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Abstract

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Methanotrophic microbial communities associated with bubble plumes above gas seeps in the Black Sea

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Bubbles evolving from active gas seeps can be traced by hydroacoustic imaging up to 1000 m high in the Black Sea water column. Although methane concentrations are not distinguishable between the water column above the deep seep and reference sites, atmospheric noble gas measurements clearly show the constant input of gases (mainly methane) via seepage into the Black Sea. Archaea (ANME-1, ANME-2) and methanotrophic bacteria detected with specific 16S rRNA-targeted oligonucleotide probes are related to active gas seeps in the oxic and anoxic water column. It is suggested that methane seeps have a much greater influence on the Black Sea methane budget than previously acknowledged and that ANME-1 and ANME-2 are injected via gas bubbles from the sediment into the anoxic water column mediating methane oxidation. Our results show further that only minor amounts of methane evolving from Black Sea gas seeps reach the atmosphere due to the very effective microbial barrier. Hence only major thermodynamically and/or tectonically triggered gas hydrate dissociation has the potential to induce rapid climate changes as suggested by the “clathrate gun hypothesis.”
1. Introduction

Methane, the second most important greenhouse gas after carbon dioxide, has been held responsible for dramatic climate changes in the past [Dickens, 2003; Kennett et al., 2000]. Owing mainly to anthropogenic production, atmospheric methane concentrations have doubled from 850 ppb to approximately 1750 ppb over the last 150 years [Rasmussen and Khalil, 1984]. A significant proportion of the methane emitted to the atmosphere (30% of the total [Cicerone and Oremland, 1988]) is of natural origin; however, atmospheric methane concentrations would be much higher if the huge methane pool that is stored in ocean sediments were to be released to the atmosphere. In ocean sediments, the anaerobic oxidation of methane (AOM) and the formation of gas hydrates generally hinder its release from the seafloor, since methane is almost quantitatively converted to bicarbonate [Reeburgh, 1976; Valentine and Reeburgh, 2000]. Recent observations, however, show that an unknown number of gas seeps exist at the seafloor, through which methane is emitted, thus escaping microbial transformation as streams of gas bubbles and floating hydrates. The few recent investigations that have been conducted have found neither substantial methane oxidation nor a methanotrophic community existing in the gas plumes above seeps [Damm and Budeus, 2003].

The Black Sea is the largest anoxic water body on earth and serves as a model for both modern and ancient anoxic environments. Stable stratification of the water column suppresses substantial deep-water renewal, fostering anoxia below the chemocline, which is located at 90–170 m. Methane concentrations in the deep water (below 500 m) are relatively constant at a very high value (11–12 μM, i.e., ~5000 times the atmospheric equilibrium concentration), and gradually decrease to only 10 nM in surface waters [Reeburgh et al., 1991]. In the north-western Black Sea, hundreds of active gas seeps, emitting mainly methane [Blinova et al., 2003], exist along the shelf and slope of the Crimean Peninsula at water depths of between 35 and 800 m [Ivanov et al., 1989]. The EU project CRIMEA has the objective to investigate the methane seeps located on the north-western Black Sea shelf and their role as a source for methane to the atmosphere. In this paper we describe the involvement of microorganisms in the transformation of methane in the water column from the seafloor to the upper water layer with a special focus on methane seeps.

2. Methods

In the present study, we explored the biogeochemical dynamics at a shallow seep site (44°50'N, 31°59'E, 92 m water depth) and at a deep seep site (44°17'N, 35°02'E, 1985 m), together with two reference sites (44°51'N, 32°01'E, 76 m and 44°14'N, 32°30'E, 1658 m) that were unaffected by active gas emissions. Samples were taken from the R/V Professor Vodyanitskiy during the CRIMEA project cruise in May/June 2003. Sampling gears involved a CTD (Seabird SBE-9) connected with a rosette of 12 water bottles each able to sample 10 L of water at a specific water depth. Water samples were taken immediately when the rosette came onboard for noble gases, methane concentration and methane isotopic composition, and fluorescence in-situ hybridization (FISH).

