

Anaerobic Oxidation of Methane: Mechanisms, Bioenergetics, and the Ecology of Associated Microorganisms

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Microbially mediated anaerobic oxidation of methane (AOM) moderates the input of methane, an important greenhouse gas, to the atmosphere by consuming methane produced in various marine, terrestrial, and subsurface environments. AOM coupled to sulfate reduction has been most extensively studied because of the abundance of sulfate in marine systems, but electron acceptors other than sulfate are more energetically favorable. Phylogenetic trees based on 16S rRNA gene clone libraries derived from microbial communities where AOM occurs show evidence of diverse, methanotrophic archaea (ANME) closely associated with sulfate-reducing bacteria, but these organisms have not yet been isolated as pure cultures. Several biochemical pathways for AOM have been proposed, including reverse methanogenesis, acetogenesis, and methylogenesis, and both culture-dependent and independent techniques have provided some clues to how these communities function. Still, questions remain regarding the diversity, physiology, and metabolic restrictions of AOM-related organisms.

Introduction

Anaerobic oxidation of methane (AOM) is one of the most scientifically intriguing, controversial, and technically challenging subjects of microbial ecology. As recently detailed (1), early investigations of AOM showed repeatedly that methane is consumed within the zone of microbially mediated sulfate reduction in marine sediments (2–9). More recently, the search for microorganisms responsible for AOM, historically limited by the lack of suitable molecular and

cultivation techniques, has led to the phylogenetic identification of multispecies consortia (10, 11) that cycle single-carbon compounds in tightly coupled metabolic reactions. These consortia typically consist of the archaeal clades ANME-1 (distantly related to the *Methanosarcinales* and *Methanomicrobiales*), ANME-2 (within the *Methanosarcinales*), or ANME-3 (closely related to the *Methanococoides*), and sulfate-reducing bacteria (SRB) closely related to the *Desulfosarcina-Desulfococcus* branch of the *Deltaproteobacteria*. However, there remain considerable gaps in the knowledge of the genetic and physiological pathways, thermodynamic constraints, and detailed interactions among these microorganisms.

Methane is the most abundant hydrocarbon in the atmosphere and is an important greenhouse gas with a 20-fold greater relative radiative effectiveness compared to CO₂ on a per-molecule basis. The net release of methane to the atmosphere is approximately 500 Tg year⁻¹ (1) and may be increasing by ~1% annually (12, 13). However, despite restrictive thermodynamic limitations, microbially mediated AOM reduces the release of methane from marine environments to 2% of the global methane flux (14).

Many studies concerning environmental AOM are carried out in anoxic marine waters and sediments near the interface where methane and sulfate concentrations approach zero (reviewed in refs 1 and 15). However, other anoxic environments with active methanogenesis and where methane comes in contact with oxidants suitable for AOM (SO₄²⁻ and, theoretically, NO₃⁻, Fe³⁺, or Mn⁴⁺, among others) may also harbor abundant AOM communities.

Reeburgh (1) thoroughly considered the biogeochemistry of oceanic methane and the role of AOM as a sink for the large amounts of methane produced in marine sediments. Other reviews focused on the ecology of AOM communities (16, 17), methane in the deep subsurface biosphere as a microbial energy source (18), and the comparison between AOM and anaerobic ammonia oxidation (19). Here, we assess current knowledge about the bioenergetics of AOM and the proposed metabolic processes, molecular methods used to

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analyze AOM communities, and opportunities for progress in this field.

Proposed Mechanisms of AOM

The mechanism for AOM is still unknown. Accordingly, most studies focus on understanding how these communities survive on the minimal energy available for this lifestyle and the identification of an interspecies electron carrier that can mediate energy transfer between consortia members.

Reverse Methanogenesis. The most thoroughly investigated biochemical hypothesis for AOM is reverse methanogenesis using sulfate as the terminal electron acceptor. Zehnder and Brock (20) provided circumstantial evidence linking methanogens to anaerobic methane oxidation using radiotracer studies designed to demonstrate pure culture uptake of $^{14}\text{C}-\text{CH}_4$. All nine of the methanogens studied anaerobically oxidized small quantities of methane simultaneously with methane production and produced CO_2 , methanol, or acetate, depending on the strain. Short-term ^{14}C -labeling experiments further indicated that the observed AOM involved a mechanism with intermediates unique to methanogenesis (20). These results were initially disregarded by many researchers since the culture conditions used were not likely to exist naturally.

AOM observed in anoxic, freshwater sediments was subsequently attributed to a consortium of active methanogens and a nonmethanogenic group (21). However, the results of this study did not support previous geochemical models (4, 5, 7, 22, 23). Alperin and Reeburgh (23) used specific inhibitors for sulfate reduction, methanogenesis, and acetate utilization on radiolabeled sediment samples from Skan Bay, Alaska, to show that AOM in this environment is mediated by either an unknown organism or a consortium of SRB and an unidentified methane oxidizer.

