates show minor incorporation of $^{13}C$. However, our data indicate that the more active aggregates (shown in an oval) are also slightly enriched in $^{13}C$ as compared to other active aggregates in our incubation, suggesting that they have incorporated minor amounts of $^{13}C$ from methane.
aggregates (Figure 5-4a). However, most of the targeted mono-specific bacterial clusters showed virtually no or minor incorporation of $^{15}$N (only the 1 out of 5 mono-specific bacterial clusters showed $^{15}$N incorporation).

In contrast, shell AOM aggregates in the manganese incubation showed little to no $^{15}$N incorporation (Figure 5-4b). Archaeal and bacterial clusters also showed minor $^{15}$N enrichment, at a much lower level than observed in the sulfate incubation. Mixed/mixed cluster aggregates, in addition to archaean aggregates of sarcina morphology, show more $^{15}$N incorporation than all of the other targeted aggregates in the manganese incubation (Figures 5-3a-f, Figure 5-4b). Surprisingly, the larger aggregates found in the manganese incubation (such as Figures 5-3c-d) showed less $^{15}$N incorporation than the smaller aggregates. The two targeted mixed/mixed cluster aggregates greater than 20 μm in diameter had $\delta^{15}$N = 43 ‰ and $\delta^{15}$N = 300 ‰, while the $\delta^{15}$N of the smaller mixed and mixed-cluster aggregates were 830 ‰ and 890 ‰.

Because of the 10 month incubation period with $^{13}$CH$_4$, the carbon isotopic composition of all target aggregates was also measured to reveal $^{13}$C incorporation indicative of methanotrophy. Shell and mixed aggregates in the sulfate incubations have $\delta^{13}$C values ranging from -29 – 0.5 ‰ (Figure 5-4a). The $\delta^{13}$C of bacterial clusters range from -47 to -16.5 ‰. Archaea (rods, sarcina, and cocci) range from $\delta^{13}$C = -25 to +39 ‰. Mixed aggregates in the manganese incubation have $\delta^{13}$C values ranging from -49.5 – -3.5 ‰ (Figure 5-4b). Shell aggregates are slightly less enriched in $^{13}$C, with $\delta^{13}$C values ranging from -73 – -17 ‰. Archaea (sarcina and cocci) have $\delta^{13}$C = -90.5 – -19. Bacteria range from $\delta^{13}$C = -78 – -34 ‰.
We attempted to conduct a similar experiment with the ferrihydrite incubation and its associated sulfate control. These experiments were carried out approximately 1 year after the birnessite incubations. Although we used the same $^{15}$NH$_4$Cl as for the birnessite incubation, the results of our $^{15}$N analysis using SIMS indicated all targeted aggregates (including shell and mixed aggregates from both the ferrihydrite and sulfate incubations) incorporated insignificant amounts of $^{15}$N. Out of the 7 targeted aggregates, the highest measured value was a mixed aggregate in the ferrihydrite incubation $\delta^{15}$N = 113 ‰. The targeted shell aggregates with the sulfate incubations showed very minor incorporation of $^{15}$N with $\delta^{15}$N = 30 ‰ or less, as compared to the $\delta^{15}$N values of shell aggregates in the sulfate incubation associated with manganese incubation ($\delta^{15}$N = 1190 ‰ or greater). The most probably reason that the injection of $^{15}$NH$_4$Cl caused our ferrihydrite experiment to become inactive is due to oxidation of the $^{15}$NH$_4$Cl to nitrate during the year between the manganese and iron experiments (which included exposure to hot environmental conditions during field work). It has been shown that the addition of nitrate to AOM incubations completely shuts of the process (3).

The non-active sulfate control described above provides an addition means to determine the amount of abiotic adherence of $^{15}$N to cells. The highest incorporation seen in the non-active control incubation that contained sulfate was 113 ‰. This value is significantly lower than the abiotic incorporation suggested by Orphan et al. (31), but appears appropriate for our study where some potentially active cells reach values of only a few hundred per mil.
Discussion

Although the processes of manganese and iron dependent AOM have been demonstrated, the microorganisms and details underlying these processes remained undetermined (3). Analysis of changes in archaeal communities based on 16S rRNA and merA analysis indicate an increase in ANME-1 and ANME-3 in the manganese incubation (3) and an increase in anaerobic methanotrophic diversity in the iron incubation (Appendix B). Characterization of the bacterial communities using 16S rRNA show the almost complete absence of Desulfosarcina, an increase in Desulfobulbus, and an increase in putative metal reducers (mainly γ-Proteobacteria, δ-Proteobacteria, and Bacteriodetes) in the manganese incubation relative the associated sulfate incubation (3). The iron incubation show a large increase Desulfobulbus, a slight decrease in Desulfosarcina and an increase in putative metal reducers (mainly γ-Proteobacteria and Bacteriodetes) relative to the sulfate control (Appendix B).