2.1. Methane Concentration and Isotopic Composition

For CH₄ analysis, a vacuum degassing method was used whereby 1600 mL of water was injected...
into a pre-evacuated 2200 mL glass bottle, which leads to almost quantitative degassing [Rehder et al., 1999]. The gas phase was subsequently recompressed to atmospheric pressure and the CH$_4$ concentration of the extracted gas was determined by gas chromatography on a Shimadzu GC14A equipped with a flame ionization detector. Nitrogen was used as the carrier gas, and separation was performed using a 4 m 1/8" SS column packed with Porapack Q (50/80 mesh) at 50 °C. The methane carbon isotopic composition was determined for the shallow stations using a Trace Gas linked to an Isoprime mass spectrometer (GV Instruments).

2.2. CH$_4$ Oxidation Rates

[6] Microbial methane oxidation was measured using triplicate 20 mL crimp-seal bottles that were filled with sampled water and sealed gas-tight. From each triplicate, one sample was treated with 50 μL formaldehyde (37%) to function as a blank. Oxygen-free aliquots of 50 μL tritiated methane ($^3$HCH$_3$) with a specific activity of 20 Ci mmol$^{-1}$ (Biotrend, Germany) were added to the bottles and incubated in the dark at ambient water temperatures for 4 days. After incubation, samples were killed with formaldehyde (37%), left opened overnight, and stripped with nitrogen prior to counting, to remove all non-reacted tracer. The product of methane oxidation ($^3$HHO) was measured on a Wallac 1209 Rackbeta (Pharmacia) using a liquid scintillation cocktail (Insta-gel Plus, Perkin Elmer). Blank values were always lower than 15–50% of the sample. Values were only approved as real microbial turnover rates if the subtraction of twice the standard deviation of all the blanks of one batch from each sample value still resulted in positive counts.

2.3. Noble Gas Measurements

[7] Water samples for noble gas analysis were collected immediately when the rosette arrived on deck and stored in gas-tight copper tubes for analysis after the expedition. Noble gas measurements were performed using a specialized vacuum extraction and purification line combined with mass spectrometric analysis [Beyerle et al., 2000].

2.4. Bacterial Abundance and FISH

[8] Bacterial abundance was determined by epifluorescence microscopy (Zeiss Axioscope 2, 1,000 magnification) of DAPI (4', 6-diamidino-2-phenylindole)-stained cells. For fluorescence in-situ hybridization (FISH), bacterial cells were fixed by the addition of concentrated formaldehyde solution (5% final concentration) for 15 min at room temperature and thereafter recovered by gentle vacuum filtration (20 and 50 mL for each sample) on to polycarbonate filters with a pore size of 0.2 μm (GTPB, Millipore). After washing with PBS and water, the filters were transferred into sterile PP petri dishes, sealed and stored frozen at −20°C for FISH. The protocol of Pernthaler et al. [2002] was used for the hybridization procedure. The following oligonucleotide probes (MWG, Germany) were used to describe the microbial communities: Arch915 for members of the domain Archaea; Eel MS 932 (ANME-2 group); ANME-1 862 (ANME-1 group), distantly related to Methanosarcinales [Boetius et al., 2000]; and MG84/705 and MA450, describing methanotroph groups I and II [Eller et al., 2001], respectively. Probes were labeled with the indocarbocyanine fluorescent dye CY3 and fluorescein (MWG, Germany).

3. Results and Discussion

[9] At the newly discovered active Vodyanitskiy mud volcano in the Sorokin Trough, at 1985 m water depth, a very prominent and constant bubble plume was found. Although the bubbles could be followed vertically by the echosounder system for more than 1000 m, methane concentrations measured in the water column in the vicinity of the plume were not significantly different from those measured in the water column at the reference sites (Figure 1). The reason for not being able to discriminate between the two sites is most likely the already very high background values. This confirms earlier results from deep Black Sea sites indicating that elevated methane concentrations above methane seeps occur only very close to the sediments [Bohrmann et al., 2003].

[10] To determine the methane partitioning between the bubbles and the surrounding water, we measured the distribution of noble gases in the water column. Except for helium, which can be supersaturated due to terrigenic input, the concentrations of dissolved atmospheric noble gases in lake and ocean water correspond closely to the equilibrium concentrations defined by the surface water temperature and salinity that prevailed during gas exchange with the atmosphere [Craig and Weiss, 1971; Kipfer et al., 2002]. Noble gases are chemically inert, and therefore any observed deviations from the initial equilibrium concentrations are interpretable in terms of purely physical processes. The presence of gas bubbles in the water
column stimulates gas exchange between the ascending gas phase and the surrounding water by gas stripping and dissolution (see Figure 2, right-hand panel) and therefore affects the local noble gas concentrations [Clark et al., 2003; Leifer and Patro, 2002; Wüst et al., 1992].

