Imaging anaerobic methane oxidation at environs near Woods Hole

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Abstract

CARD-FISH was performed to determine the presence of S-DAMO and N-DAMO in the Cape Cod Area. Samples were taken from marine sediments at Trunk River and Great Sippewissett Salt Marsh for S-DAMO studies, while freshwater samples were collected from Cedar Swamp and the Cape Cod Aquifer for N-DAMO. Comparisons of the epifluorescence micrographs found at the marine sites indicate that ANME/SRB consortia are more prevalent at Great Sippewissett Salt Marsh and more free-living members of the ANME clades are found at Trunk River. On comparing Cedar Swamp and the Cape Cod Aquifer, it seems that the *M. oxyfera* at the Cape Cod Aquifer are in consortia with methanogenic archaea, and that those at Cedar Swamp are primarily free-living.

Introduction

The process of anaerobic methane oxidation (AMO) was first discovered over 35 years ago [3, 18, 25] in the marine environment. Early in vitro activity assays, radiotracer experiments, and stable carbon isotope analysis led to the hypothesis that AMO is mediated by methanogenic archaea conducting reverse methanogenesis and sulfate-reducting bacteria [1, 10, 30].

Evidence for this hypothesis was found in 1999 with in situ detection of isotopic biomarkers [8]. Lipid biomarkers that are commonly characteristic of archaea were strongly depleted in ^{13}C. Therefore, the archaea that contain these lipids must use CH_4 as a carbon source, not as its metabolic product. With the advent of fluorescence in situ hybridization (FISH), a structured consortium of archaea and sulfate-reducing bacteria (SRB) [4] was discovered. When coupled with secondary ion mass spectrometry (SIMS) it was unambiguously demonstrated that methanotrophic archaea coupled with SRB, were negative in $\delta^{13}C$, down to -96% [21] and were performing sulfate-dependent anaerobic methane oxidation (S-DAMO). There are three archaeal clades (ANME-1, ANME-2, and ANME-3) that mediate this process under anoxic marine conditions that are dis-

tantly related to *Methanosarcinales* and *Methanomicrobiales* [19, 20] and can occur in sediments as single cells or associated with SRB [12, 19, 21, 22].

The methanogenic archaea in the consortium achieve reverse methanogenesis through the use of the same nickel-containing methyl-coenzyme M reductase (MCR) that occurs in methanogenesis, yet in the endergonic process [14, 26]. Initially, it was hypothesized that nitrite-dependendent anaerobic methane oxidation (N-DAMO) with archaea conducting reverse methanogenesis in consortia with denitrifying bacteria [23].

The first study that studied N-DAMO *in vitro* used a laboratory-scale sludge digestor [9]. Later, an enrichment culture was obtained that demonstrated both methane oxidation and denitrification occurring. Freshwater canal sediment was incubated over a year a sequencing batch reactor until measurable CH_4 and $NO_2{}^-$ turnover was obtained. 80% of the population from the enrichment culture belonged to the candidate phylum NC10 [23]. The division had been defined, up until that point, by environmental sequences only [24]. A smaller fraction of the population found in the bactch reactor consisted of Archaea (up to 10%). These belonged to the order *Methanosarcinales* and were distantly related to *Methanosaeta* and ANME-II (anaerobic methanotrophs). Labeling experiments and $\delta^{13}C$ measurements indicated that both of these microorganisms were involved in the anaerobic oxidation of methane, although the bacterial lipids were more strongly labeled. The same enrichment culture was grown for a year more and the archaea were phased out of the culture. The bacteria within the culture could perform the entire process [7].

This bacterium. the only member of the NC10 phylum, now recognized as *Candidatus Methylomirabilis oxyfera*, bypasses nitrous oxide (N_2O; a denitrification intermediate), but instead, converts two nitric oxides (NO) to nitrogen (N_2) and oxygen (O_2) gas, which then was used to oxidize CH_4 [29]. This was a ground-breaking discovery as it proposed a new pathway for the production of oxygen and helped extend the knowledge of how hydrocarbons are degraded. Most important for early-earth implications, however, is the fact that this opens the possibility that oxygen was available to microbial metabolism before the evolution of oxygenic photosynthesis.