The characterization of bacterial diversity in the incubation experiments (described above) suggests that the oxidation of methane by Fe or Mn may occur through different mechanisms and/or microbial assemblages as compared to sulfate dependent AOM. Here we mainly focus on mechanisms and microbial assemblages responsible for manganese dependent AOM. Phylogenetic analysis of the manganese incubation, summarized above, suggests that manganese reduction and methane oxidation were directly linked, either by a consortium of bacteria (metal reducers) and archaea or solely by bacteria. However, it is also possible that the addition of manganese and iron to our incubation stimulated the (biotic or abiotic) oxidation of sulfide to sulfur (1, 8), followed
by biologically mediated sulfate production (either by sulfur oxidation or sulfur
disproportionation) linked to metal reduction (3), indirectly linking AOM to manganese
and iron reduction. Organisms involved in sulfur oxidation and disproportionation use
iron and manganese either directly as an electron acceptor (6, 8, 22, 23), or as a sulfide
scrub (where the reduced forms of iron and manganese react with sulfide effectively
removing it) (1, 8, 22, 37).

The shift of bacterial populations observed during the manganese incubation is
quite different than that observed during the iron incubation. The iron incubation shows
an increase in both metal and metal/sulfur metabolizers, along with a decrease in
microorganisms involved in sulfur cycling, relative to the associated sulfate control
(Figure 5-1). However, relative to its original sediment, the iron incubation shows a
substantial increase in organisms involved in sulfate reduction, mainly related to
members of the Desulfosarcina (3). In contrast, the manganese incubation shows an
increase in putative metal reducers, as well as a decrease in sulfur and sulfur/metal
metabolizers relative to the associated sulfate incubation (Figure 5-1) and the original
sediment sample (3).

The fact that there is a large increase in metal/sulfur reducers, specifically
Desulfobulbus, in the iron incubation relative to its sulfate control is of significance
because Desulfobulbus propionicus is known to be able to disproportionate sulfur, with
its rate of sulfate production stimulated by the addition of iron, but not manganese (23).
Also of interest in our incubation, is that there is relatively less enrichment of putative
metal reducers in the iron incubations as compared to the manganese incubations (Figure
5-1). These results, in combination with the relatively small change in archaeal diversity,
suggest a scenario where *Desulfobulbus* is disproportionating sulfur using iron as an electron acceptor/sulfide sink followed by methane-oxidation by the ANME/DSS consortia using the produced sulfate. Therefore, this, in conjunction with the presence of *Desulfosarcina*, suggests that iron reduction may be indirectly coupled to AOM.

Although *Desulfobulbus* is the dominant δ-Proteobacteria in the manganese incubation (3), our results are somewhat inconsistent with *Desulfobulbus* acting mainly as a sulfur disproportionator in the manganese incubation. Because iron stimulates the production of sulfate by *Desulfobulbus*, and the addition of manganese does not affect the rate, we would expect the rate of iron dependent AOM to be greater than manganese dependent AOM if *Desulfobulbus* is acting as a sulfur disproportionator in both incubations. In contrast, the rate of manganese dependent AOM observed is substantially faster than iron dependent AOM (2). Additionally, the almost complete absence of *Desulfosarcina* in the manganese incubation (based on 16S rRNA analysis) suggests that transient production of sulfate is not primarily driving manganese dependent AOM.

The FISH data further support the preliminary conclusions drawn from the phylogenetic analysis. The prevalence of AOM shell aggregates present in our iron incubation supports the idea that transient amounts of sulfate are being produced, mediated either biotically and/or abiotically by iron, and then AOM proceeds in the typical sulfate-dependent manner. These data do not discount the possibility that iron reduction and AOM can also be directly coupled, considering the observed large 20-40 μm mixed and mixed-cluster aggregates that were not observed in the sulfate incubation. The manganese incubation, however, revealed larger changes in bacterial and archaeal association, containing very few shell aggregates, with substantially more mixed and
mixed-cluster aggregates (up to 40 μm in diameter), suggesting that this incubation stimulated a different assemblage of organisms, and possibly a different mechanism, from that operating in our sulfate dependent AOM incubations.