Figure 2 shows the observed atmospheric Neon (Ne) and Helium ($^4$He) concentrations for the deep reference and deep seep sites (depths > 800 m). Ne concentrations in the deep water are approximately constant with depth for each of the two profiles, but the mean Ne concentration determined in the plume is 3.2% lower than that determined for the reference profile. Since a similar depletion was observed in the $^4$He measurements, we conclude that gas exchange takes place between the rising bubbles and the surrounding water; i.e., that the gas bubbles strip dissolved Ne and He from the water. At the seep site, this stripping directly affects up to 1000 m of the water column and is in accordance to bubble processes that have been clearly documented at Coal Oil Point, off California [Clark et al., 2003]. It is important to note that the Ne depletion and methane dissolution observed are dependent on the timescales associated with horizontal and vertical mixing in the deep water of the Black Sea, and hence can be viewed as an integrative measure of the gas release occurring on these timescales.

Recent publications have shown high concentrations of $^{13}$C-depleted biphytanes and glyceryl dialkyl glyceryl tetraethers in the anoxic Black Sea water column below 700 m, which clearly indicate the involvement of archaea in the anaerobic oxidation of methane [Schouten et al., 2001; Wakeham et al., 2003]. However, the actual organisms mediating the anaerobic oxidation of methane in the Black Sea water column are still unknown.

To identify the community responsible for the oxidation of methane in the gas plume we used 16S rRNA-targeted oligonucleotide probes. Using Arch 915, a general archaeal probe, a 25% higher total archaeal cell count was found in the anoxic water column in the vicinity of the bubble plume than at the equivalent reference site. An archaeal community closely related to *Methanosarcina*, called ANME-2, together with sulfate-reducing bacteria, was found to be responsible for the anaerobic oxidation of methane in sediments from Hydrate Ridge off the coast of Oregon [Boetius et al., 2000]. ANME-1 and ANME-2 cells have been detected in the sediments at seep sites in the Santa Barbara Basin and the Eel River Basin off the coast of California [Hinrichs et al., 1999; Orphan et al., 2001], whereas in a Black Sea reef structure, mainly ANME-1 was found [Michaelis et al., 2002]. Methane oxidation took place at both sites with similar methane oxidation rates between
0.03 to 3.1 nM d^{-1} and we therefore tried to identify the community involved in anaerobic methane oxidation. Using 16S rRNA-targeted oligonucleotide probes specific to both groups, we were able to detect ANME-1 and ANME-2 cells in the water column, which had previously been described only in sediments. Cell counts of filters from the water column above the methane seep site revealed ANME-1 and ANME-2 cells at concentrations of up to 3% of all DAPI stained cells (Figure 1). At the deep reference site, cell counts detected ANME-1 and ANME-2 cells at lower concentrations of only 1 to 2%. Additionally, the distribution of the archaeal cells over the water column was different between the reference and the seep site. Whereas at the reference station the cells are rather homogenously distributed over the whole anoxic water column, at the seep site the archaeal abundance is higher exactly at the depth horizon where the methane plume could be observed with acoustical means, i.e., from the seafloor to approximately 1000 m water depth (Figure 1). In general, bacteria are attached to interfaces, i.e., to solid/liquid or water/gas phase boundaries. Of these, the gas/water interface is especially important for bacteria, since enrichment of hydrophobic molecules like proteins, lipids, and carbohydrates also occurs there [Kjelleberg and Stenstrom, 1980; Norkrans and Sorensson, 1977]. Our data imply that in the Black Sea water column, ANME-1 and ANME-2...
cells (and presumably also other archaea attached to the gas bubbles) are released from the seep-related sediment and injected into the water column. Such interpretation is supported by the fact that the abundance of total archaeal cells, and specifically ANME-1 and ANME-2 cells, found in the water column in the vicinity of the bubble plume is higher than that found at the reference site and that the higher abundance exactly follows the height of the methane plume.