Hoehler (10) substantiated the reverse methanogenesis theory by monitoring seasonal in situ rates of methane oxidation and CO_2 reduction in sulfate-depleted, anoxic sediments from Cape Lookout Bight, North Carolina, using sediment incubations and inhibitors of methanogenesis and AOM. Results from this survey showed that a very low rate of methane oxidation is maintained in sulfate-depleted sediments (by methanogens) during the summer and occurs at the base of the sulfate-reducing zone in the winter. Hoehler (10) hypothesized that methane at this methane/sulfate transition is most likely oxidized by CO_2 reducers via a reversal of methanogenesis that uses water as an electron acceptor to produce H_2 , which is efficiently consumed by sulfate reducers. Net AOM is, therefore, possible as long as sulfate is present and the concentration of H_2 remains sufficiently low (~10-fold lower than in sulfate-depleted sediments). Direct measurements of H_2 concentrations in sulfate/temperature manipulation experiments show that this reaction should produce a greater energy yield than CO_2 reduction, suggesting that reverse methanogenesis is possible.

Widdel and Rabus (24) also argued in favor of reverse methanogenesis with sulfate as the electron acceptor. The high activation energy of the C–H bond in methane causes the final protonation step in methanogenesis (the initial step in reverse methanogenesis, catalyzed by methyl coenzyme M reductase) to be irreversible. Therefore, an additional enzymatically catalyzed step or a coupled energy-conserving mechanism is likely required to activate methane and initiate the process. Kruger et al. (25) extracted a 951 Da, nickel-containing protein (Ni–protein-I) with an absorption spectrum similar to the F430 cofactor of methyl-coenzyme M reductase (Ni–protein-II) from microbial mats associated with anoxic methane seeps in the Black Sea. Another protein with a mass and absorption spectrum identical to F430 was purified from the same sample (25) and may be the protein needed to initiate reverse methanogenesis. Furthermore, the

presence of an F430-like cofactor (an indicator of methanogenesis) in these samples also suggests that AOM may co-occur with methanogenesis in ANME-1 (25). This co-occurrence was suggested by previous workers (15, 20, 21). Demonstrating that both proteins are from the same organism is difficult; however, the observation that ANME-1 represents 70% of the detectable cells in the samples supports this conclusion (25).

Methane produced during AOM in mat samples from a Black Sea methane seep was recently estimated at 2–17% of the total methane turnover (26). Reduction of CO_2 to CH_4 in these samples occurred only in the presence of methane and sulfate and was not affected by H_2 concentrations, suggesting that methanogenesis in this system may be related to AOM (26). A similar percentage of AOM-associated methane production was previously reported in cold seep sediments from the Gulf of Mexico (27).

Environmental genomic data provide additional support for a reverse methanogenesis mechanism of AOM. Hallam et al. (28) used whole-genome shotgun and fosmid libraries to analyze methane-oxidizing archaea enriched from a sediment pushcore interval collected from methane seeps in the Eel River Basin. Sequence data based on rRNA and *mcrA* (methyl coenzyme M reductase, subunit A) genes from these metagenomic libraries showed that the microbial community in these sediments is dominated by ANME-1, ANME-2, and SRB. Data collected from these libraries also revealed that the ANME-1 group contains all genes required for methanogenesis except the *mer* gene, which encodes methylene- H_4MPT reductase (step 5 of methanogenesis) (28). The absence of *mer* activity in this group suggests that AOM may be promoted by the consequential increase in the energy required to convert methylene- H_4MPT to methyl- H_4MPT (28).

In general, reverse methanogenesis is supported by results from prior modeling, tracer, and stable isotope experiments, and it helps to explain the low rates of AOM demonstrated by previous inhibition studies. In addition, the mechanism can be performed by known functional groups, rather than an elusive, “unknown” population. However, cultivation studies by Valentine et al. (29, 30) showed that pure cultures of methanogens transferred from a growth-supporting environment to conditions with low H_2 and high CH_4 concentrations transiently produced H_2 sufficient for energy conservation, but none of the four cultures tested were able to sustain H_2 production, and methane oxidation was never observed. ^{13}C -depleted bacterial lipid biomarkers found in samples containing both light archaeal lipids and heavier bacterial lipids (31–33) also suggest that an alternative pathway must exist for AOM, since these observations can be explained only by interspecies carbon transfer, which does not occur during reverse methanogenesis. Additionally, if representatives of the *Methanosarcinales* are responsible for methane oxidation in some environments, it is unlikely they are all performing AOM via a reverse methanogenesis pathway, since many members of this group use acetate and methylated compounds instead of H_2 during methanogenesis (34). Taken together, these results suggest that if AOM occurs via reverse methanogenesis, the mechanism is not widespread among known methanogens.