FISH-SIMS was used to further our understanding of the organisms responsible for manganese dependent AOM and to help elucidate whether it is directly or indirectly coupled to manganese reduction. The FISH analysis demonstrated the association between bacteria and archaea within our incubation experiments, while the addition of SIMS enabled the distinction between active/inactive consortia, and possible methane metabolizers. Due to the uptake of the $^{15}$N during transcription and protein synthesis, from the $^{15}$NH$_4$Cl injected into the cultures in the last two weeks of the incubations, active cells are enriched in $^{15}$N as compared to the inactive cells, as revealed by measuring the nitrogen isotopic composition of aggregates. This method has successfully been used in previous AOM culture studies (31).

We also measured the carbon isotopic values to help determine which active aggregates were the ones that were also metabolizing methane, and thus had incorporated $^{13}$C into their biomass over the entire 10-month incubation period. Due to the extremely low rate of carbon assimilation by methanotrophs, ranging from 1-3% of methane oxidized (4, 14, 25, 38, 40) very little of the $^{13}$CH$_4$ was incorporated into cells, making it difficult to distinguish between aggregates with and without $^{13}$C incorporation. However it is possible to determine, in some cases, which aggregates show minor $^{13}$C incorporation when we compare the values of the targeted aggregates incubated with $^{13}$C to those found in the ERB. Shell and mixed aggregates from the ERB typically have $\delta^{13}$C values ranging from -100 to -60 ‰ (29, 30), although some can be as heavy as -25 ‰ (17).
Archaeal aggregates of sarcina morphology have typical $\delta^{13}C$ ranging from -80 to -20 $\%_o$ (17). However bacteria are typically enriched in $^{13}C$, with $\delta^{13}C$ values typically between -45 $\%_o$ and -20 $\%_o$ (17, 29, 30). Targeted aggregates more enriched in $^{13}C$ than these natural values have likely incorporated labeled $^{13}C$-methane.

Our SIMS analysis of the shell and mixed aggregates found in the sulfate incubation showed that they are active in our incubation, as indicated by the incorporation of $^{15}N$ (Figure 5-4a). The carbon isotopic analysis of these aggregates suggests that a subset of these microorganisms are also metabolizing methane (Figure 5-4a), as they are enriched in $^{13}C$ reaching values as high as +40 $\%_o$ (Figure 5-4a). The bacteria in the sulfate incubation show little $^{15}N$ incorporation, with the exception of one bacterial cluster (Figure 5-4a). However, the bacterial clusters have carbon isotopic compositions in the range of bacteria reported from the ERB, suggesting that they are not metabolizing methane.

The bacterial clusters from the manganese amendment, which likely include heterotrophic manganese reducers, also showed little incorporation of $^{15}N$, indicating that they are not very active during the incubation (8 of the 12 analyzed targets $\delta^{15}N < 70 \%_o$, with the other 4 with $90 \%_o < \delta^{15}N < 150 \%_o$). In addition, the bacterial clusters show similar carbon isotopic values as bacteria found in methane seep environments (Figure 5-4b), indicating that bacteria are likely not metabolizing much methane, as observed in the sulfate control. These results suggest that bacteria, alone, cannot mediate manganese dependent AOM.
SIMS analysis of shell aggregates recovered from the manganese incubation were relatively inactive (Figure 5-4b), as indicated by minor incorporation of $^{15}$N ($\delta^{15}$N < 100 ‰). In addition, the $\delta^{13}$C values (-100 ‰ up to -60 ‰) of the shell aggregates are comparable to typical shell aggregates found in the ERB (29, 30), indicating little to no incorporation of $^{13}$CH$_4$ throughout our incubation. One shell aggregate from the manganese incubation has a slightly higher $\delta^{13}$C value (-17 ‰), however this aggregate was not enriched in $^{15}$N (Figure 5-4b). This suggests that the shell aggregates originated from the starting sediment and were inactive during the incubation (as indicated by the lack of incorporation of $^{15}$N and $^{13}$C).