Although the seeps appear to currently add microorganisms to the water column, it remains an open question whether the deep bubble plumes had in the past, and still have today, the capacity to inoculate the entire anoxic water body of the Black Sea with methane-oxidizing archaea. The $^{13}$C-depleted biomarkers mentioned above as having been detected in the anoxic water column of the central Black Sea [Schouten et al., 2001; Wakeham et al., 2003], an area where no seeps have yet been found, suggest that there are other methane-transforming archaea in addition to ANME-1 and ANME-2 awaiting discovery.

In contrast to the deep sites, where the additional methane influx from seeps is difficult to detect, the methane excess at the shallow seep site was very pronounced, with concentrations that were on average 10 times higher than at the reference site (Figure 3a). This excess is comparable to the situation at Coal Oil Point seep [Clark et al., 2000]. Additionally, methane oxidation rates at the seep site (0.02 to 1.6 nM d$^{-1}$) were approximately 30 times higher than at the reference site (0.001 to 0.05 nM d$^{-1}$), indicating that a methanotrophic community lives in the oxic environment affected by the bubble plume. Further evidence for enhanced microbial activity comes from the carbon isotope composition of the dissolved methane. Carbon isotope values at the shallow seep site ($-66.1\%$ VPDB) are characteristically depleted with respect to those at the reference site ($-58\%$ VPDB, Figure 3b), clearly indicating that gas seeps inject methane into the overlying water. Additionally, the decrease in methane concentration due to oxidation is associated with a carbon isotope effect that results in the remaining methane being enriched with $^{13}$C [Barker and Fritz, 1981] which can be clearly seen in Figure 3b ($-66.1$ to $-48.7\%$ VPDB from above the sediment to the uppermost 5 m water depth). Accordingly, using oligonucleotide probes for methanotrophic bacteria types I and II [Eller et al., 2001], up to 4% and 2.2%, respectively, of all DAPI stained cells could be identified as methanotrophic bacteria (Figures 3c and 3d). Methanotrophic bacteria of type II were detected only in the deepest sample at the seep site. Type I methanotrophs were rather uniformly distributed with numbers $< 0.5\%$ at both shallow sites with the exception of one sample. Since no higher methanotrophic bacterial numbers were detected in the water column in the vicinity of the bubble plume, higher oxidation rates must be a result of higher metabolic rates in the community above the seep site.

Reeburgh et al. [1991] have estimated that $2.9 \times 10^{11}$ mol CH$_4$ are formed in the sediments of the shelf and slope of the Black Sea and that $\sim 99\%$ is oxidized anaerobically in the euxinic water column. In contrast, pore water methane in sediment cores from the NW shelf and slope is anaerobically oxidized by sulfate and never reaches the water column [Jørgensen et al., 2001]. Furthermore, flux chamber experiments showed only a negligible contribution of methane from the NW shelf sediments [Friedl et al., 1998; Friedrich et al., 2002]. The methane contribution from the sediments would therefore seem to be rather small, implying that methane emanating from seeps located on the abyssal plain, shelf and slope regions must be an important term in the methane budget of the Black Sea.

Twice as much methane was detected in the uppermost 20 m of the water column at the shallow seep site than at the reference site (Figure 3a, inset). Surface waters above shallow seeps are therefore methane-enriched, and emit this greenhouse gas directly into the atmosphere. An enrichment of methane in the water column above seep stations with a subsequent release to the atmosphere has also been described recently by Schmale et al. [2005]. However, higher methane oxidation rates demonstrate that microbes associated with the bubble plume are catching up with the higher methane concentrations. Our findings therefore suggest that slow dissociation of gas hydrates due to warmer bottom waters will not result in dramatic climate changes. On the other hand, the huge amount of methane released from gas hydrates as the result of a sudden tectonic event as proposed by the “clathrate gun hypothesis” [Kennett et al., 2000] will certainly exceed the oxidation capabilities of the microbial community, thus leading to an increase in atmospheric methane concentrations.

Higher mean surface water methane concentrations at the deep seep site, and hence higher rates
of methane emission to the atmosphere are not observed and are also not expected due to modeling results (D. F. McGinnis et al., The fate of rising methane bubbles in stratified waters: What fraction reaches the atmosphere?, submitted to *Journal of Geophysical Research*, 2005). Although caution has to be exercised in drawing any final conclusions, we conclude that as long as the mixing dynamics of the Black Sea are strongly reduced by the prominent chemocline, methane from deep plumes will not reach the atmosphere and will therefore not result in higher atmospheric methane concentrations.

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