Acetogenesis. As an alternative to reverse methanogenesis, Valentine and Reeburgh (34) proposed two mechanisms of sulfate-dependent methane oxidation that are energetically more favorable and are consistent with results from prior cultivation studies, analyses of bacterial lipid isotopic signatures, and the phylogenies of methane-oxidizing archaea. One mechanism hypothesizes that methane-oxidizing archaea produce H_2 and acetic acid from two molecules of methane, which are subsequently consumed by SRB. The net reaction between methane and sulfate should provide

twice the amount of free energy as reverse methanogenesis for both members of the consortium. Furthermore, this model helps to explain why only a fraction of the SRB lipids found in the same sample are isotopically depleted (31–34). In the second mechanism, which had been previously suggested (10, 21), methane-oxidizing archaea produce acetate from CO₂ and CH₄ (i.e. “reverse acetoclastic methanogenesis”), and SRB subsequently consume the acetate.

These hypotheses are testable with laboratory experiments designed to detect methane consumption by known methanogens in consort with H₂- and acetate-consuming SRB or by the identification of isotopically light acetate in pore waters of dominantly methane-oxidizing sediments (34). The low concentrations and rapid turnover of acetate in most environments presents a major challenge for detecting and measuring labeled acetate produced from labeled CH₄, but accelerator mass spectrometry may provide the means to do so (1). Nauhaus et al. (35) examined the potential for H₂, formate, acetate, and methanol to act as exogenous electron donors other than methane to test the response of the indigenous methane-oxidizing community to possible AOM intermediates within a sediment sample from Hydrate Ridge. However, sulfate reduction in these samples was much slower than in samples incubated with methane and was unaffected by the subsequent addition of methane. This suggests that none of these compounds, including acetate, stimulate methane oxidation in the studied consortia and are unlikely intermediates of the AOM pathway.

Methylogenesis. Recently, Moran et al. (36) proposed a new model (methylogenesis) for substrate transfer in methane-oxidizing communities in which methyl sulfides produced by methane-oxidizing and CO₂-reducing archaea are transferred to SRB. In this study, ¹³C-CH₄ was added to sediment incubations from the Eel River basin and the Hydrate Ridge to track the fate of CH₄ in the presence and absence of H₂ within active AOM communities. In this experiment, AOM was not inhibited by the presence of high H₂ concentrations (0.43 mM). This suggests that H₂ cannot be an exchanged intermediate of AOM, since H₂-mediated AOM is not thermodynamically feasible at H₂ concentrations above 0.29 nM under standard marine sediment conditions (10, 36). This is also supported by prior cultivation experiments with SRB in which the addition of H₂ failed to support sulfate reduction (37). Cultivation of an ANME-2-related methanogen to investigate trace methane oxidation, described previously (20), resulted in the production of methyl sulfides at ~10 times the amount observed in the original study (38), suggesting the presence of a novel enzymatic pathway.

In the dominantly reductive pathway proposed by Moran and colleagues (36), methane is activated as it binds to coenzyme M (CoM) and releases electrons for ATP synthesis in ANME. CO₂ is simultaneously reduced to CH₃ bound to CoM (following a pathway nearly identical to CO₂ reductive methanogenesis) using electrons made available by methane or CO oxidation. The subsequent transfer of these methyl groups to sulfide regenerates CoM to continue methane activation and CO₂ reduction. Considering all exchanges between consortia members, the net stoichiometry of this mechanism agrees with previously established AOM reactions. The net products of sulfate reduction by SRB, HCO₃⁻ and HS⁻, are used in the initial steps of methanotrophy, which, in turn, provides H₃CSH as a substrate for sulfate reduction. Consumption of the intermediate maintains the low concentrations required for net energy generation. Furthermore, the limited number of SRB that are known to use methyl sulfides, as well as the lack of support for other proposed intermediates (H₂ and acetate), may account for the limited diversity of SRB observed in AOM consortia (36).

The methylogenesis model is distinct from other proposed

mechanisms of AOM in that the archaeal member of the consortium is able to conserve energy by reducing CO₂ to a methyl group. This also permits the dominance of methanogenesis under high CH₄ concentrations which normally do not favor CH₄ as an electron acceptor (36). This is supported by incubation and enrichment experiments using sediments from Eel River basin and Hydrate Ridge, which are unique for having high methane concentrations and fluxes. However, a more thorough test of this model would be to use sediment incubations similar to those performed by Moran et al. (36) under the low methane concentrations and fluxes that exist in diffusion-controlled sediments from Skan Bay, Chesapeake Bay, Cape Lookout Bight, and the Santa Barbara Basin, where the first studies of AOM were conducted, or in the anoxic marine waters of the Black Sea or the Cariaco Basin, where AOM has been observed.

Thermodynamic Considerations for AOM

The ultimate thermodynamic constraint on metabolic reactions is the need to couple the reaction to adenosine triphosphate (ATP) formation. The minimum amount of energy required to form one mole of ATP under normal cellular conditions is ~50 kJ. However, because some inefficiency is inevitable, this should be regarded as the minimum amount; a more realistic estimate is 60–70 kJ (39). Generally, three protons are used to produce a single ATP molecule (40), although some organisms can use up to four (41–43) and possibly more (44).