In contrast to the shell aggregates, the mixed and mixed-cluster aggregates showed substantial $^{15}$N assimilation ($\delta^{15}$N > 300 ‰) (Figures 5-3a-d, Figure 5-4b), indicating that these aggregates were active in the manganese incubation. In addition, the two archaeal aggregates of sarcina morphology, likely members of the ANME-2, show incorporation of $^{15}$N, and thus are active in our incubation. The $^{15}$N data is independently supported by the $\delta^{13}$C data, which shows that the mixed and mixed-cluster and archaeal sarcina aggregates, in contrast to the shell aggregates, have assimilated $^{13}$C-labeled methane (Figure 5-4b). Intriguingly, the organisms in these mixed aggregates are morphologically similar to ANME and Desulfobulbus (21). The fact that archaea sarcina (putatively identified as ANME-2a based on morphology and phylogentic analysis (3)) are active in our manganese incubation and that tightly-bound shell aggregates (likely ANME-2/DSS) are not, may indicate that DSS are not capable of Mn reduction.
The most active aggregates from the manganese incubations (Figures 5-3a and 5-3d) incorporated between 30 - 70 % less $^{15}$N than the active aggregates in the associated sulfate incubation (Figure 5-4). This result was expected since during our parallel experiments manganese dependent AOM occurred at a quarter of the rate of sulfate-dependent AOM (2). Interestingly, the larger mixed aggregates (Figure 5-3c-d) show less $^{15}$N incorporation ($\delta^{15}$N = 43 ‰ and 300 ‰) than the smaller aggregates ($\delta^{15}$N =830 ‰ and 890 ‰) (Figure 5-3a-b). This was an unexpected result since one might expect that to grow so large, an aggregate must be extremely active. One possibility is that the size of these aggregates may limit diffusion of methane and/or $^{15}$NH$_4$Cl into the cluster, and thus activity of the aggregate (as observed with sulfate dependent AOM in Orphan et al. 2009 (31)). An additional possibility is that these aggregates were active for much of our incubation period, but had become less active towards the end (during which the $^{15}$N study was conducted). Thirdly, it is feasible that these aggregates did not grow within our incubation, but rather originated from the starting sediment, and thus were never very active in our incubation. It must be noted, however, that no similar aggregates were observed in either of the sulfate incubations.

The lack of active shell aggregates in the manganese incubation could indicate the absence of sulfate, and thus argue against transient sulfate production as the main process driving AOM. Alternatively, it is feasible that transient production of sulfate is occurring in our incubations and that this transient sulfate creates conditions that favor mixed aggregates over active shell aggregates in the manganese incubations. However, approximately one fourth of the aggregates observed in the iron incubations were shell aggregate (morphologically similar to ANME-2/DSS aggregates), as compared to 10 %
shell aggregates in the manganese incubation. In addition to this, 16S rRNA analysis revealed a substantial number of *Desulfosarcina* in the iron incubation (which were not present in the manganese incubation). It therefore seems likely that the mechanisms in manganese and iron dependent AOM are different. It is possible that manganese dependent AOM is directly coupled and iron dependent AOM is indirectly coupled through the transient production of sulfate.

**Conclusions**

Phylogenetic analysis, in combination with FISH and SIMS, has allowed for the characterization manganese-dependent AOM in a mixed enrichment culture. Phylogenetic comparison of the iron incubation, as well as FISH results suggests that the mechanism for iron dependent AOM is, at least partially, indirect coupling between methane oxidation and iron reduction. In contrast, our data suggests that manganese dependent AOM proceeds by a different mechanism, possibly directly linked to manganese reduction. Active mixed and mixed-cluster aggregates of archaea and bacteria, in addition to clusters of archaea of sarcina morphology (ANME-2) are the likely organisms responsible for this newly characterized process. Future research should focus on identifying the specific microorganisms (possibly ANME-3 and *Desulfobulbus*) in the active mixed aggregates in the manganese incubation.
References


Chapter 6

Future Directions

Although we made much progress in understanding the geochemical requirements of AOM, many questions remain. The results of our modeling, described in Chapter 2, indicate that there may be a correlation between tectonics/geophysics and methane seep location within the Eel River Basin (ERB). Future work should further explore this relationship by sampling methane throughout the ERB and measuring both carbon and hydrogen isotopes to determine its origin. In addition methane seeps throughout the ERB should be located either by an indirect means, such as using backscatter, or by direct means (using a submersible) to determine if the model predictions of areas with abundant methane seeps are correct.

The results in Chapter 3 indicate that surprisingly high rates of AOM occur at low sulfate levels. In addition, we show that AOM and sulfate reduction often do not occur in a 1:1 ratio, as often stated in the literature. However, the mechanism by which extremely high rates of AOM occur at low sulfate levels (described in Chapter 3) remains elusive. Although we propose the loss of electrons from AOM microbes to organisms not involved in sulfate reduction, we do not have data to support this argument. Future research should focus on determining what drives AOM when methane oxidation and sulfate reduction rates are uncoupled.

Our results in Chapter 4 are the first direct evidence of manganese- and iron-dependent AOM. However, the mechanism and identity of organisms responsible for
these processes remains unknown. We have evidence that mixed and mixed-cluster aggregates are active in our manganese-dependent AOM incubations (described in Chapter 5). However, we do not yet know the identity of these organisms. In addition, we were unable to perform SIMS analysis on the iron incubations, and therefore do not know which aggregates are active in these incubations. Future work should focus on using specific FISH probes (such as ANME-1, ANME-2, ANME-2, Desulfosarcina, Desulfobulbus, etc.) in combination with SIMS to determine the identity of the microbes responsible for manganese and iron AOM. In addition, a new study including SIMS analysis of aggregates from iron-dependent and manganese-dependent AOM incubations run in parallel (from the same original sediment) should also be performed. These results will help to indicate if different mechanisms and/or microbial assemblages are responsible for manganese- and iron-dependent AOM. Finally, the addition of molybdate, a sulfate reduction inhibitor, to manganese- and iron-dependent AOM incubations could help to determine if these processes are directly or indirectly coupled to methane oxidation.