Previous to these findings, at a contaminated aquifer in Cape Cod, Massachusetts a single study had unambiguously demonstrated anaerobic oxidation of methane coupled to denitrification [27]. A later study at the site also demonstrated the presence of *M. oxyfera* with primers specific for the *pmoA* gene of the NC10 phylum [16]. *M. oxyfera* were also found at another site in Cape Cod, Cedar Swamp, through using phylum-specific 16S rRNA gene primers [13]. No evidence of S-DAMO has yet been determined in the coastal environment.

In the present study, I seek to elucidate whether or not S-DAMO organisms are present at sites near Woods Hole through CARD-FISH and epifluorescent microscopy. I also want to determine if N-DAMO can be found using these techniques and if they are associated in symbioses with methanogenic archaea in the Cape Cod Aquifer, where methane concentrations are not measurable.

Methods and Materials

Sampling

The water-sediment interface was collected from pools containing berries at Great Sippewissett Salt Marsh using 50mL Falcon tubes (BD Biosciences; San Jose, CA, USA) without introduction of air. 50mL Falcon tubes were also collected at the water-sediment interface at Trunk River where bubbling of H_2S was occurring.

Water samples were collected from Cedar Swamp and the Cape Cod Aquifer. 2L bottles were collected from Cedar Swamp at 80 cm depth, right above the bottom of the swamp. Three 125mL glass bottles (Wheaton; Millville, NJ) were also collected from the water and sealed using gas-tight caps and septa. 2L and 125mL bottles were also collected from F168-M16-09Y at the Cape Cod Aquifer.

Determination of chemical concentrations

The 50mL Falcon tubes from Trunk River and Great Sippewissett Salt Marsh were centrifuged to combine the water overlying the sediment and porewater. The 125mL bottles from Cedar Swamp and the Cape Cod Aquifer were used to measure concentrations of chemical substrates within each of the samples. Dissolved oxygen (DO) was measured from one bottle by using an oxygen probe (Unisense; Aarhus, DK). Nitrate ($NO_3{}^-$), $NO_2{}^-$, and sulfate ($SO_4{}^{2-}$) were measured using a Dionex ICS-2100 ion chromatograph (Thermo Fisher Scientific, Inc.; Sunnyvale, CA, USA). CH_4 was measured with a GC-2014 gas chromatograph (Shimadzu; Columbia, MD, USA).

CARD-FISH

Great Sippewissett Salt Marsh and Trunk River sediment was fixed and diluted by doing a 1:10 dilution of the sample with a Phosphate Buffer/Ethanol solution. The fixed sample was sonicated on ice and was filtered using a 0.2μm filter with a final dilution of 1:10,000.

Water from Cedar Swamp and the Cape Cod Aquifer were pre-flitered using an 11μm filter. The water from each site was then fixed using by creating a final concentration of 1% paraformaldehyde (Electron Microscopy Sciences; Hatfield,PA, USA). 1mL, 5mL, and 10mL of water from each sample were then filtered through a 0.2μm filter to obtain cells.

All filters were then embedded in 0.1% agarose and permeabilized by both a lysozyme buffer and using a Proteinase K solution. Peroxidases were inactivated with hydrogen peroxide in methanol. The filters from marine sediments were hybridized with anaerobic methane oxidizer probes, ANME-1-350, EelMS932, ANME-2A-647, ANME-3-1249 with helper probes ANME-3-1249H3 and ANME-3-1249 (Table 1). They were also hybridized with sulfate-reducing bacteria probes DSS658 and DBB660. Freshwater samples were hybridized S-*-DBACT-0193-a-A-18 for *M. oxyfera* and Arch915 for *Archaea*. The signal of

hybridization was amplified using tyramides, Alexa488 (green) was used for bacterial groups and Alexa594 was used for archaea. The cells were also stained with DAPI and mounted to slides using a Citifluor/Vectashield mounting medium. The filters were imaged using an Axio Imager.M2 (Zeiss; Jena, Germany).