If the energetic burden of ATP formation is split among four protons, each must provide at least 15 kJ mol⁻¹, which places a lower limit on the amount of energy a reaction must provide to be used for ATP generation. Within a syntrophic consortium, such as the ANME-SRB group, the energy derived from substrate oxidation is shared between the two species and therefore must yield more than 30 kJ mol⁻¹. Given the inevitable loss of energy in generating and exchanging an electron transfer agent, the minimum energy yield is certainly higher.

Available Energy. Oxidation of organic substrates coupled to sulfate reduction yields a relatively small amount of energy, particularly when compared to oxidation with oxygen or nitrate as the electron acceptor. Standard Gibbs free energy (ΔG_r^0) values near -16.6 kJ mol⁻¹ have been reported for sulfate-coupled methane oxidation (eq 1) by several authors (10, 15, 34, 45). Calculations using environmental concentrations of substrate and products in areas where AOM is known to occur have given a net energy yield from -22 to -35 kJ mol⁻¹ (34), which is near the minimum energy needed for ATP generation using four protons per ATP molecule.



It is evident from the ΔG_r^0 value obtained that the free energy of formation (ΔG_f^0) of methane gas (CH_{4(g)}) was used in the thermodynamic calculations cited above. This implies that methane enters the reaction in the gas phase, which has two significant consequences. First is that at least the initial step in AOM is carried out at the gas/aqueous interface, since almost all cellular reactions are aqueous-phase reactions. Second, there should be a measurable effect of the large volume change from gaseous methane to aqueous bicarbonate ion that would manifest as significant changes in energy production with changes in pressure (eq 2).

$$\Delta G_{(T,P)} = \Delta H^0 - T\Delta S^0 + \int_{T^0}^T \frac{\Delta C_p}{T} dT + \int_{P^0}^P \Delta V dP \quad (2)$$

The molar volume (V^0) of methane gas is 24.466 L, whereas the molar volumes of sulfate, bicarbonate, hydrosulfide, and water are 13.9, 24.6, 20.7, and 18.1 mL (46), respectively. This

gives a ΔV of -24.416 L. Assuming ideal gas behavior for the purpose of simplification and also assuming that the change in heat capacity (C_p) is negligible over the temperatures studied, eq 2 reduces to

$$\Delta G_{(T,P)} = \Delta H^0 - T\Delta S^0 + nRT \ln \frac{P}{P^0} \quad (3)$$

where n is the net change in the number of moles of gas in the reaction (in this case, -1). This indicates that reaction 1 will yield more energy as the pressure (or depth) increases. At 100 m, this would increase the yield by ~ -5.7 kJ mol $^{-1}$. Again, this is only a first approximation, and the actual increase in reaction free energy would most likely be less because of nonideal gas behavior. However, this shows how the oxidation of methane with sulfate could generate more energy than previously suspected if the methane is entering the reaction in the gas phase.

If methane enters the reaction in the aqueous phase, the thermodynamic calculations should include the values for aqueous methane ($\text{CH}_{4(\text{aq})}$), which has a ΔG_f^0 of -34.33 kJ mol $^{-1}$, as compared to -50.72 kJ mol $^{-1}$ for methane gas. The ΔG_r^0 for reaction 1 in this case would be 32.96 kJ mol $^{-1}$. Since the ΔV would be only -12.1 mL, the effect of nonstandard pressure would be negligible at environmentally relevant pressures.

Another type of thermodynamic constraint on sulfate-coupled AOM is the concentration of the reactants and products. Seawater has a sufficient sulfate concentration (~ 28 mM) and an essentially unlimited supply, whereas most terrestrial waters do not contain significant amounts of sulfate. In marine sediments, sulfate concentrations at the depths where AOM is occurring are lower than ambient seawater due to consumption by AOM consortia. AOM activity has been found at sulfate concentrations of 2 mM (47, 48) and lower (49–51).

Sulfate-coupled AOM often occurs in natural waters where methane concentrations are high enough to form gas bubbles, indicating that methane is at saturation. However, the amount of dissolved methane is still relatively low because of its low solubility. Methane concentrations at Hydrate Ridge (47) have been measured at approximately one-half (50 mM) to one-tenth (10 mM) of the theoretical saturation concentration at that depth (52). The higher values are close to the calculated concentration of methane in seawater that is in equilibrium with methane hydrates at that depth, pressure, and salinity (66 mM) (52). Indeed, one study showed that the rate of AOM is dependent upon the partial pressure of methane, as demonstrated by a 4- to 5-fold increase in the rate of sulfide production by ANME-2 consortia when methane partial pressure was increased from 0.1 to 1.1 MPa (35).