The research presented in this dissertation has helped to elucidate new processes involved in marine AOM. The exact mechanism involved in the syntrophic association between ANME and their sulfate reducing partners, however, remains a mystery (especially at low sulfate concentrations). It is also unclear if both methane oxidation and sulfate reduction can occur within ANME (without a bacterial partner). It has been shown that AOM is a significant sink of methane in marine environments, the importance of this process in freshwater environments has yet to be quantified. Our research, which demonstrated that manganese- and iron-dependent AOM occur, has opened up new
questions concerning the mechanisms involved in AOM. What microorganisms are responsible for these processes? Can they occur within ANME without a syntrophic partner? Does transient sulfate production drive manganese- and iron-dependent AOM? Current research, by many labs groups, is working to answer many of these mysteries regarding AOM in hopes to understand the mechanisms driving this globally significant process.
Appendix A

Supplementary Information for Chapter 4

Materials and Methods

Sampling

Sediment samples were taken from two cruises to the Eel River Basin, one from July to August 2005 (R/V Western Flyer) and the second in September 2006 (R/V Atlantis). The samples used in the manganese experiments are from the first cruise: dive T-866 PC-42 (mat, 0-10 cm) and T-866 PC-73 (mat, 0-10 cm). The samples used in the iron experiments are from the second cruise: Dive A4254 PC 11 (mat, 0-6 cm) and PC-100 (Calyptogena clam, 0-12 cm). Sediment was sealed in mylar bags with argon on the ship and was refrigerated until use. Sediment used in the nitrate and ferric oxyhydroxide experiments was stored at 10º C for 6 months in sealed anaerobic mylar bags before incubation. Sediment for the birnessite and ferrihydrite experiments was stored at 10º C for 12 months in sealed anaerobic mylar bags before incubation.

Metal Synthesis

Ferric oxyhydroxide was synthesized by neutralizing a 0.4 M solution of FeCl₃ with sodium hydroxide (S1). Birnessite synthesis followed the protocol of Golden et al. (S2). 2-line Ferrihydrite was synthesized using the protocol in Cornell and Schwertmann.
Birnessite and ferrihydrite were sterilized using ethanol and were never autoclaved.

**Incubations**

Artificial sulfate-free seawater was made following the recipe of Lyman and Fleming (S4). Seawater was degassed with N2/CO2 and reduced with 0.5 g/L Na2S·9H2O. FeCl2 was added in 1:1 stoichiometric ratio to the sulfide to precipitate all of the added sulfide and then the artificial seawater was autoclaved. The FeS was allowed to settle before use of the seawater so as to minimize the amount of sulfide added to the incubations. 10 mL of homogenized sediment was distributed to 120 mL culture bottles containing 30 mL of artificial seawater. Triplicate incubations were given sulfate (5 mM), birnessite (10 mM), ferrihydrite (10 mM), nitrate (5 mM), ferric oxyhydroxide (10 mM), or no provided electron acceptor. The headspace of the bottles was then filled with 2.5 bars methane. 35 mL of CO2 and 6 mL 13C-CH4 was injected into the headspace. Isotope analysis of the CO2 followed the same procedure used for similar studies (S5, S6). After ~7 months of incubation for the birnessite experiments, the headspace was vacuumed and a new headspace (with the same proportions of CO2, 13C-CH4, and CH4) was added to all of these bottles. The data in the figures starts at the time the new headspace was added and are normalized to the 13F\textsubscript{CO2} values at this time. This makes for a total of 10 months that each bottle in the birnessite experiments was incubated. The ferrihydrite experiments also were given a new headspace (as described with the manganese experiments) after 5 months. The data shown the in the ferrihydrite figures
begins at the time when the new headspace was added and the data is normalized to this starting time point. We changed the headspace in both the birnessite and ferrihydrite experiments, and thus created a new beginning to the experiments, after months of incubation to ensure that any residual sulfate in the mud had been consumed at the start of our experiments. Additional sulfate was injected into the sulfate incubations, and birnessite and ferrihydrite into their respective incubations when the headspace was changed.