Table 1: Oligonucleotide probes used in this study

Probe	Specificity	Probe Sequence (5'-3')	%FA	Reference
ANME-1-350	ANME-1	AGT TTT CGC GCC TGA TGC	40	[4]
EelMS932	ANME-2	ACG TCC ACC CGT TGT AGT	65	[4]
ANME-2a-647	ANME-2a	TCT TCC GGT CCC AAG CCT	50	[12]
ANME-3-1249	ANME-3	TCG GAG TAG GGA CCC ATT	40	[19]
ANME-3-1249H3	Helper probe for ANME-3-1249	GTC CCA ATC ATT GTA GCC GGC		[15]
ANME-3-1249H5	Helper probe for ANME-3-1249	TTA TGA GAT TAC CAT CTC CTT		[15]
DSS658	*Desulfosarcina* spp., *Desulfofaba* spp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCC ACT TCC CTC TCC CAT	60	[17]
DBB660	*Desulfobulbus* spp.	GAA TTC CAC TTT CCC CTC TG	60	[5]
S-*-DBACT-0193-a-A-18	*Methylomirabilis oxyfera*	CGC TCG CCC CCT TTG GTC	40	[23]
Arch915	*Archaea*	GTG CTC CCC CGC CAA TTC CT	35	[28]

Results & Discussion

Site chemistry

Trunk River and Great Sippewissett Salt Marsh

The marine sites differed in the amount of dissolved oxygen found within the samples (Table). The Trunk River sediment was completely anoxic while the Great Sippewissett Salt Marsh samples had some DO present (7.18μM). A low concentration of CH_4 was found within Trunk River (3.17μM), but there was no measurable amount within Great Sippewissett Salt Marsh. Sulfate was present at both sites, but was higher at Great Sippewissett Salt Marsh (28,484.30 μM) than at Trunk River (5,839.90 μM). The lack of oxygen present at Trunk River was almost undoubtedly due to the amount of sulfides being produced within the sediment which would have also affected the ${SO_4}^{2-}$ present within the samples. The low amount of ${SO_4}^{2-}$ within Trunk River sediments may also be due to the fact that the water is brackish.

Table 2: Chemical concentrations in marine sites (μM)

Site	DO	CH_4	${SO_4}^{2-}$
Trunk River	0	3.17	5830.90
Great Sippewissett Salt Marsh	7.18	0	28484.30

Cedar Swamp and the Cape Cod Aquifer

Both freshwater sites studied were primarily anoxic (Table **??**), with the Cape Cod Aquifer containing some oxygen (1.52μM. Cedar Swamp had a large amount of CH_4 present (28096.45 μM) and no NO_3^-, while the Cape Cod Aquifer had no CH_4, but contained NO_3^- (158.23 μM). No NO_2^- was measured at either site. The fact that there is little to no oxygen present within both of the sites should lend to N-DAMO occurring at both sites. NO_2^- is a transition species and is readily converted into NO_3^-. Therefore, NO_2^- concentration is not a true measure of the capability of the denitrification to take place and NO_3^- is a better indicator. The Cape Cod Aquifer has high NO_3^- and, therefore, accessible NO_2^- whereas, whatever NO_2^-/NO_3^- is present within the system at Cedar Swamp is being immediately scavenged. There is an abundance of CH_4 at Cedar Swamp, but none present at the Cape Cod Aquifer. Consequently, if there is CH_4 being produced within the aquifer, it is also instantly used. This displays that the *M. oxyfera* detected through sequencing within these sites are under opposite chemical conditions.