Bicarbonate, a product of AOM in marine systems, can reduce the available energy if it accumulates. Precipitated calcium carbonate, which is abundant in sediments (53, 54) and water (55) where AOM is occurring or has previously occurred, decreases the concentration of bicarbonate. Since most seawater is oversaturated with calcium carbonate, small increases above the ambient bicarbonate level should cause precipitation, limiting the bicarbonate concentration. Reaction modeling shows that the maximum bicarbonate concentration in sediments is approximately 22 mM (56). This suggests that bicarbonate concentration is constrained to less than this concentration in ocean water or marine sediments.

Sulfide, another product of sulfate-coupled AOM, occurs in AOM-active sediments at concentrations between 0 and 15 mM (47) and is a source of energy for sulfide-oxidizing organisms, many of which are found on the seafloor and in sediments above areas of sulfate-coupled AOM. Microbial sulfide consumption reduces sulfide concentrations in these regions and may enable AOM to continue without thermo-

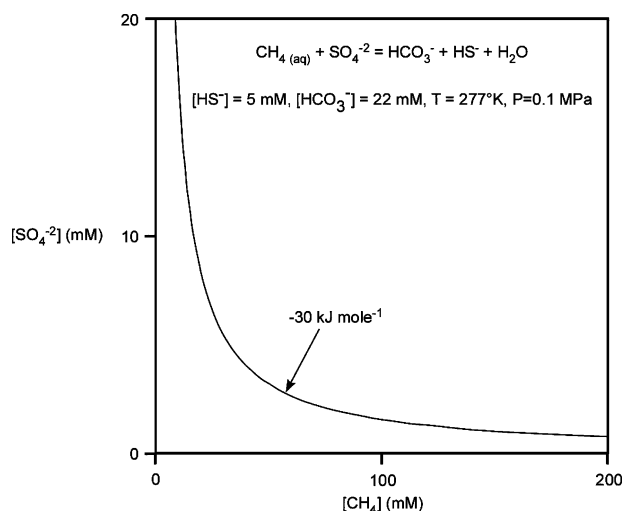


FIGURE 1. Free energy from methane oxidation with sulfate. The line represents the combinations of sulfate and methane concentrations that can yield 30 kJ mol $^{-1}$ at 277 K with $[\text{HCO}_3^-] = 22$ mM and $[\text{HS}^-] = 5$ mM. Combinations to the left of the line yield insufficient energy to produce ATP using four or fewer protons per ATP molecule.

dynamic limitation from sulfide accumulation. Additionally, sulfide inhibits the metabolism of sulfate-reducing bacteria, either by product inhibition or some other mode of toxicity, at concentrations between 10 and 15 mM (57).

Figure 1 is a plot of the free energy of reaction 1 at varying methane and sulfate concentrations. Combinations of methane and sulfate concentrations falling to the left of the -30 kJ mol $^{-1}$ line are not sufficient to support AOM, given the constraints discussed above. Since AOM has been observed at methane and sulfate concentrations below these levels (58), it is probable that the thermodynamic model of AOM presented above is not complete.

One possible explanation is that the products of AOM are not bicarbonate and hydrosulfide ions, as in ref 1, but aqueous carbon dioxide and hydrogen sulfide (eq 4).



This reaction has a ΔG_r^0 of -109.2 kJ mol $^{-1}$, which is over 3 times the free energy of the reaction as it is usually written. It provides -74.0 kJ mol $^{-1}$ at a methane concentration of 1 μM and a sulfate concentration of 2 mM. This could explain the presence of AOM at substrate or product concentrations that are unfavorable given the thermodynamics of reaction 1. Another advantage is that the reaction products, carbon dioxide and hydrogen sulfide, are able to diffuse out of the cell. Bicarbonate and hydrosulfide ions would need to leave the cell through an ion channel. Once outside the cell, the carbon dioxide and hydrogen sulfide would dissociate into bicarbonate, hydrosulfide, and protons.

Other Electron Acceptors. Although sulfate-coupled AOM has the lowest free energy yield among the possible electron acceptors, the abundance of methane-rich marine sediments available for sulfate-coupled AOM suggests that this may be the most prevalent form of methane oxidation, especially since sulfate is the first electron acceptor that methane encounters as it diffuses out of the deeper anoxic sediment.

Coupling AOM with nitrate or nitrite reduction yields significantly more energy (Table 1), even at the lower dissolved methane concentrations found in shallow waters. Nitrate is more available in nonmarine waters than sulfate, particularly in agricultural areas receiving fertilizer runoff. Freshwater AOM primarily occurs in shallow waters (e.g., swamps, rice paddies, and peat bogs) (59, 60) and, therefore, potentially at lower dissolved methane concentrations. Under