**Free Energy Calculations**

The *in situ* conditions used in the free energy calculation for the manganese experiments are: \([\text{MnO}_2] = 10 \text{ mM}, [\text{Mn}^{2+}] = 0.2 \text{ mM}, [\text{H}^+] = 10^{-8} \text{ mM}, [\text{HCO}_3^-] = 11 \text{ mM}, [\text{CH}_4] = 1.5 \text{ mM}\). The *in situ* conditions used in the free energy calculation for the iron experiments are: \([\text{Fe(OH)}_3] = 10 \text{ mM}, [\text{Fe}^{2+}] = 10 \text{ mM}, [\text{H}^+] = 10^{-8} \text{ mM}, [\text{HCO}_3^-] = 11 \text{ mM}, [\text{CH}_4] = 1.5 \text{ mM}\). The *in situ* conditions used in the free energy calculations for the sulfate incubations are: \([\text{SO}_4^{2-}] = 5 \text{ mM}, [\text{HS}^-] = 2 \text{ mM}, [\text{H}^+] = 10^{-8} \text{ mM}, [\text{HCO}_3^-] = 11 \text{ mM}, \text{ and } [\text{CH}_4] = 1.5 \text{ mM}\). Concentration of MnO2, SO42-, Fe(OH)3, HS- are based on the amount of each of these species added at the start of the incubations. The values for Mn2+ and Fe2+ are based averaged measured values of starting concentrations of these species. Values of HCO3- and CH4 were calculated using Henry’s law. The value for H+ concentration comes from pH measurements of the incubations. We estimate that each of the incubations contain 7 cm³ of sediment. The temperature in the free energy calculations was 10º C for both experiments.
Dissolved manganese and iron measurements

1 mL of sample from the incubation was taken for dissolved manganese measurements. This sample was immediately acidified to pH 2 using HCL, and filtered on a 2 μm filter. Dissolved manganese was then measured on a Leeman Labs PS3000UV ICP (inductively coupled plasma emission spectrometer). 0.5 mL samples were taken for dissolved iron measurements and acidified to pH 2 using HCL and then centrifuged for 5 minutes at 14,000 rpm to remove sediment. Dissolved iron was measured using the ferrozine assay (S7).

Clone libraries/Phylogenetic Analysis

Samples were taken from the starting sediment used in all incubations as well as at the end of the experiments (which corresponds to a total of ~10 months of incubation). Bacterial primers 27F 5'-AGAGTTTGATCCTGAG-3' and 1492R 5' - GGTTACCTGGTACG-3', archaean primers 21F 5'-TTCCGGGTGATCCYGGCGGA-3' and 958R 5'-YCCGGCGTTGAMTCCAATT-3' mcrA primer sequences are mcrA forward 5'-GGTGGTGATCCGGATCCGACAR-3' and mcrA reverse 5'-TCATTGCRTAGTTWGGRTAGTT-3' (S8) were used in PCR amplification of DNA using PuRe Taq Ready-to-Go PCR beads (GE Healthcare). Amplification conditions for archaean PCR are initial melting at 94° for 5 min, followed by 30 cycles of 94° for 1 min, 58° for 1 min, and 72° for 1.74 min, ending in a final elongation at 72° for 20 min, bacterial PCR conditions are described in (S9) and for mcrA in Moran et al. (S6). DNA
extraction, cloning and sequencing was performed as described by Moran et al. (S5). Archaea were classified based on the classification presented in Knittel et al. (2004) (S10). Possible metabolisms for bacteria were based on phylogenetic association of clones determined using the greengenes classify tool (http://greengenes.lbl.gov) as well as BLAST hits using the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bacterial neighbor joining trees were constructed using PAUP. Closest sequences to our mcrA sequences were found using BLAST and identified using mcrA sequences that had already been classified (S11). We screened 46 archaeal, 47 bacterial, 46 mcrA and clones from the original sediment, 24 archaeal, 23 bacteria and 24 mcrA clones from the live control, 31 archaeal, 31 bacterial, and 32 mcrA clones from the manganese incubations, and 23 archaeal, 24 bacterial, and 24 mcrA clones from the sulfate incubations.

**Additional manganese and iron sources to the Eel River Basin (ERB)**

Aeolian dust deposition to ocean basins is estimated at 1000 – 2000 Tg/yr (S12). If dust composition reflects the composition of the Earth, it can be estimated that 50 - 100 Tg/yr of iron and 1 - 2 Tg/yr manganese are deposited to oceans. This is a significant amount of both iron and manganese, but it is about of an order of magnitude less than the riverine input. The Gorda Ridge has the potential to deliver an additional source of both iron and manganese to the ERB. It is estimated that hydrothermal sources release 0.6 – 1.9 Tg dissolved manganese and 1.3 -11 Tg dissolved iron into oceans (S13), which, once oxidized, has the oxidative potential to consume 0.03 % – 0.7 % of methane globally.
consumed by AOM today. Although this amount of oxidative potential seems very small, especially compared to riverine input, iron and manganese can be oxidized and reduced 100 – 300 times before being buried (SI4). Thus, the hydrothermal flux of iron and manganese into the oceans has the ability to oxidize significantly more methane than a direct stoichiometric relationship would imply.