Table 3: Chemical concentrations in freshwater sites (μM)

Site	DO	CH_4	NO_3^-	NO_2^-
Cedar Swamp	0	28096.45	0	0
Cape Cod Aquifer	1.52	0	158.23	0

Epifluorescent Microscopy of Anaerobic Methane Oxidizers

S-DAMO

At Trunk River, each ANME clade was represented. However (Fig 1), most ANME were not found in consortium with SRB and were, instead, free-living. Species that were targeted by the DSS658 probe (*Desulfosarcina* spp., *Desulfofaba* spp., *Desulfococcus* spp., and *Desulfofrigus* spp.) were in extremely high numbers while those targeted by the DBB660 probe (*Desulfobulbus* spp.) were in low abundance. There were also very few ANME-3 present.

All ANME groups were also present at Great Sippewissett Salt Marsh (Fig 2). Fig 2C is an example of the types of consortia found at the salt marsh. Organisms targeted by the DSS658 probe were in less abundance than found in Trunk River. This is logical considering the amount of sulfide production that occurs at Trunk River. The fact that Great Sippewissett Salt Marsh has more consortia present is counter to published literature [2], which states that consortia are in higher abundance where there are high concentrations of CH_4. However, there was no measurable CH_4 present at Great Sippewissett Salt Marsh. When sampling from Great Sippewissett Salt Marsh, it was noted that the smell of dimethyl sulfide (DMS) was particularly strong. The ability of methanogenic archaea to access the methyl group of dimethylsulfoniopropionate (DMSP) has

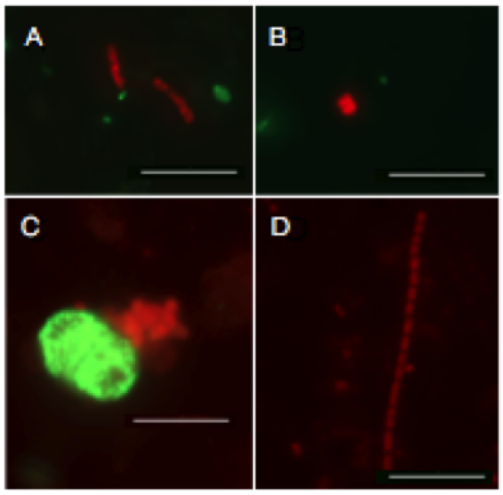

Figure 1: Epifluorescence micrographs of AMO and SRB at Trunk River visualized by CARD-FISH with probes specific for ANME-1 and DSS (A), ANME-2 and DSS (B), ANME-2a and DSS (C), and ANME-3 and DBB (D). All ANME are shown in red. DSS and DBB are green. Scale bars, 10 μm.

been documented [11]. Perhaps ANME also have the ability to access this compound for methane oxidation.

N-DAMO

Methylomirabilis oxyfera found at Cedar Swamp are primarily free-living (Fig 3). However, there a few that are found in consortia with archaea. On inspection of the labeled archaea under DAPI staining (not shown) it is clear that the archaea are *Methanosarcina* spp.. When looking at samples that were obtained from the Cape Cod Aquifer under epifluorescence, almost all *Methylomirabilis oxyfera* are found in consortia with *Methanosarcina* spp. (Fig 4). However, it is displayed in Fig 4C that *M. oxyfera* are not associated with *Methanobacterium* spp.. I hypothesize that the absence of measurable CH_4 concentrations within the aquifer may be the reason that these organisms are in consortia.