TABLE 1. Standard Free Energies of Reactions between Methane and Environmentally Relevant Electron Acceptors

reaction	ΔG_r° (kJ mol ⁻¹ CH ₄)	
	CH ₄ (g)	CH ₄ (aq)
CH ₄ + SO ₄ ²⁻ → HCO ₃ ⁻ + HS ⁻ + H ₂ O	-16.6	-33.0
CH ₄ + SO ₄ ²⁻ + 2H ⁺ → CO ₂ + H ₂ S + 2H ₂ O	-92.8	-109.2
CH ₄ + 2O ₂ → HCO ₃ ⁻ + H ₂ O + H ⁺	-806.0	-822.4
CH ₄ + 2O ₂ → CO ₂ + 2H ₂ O	-842.3	-858.7
CH ₄ + 4NO ₃ ⁻ → HCO ₃ ⁻ + 4NO ₂ ⁻ + H ⁺ + H ₂ O	-467.0	-483.4
CH ₄ + 4NO ₃ ⁻ → CO ₂ + 4NO ₂ ⁻ + 2H ₂ O	-503.4	-519.8
5CH ₄ + 8MnO ₄ ⁻ + 19H ⁺ → 5HCO ₃ ⁻ + 8Mn ²⁺ + 17H ₂ O	-991.7	-1008.1
5CH ₄ + 8MnO ₄ ⁻ + 24H ⁺ → 5CO ₂ + 8Mn ²⁺ + 22H ₂ O	-1028.1	-1044.5
CH ₄ + 8Fe ³⁺ + 3H ₂ O → HCO ₃ ⁻ + 8Fe ²⁺ + 9H ⁺	-418.3	-434.7
CH ₄ + 8Fe ³⁺ + 2H ₂ O → CO ₂ + 8Fe ²⁺ + 8H ⁺	-454.6	-471.0
CH ₄ + ClO ₄ ⁻ → HCO ₃ ⁻ + Cl ⁻ + H ⁺ + H ₂ O	-895.9	-912.3
CH ₄ + ClO ₄ ⁻ → CO ₂ + Cl ⁻ + 2H ₂ O	-932.2	-948.6
CH ₄ + 4HAsO ₄ ²⁻ + 3H ⁺ → HCO ₃ ⁻ + 4H ₂ AsO ₃ ⁻ + H ₂ O	-299.6	-316.0
CH ₄ + 4HAsO ₄ ²⁻ + 4H ⁺ → CO ₂ + 4H ₂ AsO ₃ ⁻ + 2H ₂ O	-263.3	-279.7

these conditions, AOM coupled to nitrate reduction is more likely to occur than sulfate-coupled AOM. Therefore, nitrate-coupled AOM may play a significant role in global methane oxidation according to the contribution of freshwater environments to the global methane budget.

The discovery of a consortium performing AOM with nitrate as the electron acceptor (61) prompts the consideration of other electron acceptors. For example, the association of Fe(III) reduction with hydrocarbon oxidation in anoxic environments (24) suggests that some organisms, or syntrophic consortia, may be able to use Fe(III) as an electron acceptor for methane oxidation. In other situations, it might be possible that other electron acceptors (e.g., arsenate, selenate, perchlorate) could be coupled to anaerobic methane oxidation. Table 1 gives the standard free energies for methane oxidation with various environmentally relevant electron acceptors.

Physiology, Growth, and Strategies for Cultivating AOM Consortia

Fluorescent in situ hybridization (FISH) has provided microscopic evidence for a close, physical association between members of the ANME-1 and ANME-2 clades with SRB (62, 63). ANME-2 and ANME-3 observed in these assays appear to form highly organized clusters, tightly surrounded by SRB, whereas ANME-1 and SRB cells seem more loosely organized (63, 64). Nauhaus et al. (65) also observed in vitro propagation of small ANME-2 and SRB clusters using fluorescence imaging. Approximately 3% of the total biovolume analyzed consisted of these small aggregates, whereas the remaining 60–70% formed larger clusters, <25 μm in diameter (65). These results support the likelihood of an exchanged intermediate during AOM and suggest that sulfate-coupled AOM may require consortia development, but many questions remain regarding the genetic activation, regulation, and physiology of these distinct, physical associations.

Physiological parameters associated with AOM consortia, such as specific growth rates, substrate utilization rates, and responses to environmental factors (e.g., temperature, pressure, and pH) have recently been investigated using anoxic sediment incubation and enrichment experiments as well as a continuous flow bioreactor to allow in vitro rates of methane consumption and sulfate reduction to be monitored and quantified (35, 36, 58, 65, 66). The specific growth rate of indigenous ANME-2 within anoxic sediment slurries from the Hydrate Ridge was measured by incubating sediment samples within pressure-proof steel cylinders under a methane partial pressure of 1.4 MPa (65). By determining changes in biovolume over 24 months, the specific growth rate of this AOM consortium was estimated to be 0.003 day⁻¹ (65). “Seep” and “nonseep” sediments from within and outside a hydrocarbon seep in Monterey Canyon, California, were incubated in a novel continuous-flow anaerobic methane incubation system to simulate in situ conditions for metabolism and growth of methanotrophic archaea (58). In this study, Girguis and colleagues estimated growth rates of 0.02–0.4 day⁻¹ in the “nonseep” sediment during the early phase of the experiment, and rates decreased to ≤0.01 day⁻¹ during the later phase (58).