**Presence of dissolved manganese**

The incubations with birnessite show an increase in the amount of dissolved manganese with time. They also have a greater amount of dissolved manganese than the autoclaved control, which shows no change in dissolved manganese with time. The live controls, with no electron acceptor addition, show no measurable dissolved manganese. The concentration of dissolved manganese only increases by approximately 5 μM, which is \( \frac{1}{20} \) of what one would expect to see based on stoichiometric calculations. However, one expects that most of the dissolved manganese in our incubations will precipitate as manganese carbonates (such as rhodochrosite) (SI5). If the precipitation of manganese carbonates is taken into account, the lower concentration of dissolved manganese observed in the incubations is not surprising. Similarly, it is expected that iron carbonates (such as siderite) (SI5) will precipitate in our incubations as ferrihydrite is reduced. Thus, we would also expect to see a small change in the amount of dissolved iron in our cultures. The starting concentration of dissolved iron was 10 mM and thus the expected change of the dissolved iron concentration (on the order of a few μM) is only a few tenths of a percent change. It is likely for this reason that we did not observe a significant increase in
dissolved iron in the ferrihydrite incubations.

Figure A-1. $^{13}$C enrichment of CO$_2$ reported in $^{13}$F$_{CO2}$ ($^{13}$C/$^{13}$C+$^{12}$C) values and converted to moles methane oxidized. The incubations with ferric oxyhydroxide, nitrate, and nitrate with sulfate oxidize less methane than the live control (sulfate free, no provided electron acceptor), indicating that ferric oxyhydroxide and nitrate cannot be used as an electron acceptor in AOM. In addition, because there is no methane oxidation with nitrate and sulfate, these data indicate that nitrate inhibits AOM. Sediment was stored at 10°C for 12 months in sealed anaerobic mylar bags before incubation. Error bars represent the range of data from the triplicate incubations.
Figure A- 2. Percentages of archaeal clones from the initial sediment, live control, manganese, and sulfate incubations, based on 16S rRNA and the mcrA gene (insert). MBGD=Marine Benthic Group D, MBGB=Marine Benthic Group B, MBGC=Marine Benthic Group C. The starting sediment was stored anaerobically for approximately a year before it was used, and therefore this microbial characterization does not accurately reflect the proportions of archaea when it was sampled from the Eel River Basin.

Figure A- 3. (Shown on pg. 127) Neighbor joining phylogenetic tree of bacterial clones associated with the manganese incubations (Mn3b-B clones). Phylogenetic analysis included the top BLAST matches and other nearest neighbors (with emphasis on metal-associated sequences).
References


Appendix B

Supplementary Information for Chapter 5: Phylogenetic Analysis of Iron Dependent Methane Oxidation

Changes in microbial assemblages associated with iron dependent AOM were assessed based on characterization of 16s rRNA and mcrA gene diversity. DNA was extracted from one of each of the following triplicate incubations (see Beal et al. (2009)): a sulfate control, a ferrihydrite incubation (sulfate free), and a live control (sulfate free, no added electron acceptor). All incubations, which were monitored for a total of 10 months, contained methane seep sediment from the ERB, artificial sulfate-free seawater, methane, \(^{13}\)C-labelled methane, carbon dioxide and an added electron acceptor (sulfate, ferrihydrite, or no added electron acceptor). In addition, DNA was extracted from the original methane seep sediment used for all of the incubations.

The percentage of methanotrophs, based on archaeal 16s rRNA gene diversity, increase greatly in both the ferrihydrite and sulfate incubations as compared with the starting sediment and live controls (Figure B-1, Table B-1). In particular, 47% of the recovered archaeal phylotypes from the sulfate incubations are methanotrophs, while 26% of the phylotypes from the ferrihydrite incubations are known methanotrophs. In contrast, only 10% and 13% of the recovered archaeal phylotypes from the original sediment and live control, respectively, are known methanotrophs. The ferrihydrite incubation showed the most diversification in the methanotrophic population, based on
16s rRNA analysis, with ANME 1b, 2a, 2b, 2c, and 3 all present in the incubation (Figure B-1).