Concluding remarks

The discovery of ANME within the sediment of the marine sites shows the need for more CARD-FISH surveys. Both of the marine sites used in this study had very little CH_4 present within the samples. However, not only was the presence of organisms that utilize methane for anaerobic oxidation detected, they were found in abundance. Therefore, the ability of microorganisms to

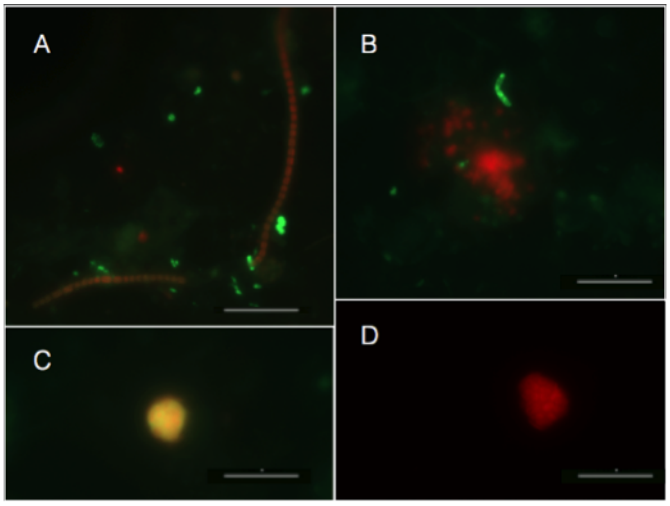

Figure 2: Epifluorescence micrographs of AMO and SRB at Great Sippewissett Salt Marsh visualized by CARD-FISH with probes specific for ANME-1 and DSS (A), ANME-2 and DSS (B), ANME-2a and DSS (C), and ANME-3 and DBB (D). All ANME are shown in red. DSS and DBB are green. Scale bars, 10μm.

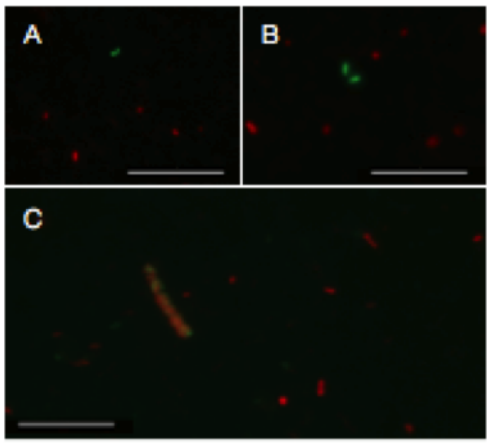

Figure 3: Epifluorescence micrographs from samples collected at Cedar Swamp visualized by CARD-FISH with probes specific for *Methylomirabilis oxyfera* (green) and *Archaea* (red). Scale bars, 10μm.

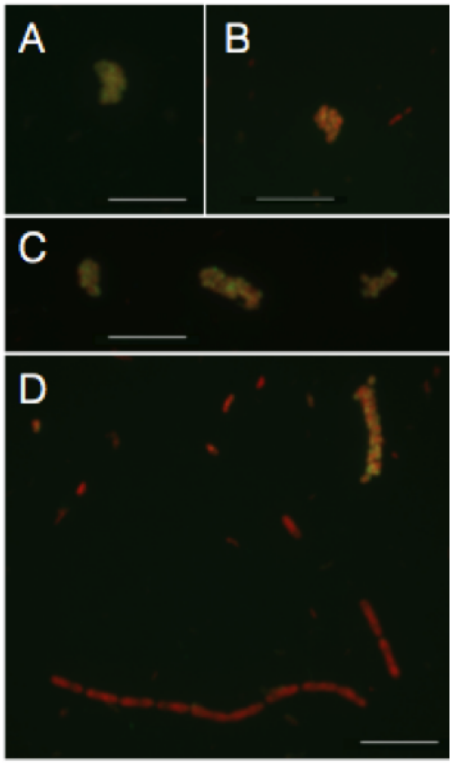

Figure 4: Epifluorescence micrographs from samples collected from the Cape Cod Aquifer visualized by CARD-FISH with probes specific for *Methylomirabilish oxyfera* (green) and *Archaea* (red). Scale bars, 10μm.

make a living on next to nothing is displayed in this study. *Methylomirabilis oxyfera* was shown to have a close association with methanogenic archaea in an environment that has extremely limited CH_4 concentrations, which displays the adaptability of microorganisms when conditions are unfavorable.

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