The availability of methane and rates of sulfate reduction in AOM communities are highly influenced by temperature, pH, and salinity. Temperature is a significant determinant of dissolved methane concentration and, therefore, may be one of the most important abiotic factors influencing ANME-SRB consortia activity. For example, enrichment cultures of AOM consortia containing ANME-1 from different locations exhibited a maximum rate of sulfate reduction between 16–24 °C, but ANME-2 consortia were most active between 5–10 °C (37). Below pH 7 and at salinities below 20 ‰ or above 40 ‰, sulfate reduction in ANME consortia is significantly decreased (37). However, since these values are based on rates of sulfate reduction, it is not possible to determine if these limitations are inherent to either the ANME or SRB consortium members or if they affect both AOM and sulfate reduction. Smemo and Yavitt (67) suggest a possible annual or seasonal cycle of electron acceptor availability for AOM in freshwater peatlands. In this study, continuous additions of CH₄ during closed-system peat incubations decreased net oxidation and production rates once net rates of AOM reached a certain threshold. Therefore, if climatic factors, fluctuations in redox state, and associated hydrology influence the supply of potential electron acceptors, such environmental conditions may, likewise, influence the spatial and temporal patterns of AOM (67).

Methods for Analyzing AOM Consortia

Fluorescent In Situ Hybridization (FISH). The use of FISH (reviewed in ref 68) has played a unique role in the discovery and characterization of AOM-related microbial communities, beginning with their original identification (69). FISH has also been combined with other techniques, such as secondary ion mass spectrometry (SIMS), to help link phylogeny with function within the archaeal component of an AOM consortium (62). FISH enables the distinction between archaeal and bacterial cells; SIMS reveals extremely depleted carbon isotope signatures associated with *Methanosarcinales* (ANME-2) cells, which is best explained as an assimilation of carbon from ¹³C-depleted methane (62).

Stable isotope probing (SIP) labels actively metabolizing communities by supplying them with substrates enriched in stable isotopes (70). To our knowledge, strategies that use SIP or FISH combined with other molecular tools, such as catalyzed reporter deposition (CARD-FISH) and microautoradiography, have not yet been used to characterize AOM communities. However, these tools may help trace the

movement of carbon in AOM consortia and determine where AOM activity is unusually high, low, or only transiently occurring.

Biomarkers. Along with FISH, analyses of molecular biological markers, or biomarkers (reviewed in refs 1, 34, 71), have provided the primary evidence for the presence of AOM, particularly in and around cold seeps where direct measurements of methane distribution and rates of AOM are not possible (1, 62). In brief, cell membrane lipids that are taxonomically specific to members of AOM consortia can be detected and identified by mass spectrometry. Evidence for the consumption of highly ^{13}C -depleted methane can also be detected in these lipids (72). Therefore, the incorporation of ^{13}C -depleted carbon from methane into microbial biomass can be used as an indicator for microbially mediated methane oxidation and permits reliable identification of the bacterial (73) and archaeal (74) groups involved. Variations of these studies that target lipid biomarkers preserved in the rock record have been used to implicate AOM in the formation of carbonate systems approximately 2.7 billion years old (75) (the first definite occurrence of AOM chemofossils are ~150 million years old (76)).

Genomics and Metagenomics. Another approach for predicting AOM activity is the identification of whole-community genomes using large-fragment DNA libraries. Gene libraries can be queried to address hypotheses regarding physiological processes or cellular structures, such as attachment molecules that assist electron transfer at membrane surfaces, that are unique to the communities in question and can be further scrutinized to identify genetic clues to how AOM consortia are able to survive at their bioenergetic limits. For example, careful inspection of a syntrophic proteobacterium genome recently revealed clues to how that organism survives at the thermodynamic limits of life (77). These and other genomic databases can also be used to develop mRNA-based microarrays to analyze gene expression, community proteomic tools for studying functional enzyme fluxes, and new strategies for cultivating consortia members.

Magneto-FISH. High-throughput sequencing and metagenomics have provided significant insight into the phylogeny and metabolism of the communities studied, but relating whole-community sequence data to specific microbial groups and their interactions can be challenging. Single cells may be isolated from their native community prior to genome sequencing using optical trapping (78), immunomagnetic capture (79), flow cytometry (80), or microfluidics (81), but these techniques are limited in their ability to characterize interspecies associations in complex environments. Magneto-FISH technique was developed to selectively isolate whole cells and cell aggregates from an environmental sample using phylogenetic probes (82). This technique combines CARD-FISH with immunomagnetic capture of hybridized cells using paramagnetic beads coated with a fluorochrome-specific antibody. The method can also be combined with high-throughput sequencing and isotope labeling to characterize otherwise "hidden" microbial groups and simplifies metagenomic sequencing by reducing community complexity (82).