Analysis of the methanotrophic population in all the incubations, based on mcrA analysis, are fairly consistent with the results from 16s rRNA analysis (Figure B-1). The main differences between the mcrA screen and the 16s rRNA screen are larger proportions of ANME-1 as compared to ANME-2 in all incubations. We believe this inconsistency is due to a PCR bias for ANME-1 associated with mcrA amplification and/or an ANME-2 PCR bias associated with 16s rRNA amplification. In addition, we recovered phylotypes associated with ANME-3 and methanogens in the sulfate incubations, revealing a greater diversity of phylotypes associated with the mcrA gene than the original 16s rRNA screen showed. No ANME-3 phylotypes were recovered in the live control in the mcrA screening, although we saw the presence of this phylotypes in the 16s rRNA screening (Figure B-1).

Analysis of the bacterial population of the incubations based on 16s rRNA analysis showed substantial shifts in bacterial diversity, on the species level, between the different incubation conditions. In contrast, the proportions of different phyla between the iron incubations, its controls, and the original sediment are very similar (Figure B-2), although Proteobacteria are slightly enriched in the iron incubation relative to the other incubations. The most variation seen between the incubation occurs within the phylum Proteobacteria. Specifically, only 4% of the recovered phylotypes from the iron incubations belong to the class ε-Proteobacteria (Figure B-2). In comparison, the original sediment used in the experiments contains 33 %, the live control contains 35%, and the
sulfate incubation contains 17 % ε-Proteobacteria. In addition, there is a small increase in phytotypes belonging to δ-Proteobacteria in the iron incubations (Figure B-2).

Although there is not a large shift in proportions of phyla between the incubations, we see a change in the percentage of potential iron reducers and organisms involved in sulfur cycling between the incubations. Approximately 30 % of the recovered phytotypes from the iron incubations are potential iron reducers (Figure B-2). The original sediment, the live control, and the sulfate incubation support a much smaller percentage, with only 10-20 % of the recovered phytotypes associated with iron reducers. The potential iron reducers include clones from the phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (Peptococcaceae). Additional clones putatively involved in iron cycling were identified based on their association with microorganisms found in heavy metal contaminated sites and hydrothermal systems.

All of the incubations contain phytotypes that are likely involved in both iron and sulfur cycling. Around 20 % of the bacterial clones, from both the original sediment and the iron incubation, are potentially involved in both iron and sulfur cycling (Figure B-2). The main phytotype, found in all incubations, associated with both sulfur and iron cycling is Desulfobulbus. In addition to Desulfobulbus, clones related Geobacter, Desulfuromonas acetoxidans, Desulfuromonas svalbardensis, Desulfovibrio ferri reducens are present in the iron incubation.

The sulfate and iron incubations support a large proportion of sulfate reducer related to Desulfo bacteraceae, specifically Desulfosarcina and Desulfobacter, in addition to smaller proportions of Sulfurovumales. In fact, of the 20 % of phytotypes associated with sulfur cycling from the iron incubations, ~15 % are sulfate reducers. This is in
contrast to the manganese incubation which show a significant proportion of

*Sulfurovumales*, but almost no δ-Proteobacteria without the ability to reduce sulfate
and/or manganese and iron (see Beal et al. 2009).
Figure B-1. Percentages of archaeal clones from the initial sediment used in the incubations (starting sediment), live control (no added electron acceptor, sulfate free), ferrihydrite, and sulfate incubations, based on 16S rRNA and the mcrA gene (insert). MBD=Marine Benthic Group D, MBGB=Marine Benthic Group B, MBGC=Marine Benthic Group C. The starting sediment was stored anaerobically for approximately a year before it was used. Therefore the microbial characterization does not reflect the proportions of archaea when it was sampled from the Eel River Basin.
Figure B-2. Percent distribution of recovered bacterial clones based on 16S rRNA genes in the starting sediment (before any incubations), live control (sulfate free, no added electron acceptor), iron (ferrihydrite), and sulfate incubations. Sulfur-metabolism indicates phylotypes putatively involved in sulfur cycling. Metal associated represent phylotypes that are possible manganese reducers. Metal/Sulfur are the phylotypes that have the potential to partake in sulfur and/or metal cycling. The starting sediment was stored anaerobically for approximately a year before use, and therefore does not reflect the proportions of bacteria when it was sampled.

Figure B-3. (shown on next page). Neighbor joining phylogentic tree of bacterial clones associated with the iron incubations (Fe B clones). Phylogenetic analysis included the top BLAST match as well as other nearest neighbors (which focus on organisms known to be involved in metal and/or sulfur cycling).
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PUBLICATIONS


Beal, Emily J. Mark Claire, and Christopher House, High rates of anaerobic methanotrophy at low sulfate concentrations with implication for the past a present methane cycle. Paper, in review Geobiology.