Functional Gene Indicators of AOM Consortia. Functional gene analyses have also contributed to the metabolic characterization of these consortia. Subunit A of the methyl-CoM reductase (*mcrA*) gene is commonly used to determine the presence of ANME in AOM consortia. This gene, which was originally used as an exclusive indicator of methanogenic microorganisms, exists in the ANME-1 and ANME-2 genomes, where it hypothetically performs the initial step of methane oxidation (83). Studies using quantitative PCR (qPCR) or minimum cycles for detectable products PCR (MCDP-PCR) to determine the relative abundances of *mcrA* and the 16S rRNA gene, respectively, have been used to enumerate

archaeal cells within AOM consortia and to distinguish between different ANME groups present in a given sample (84, 85). Similarly, the dissimilatory (bi)sulfite reductase (*dsrAB*) gene has been used to detect the bacterial component of an AOM consortium (86, 87). Phylogenetic trees generated from partially translated amino acid sequences of PCR-amplified *mcrA* and *dsrAB* gene sequences from various environmental samples generally agree with the 16S rRNA gene phylogeny and further indicate the relative diversity of identified sediment consortia and the distribution of different community members (83, 87).

On-Going and Future Work

The successful cultivation of individual ANME would allow researchers to describe the physiological processes and regulation of AOM metabolism, thereby yielding a more complete understanding of the parameters within which AOM-associated microorganisms function. Valentine et al. (29) designed a microbial culture apparatus for studying interspecies hydrogen transfer that, in general, operates by sparging a liquid culture with purified gases. However, the apparatus was used to study only pure cultures. Similar devices may be used with environmental samples to study the metabolic interactions of microbial communities. Moran et al. (36) used sediments from Hydrate Ridge and Eel River basin to implicate the possibility of AOM through a methylogenes pathway. However, this and similar incubation studies should be extended to simulate more diverse environmental conditions where AOM may occur or is known to occur. These and related studies should also be combined with future and ongoing genomic, biomarker, and FISH-related work to better understand the mechanism(s) of AOM and related microorganisms in more diverse in situ conditions and to greatly enhance our knowledge of AOM overall.

The sulfate–methane interface (SMI) in marine sediments where the concentrations of both sulfate and methane coincidentally diminish and which is created and maintained by AOM consortia, may be studied with geophysical tools. Such measurements would allow rapid determination of SMI depth and by proxy, the amount of methane migrating upward in the sediments without the need to core and analyze porewater sulfate and methane concentrations. A shallow SMI indicating a high methane flux could be a reason to locate man-made seafloor structures elsewhere because of the tendency for such sediments to be unstable. These measurements may also help to refine models used for analyzing the SMI depth, the dissolved and hydrate–methane dynamics in marine sediments and, therefore, the degree to which methane is likely moving upward in the sediments toward the sediment–water interface. This new field of biogeophysics has detected microbially induced changes in mineral surface resistivity and electrical conductance based on the formation of metal sulfides where SRB are active (88). In an AOM application, the approach may key on the remote, nondestructive detection of magnetic iron sulfides left by AOM communities (89, 90). The rate of bacterial sulfate reduction ($0.93 \mu\text{M SO}_4/\text{g sediment/day}$) in laboratory columns that could be detected using this geophysical approach (88) is considerably lower than laboratory-derived rates of sulfide production by AOM consortia (65) ($20\text{--}230 \mu\text{m S}^{2-}/\text{g sediment/day}$). Although metal sulfides are present in typical marine sediments, high levels of these minerals appear to be associated with AOM activities (91). Therefore, in principle, AOM activities may be similarly detectable using geophysical measurements, although making such measurements on the seafloor is yet to be accomplished.

Measurements of natural $^{14}\text{C-CH}_4$ may provide a clean, but challenging, method for tracing the consumption of large quantities of methane in open ocean and coastal waters (1).

Recent estimates of fossil methane contributions to the ocean (92) and radiocarbon measurements of methane hydrates (93) suggest that the global influx of methane to marine waters may be larger than previously predicted. However, despite this large input, the concentration of methane in the open ocean is at a nanomolar scale, presumably due to extensive oxidation. Commercially available membrane gas exchange technology may be used to extract sufficient concentrations of methane to analyze the natural abundance of marine ^{14}C - CH_4 to constrain the fossil methane contribution and obtain a better understanding of the global impact of methane oxidation in marine environments (1).

Understanding AOM communities and the environmental conditions under which they consume methane may help to refine computational models for methane cycling on Earth and should improve the accuracy of long-term climate change projections.

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Supporting Information Available

A detailed listing and schematic representation of natural methane sources on Earth (as potential habitats for microbially mediated AOM) and a phylogenetic tree based on 16S rRNA gene sequences showing the approximate evolutionary relationships of ANME-1, ANME-2 and ANME-3 are presented in the Supporting Information. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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