Anaerobic methanotrophic community of a 5346-m-deep vesicomyid clam colony in the Japan Trench

J. Felden, S. E. Ruff, T. Ertefaï, F. Inagaki, K.-U. Hinrichs and F. Wenzhöfer

Abstract

Vesicomyidae clams harbor sulfide-oxidizing endosymbionts and are typical members of cold seep communities where active venting of fluids and gases takes place. We investigated the central biogeochemical processes that supported a vesicomyid clam colony as part of a locally restricted seep community in the Japan Trench at 5346 m water depth, one of the deepest seep settings studied to date. An integrated approach of biogeochemical and molecular ecological techniques was used combining in situ and ex situ measurements. In sediment of the clam colony, low sulfate reduction rates (maximum 128 nmol mL\(^{-1}\)/day) were coupled to the anaerobic oxidation of methane. They were observed over a depth range of 15 cm, caused by active transport of sulfate due to bioturbation of the vesicomyid clams. A distinct separation between the seep and the surrounding seafloor was shown by steep horizontal geochemical gradients and pronounced microbial community shifts. The sediment below the clam colony was dominated by anaerobic methanotrophic archaea (ANME-2c) and sulfate-reducing Desulfobulbaceae (SEEP-SRB-3, SEEP-SRB-4). Aerobic methanotrophic bacteria were not detected in the sediment, and the oxidation of sulfide seemed to be carried out chemolithoautotrophically by Sulfurovum species. Thus, major redox processes were mediated by distinct subgroups of seep-related microorganisms that might have been selected by this specific abyssal seep environment. Fluid flow and microbial activity were low but sufficient to support the clam community over decades and to build up high biomasses. Hence, the clams and their microbial communities adapted successfully to a low-energy regime and may represent widespread chemosynthetic communities in the Japan Trench. In this regard, they contributed to the restricted deep-sea trench biodiversity as well as to the organic carbon availability, also for non-seep organisms, in such oligotrophic benthic environment of the dark deep ocean.

Received 30 August 2013; accepted 6 January 2014

Corresponding author: J. Felden. Tel.: +49 421 218 65598; fax: +49 421021865505; e-mail: jfelden@marum.de

*Present address: MARUM Center for Marine Environmental Sciences, University of Bremen, Bremen, Germany

Introduction

Cold seep communities establish where tectonic or gravitational forces push free gas, methane-rich pore water, and/or mud upward into sulfate-penetrated surface sediments (Boetius & Wenzhöfer, 2013). High energy availability at and near the sediment surface thereby supports enormous biomasses of chemosynthetic organisms such as siboglinid tubeworms, mytilid and vesicomyid bivalves, and giant sulfide-oxidizing bacteria (Sibuet & Olu, 1998; Levin, 2005; Grünke et al., 2012). These organisms are well adapted to access and use reduced compounds in seep sediments. For
instance, most vesicomyid clams have a reduced gut system and thus rely almost entirely on their autotrophic sulfide-oxidizing endosymbionts for nutrient and energy supply (Childress et al., 1993; Goffredi & Barry, 2002, and references therein). To access the sulfide, they dig with their foot several centimeters into the sediment (Dubilier et al., 2008), take the sulfide up, and transport it with their blood to the endosymbionts (Childress et al., 1993). Some vesicomyid species are able to accumulate amounts of sulfide in their body that exceed ambient concentrations more than 60-fold (Childress et al., 1993; Barry & Kochevar, 1998) and are thus found in habitats with a wide range of sulfide concentrations (0.6–20 mM; Barry et al., 1997; Decker et al., 2012; Pop Ristova et al., 2012). Bioturbation by the clams enhances the sulfate transport from the water column into the sediment, resulting in sulfate reduction (SR) at sediment depths that otherwise would be sulfate-limited (Wallmann et al., 1997; Levin et al., 2003; Treude et al., 2003). Hence, vesicomyid clams are able to populate seep sites of low geological activity, where sulfide is not found close to the sediment surface (Fischer et al., 2012).

In methane-enriched seep sediments, sulfide is a product of bacterial SR that is often coupled to the anaerobic oxidation of methane (AOM) mediated by consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB; Boetius et al., 2000). High densities of these microbial consortia have been described in seep sediments of all continental margins from shallow waters to the deep sea (Knittel & Boetius, 2009, and references therein). The occurrence, distribution, and activity of the microbes involved in AOM have been intensively studied using different molecular ecological tools and biogeochemical measurements (Boetius et al., 2009; Knittel & Boetius, 2009). So far, there are three main ANME clades ANME-1, ANME-2, and ANME-3 (Hinrichs et al., 1999; Niemann et al., 2006b), which contain several sub-clades, such as thermophilic ANME-1 (Holler et al., 2011), ANME-2a-c (Orphan et al., 2001), and the recently described Methanoperedenaceae (Haroon et al., 2013). The involved SRB are close relatives of either Desulfovocrina/Desulfococcus or Desulfobulbus (Knittel et al., 2003; Schreiber et al., 2010; Kleindienst et al., 2012). The different ANME clades can be distinguished using methods based on nucleic acids (Orphan et al., 2001; Knittel et al., 2005; Pernthaler et al., 2008) and membrane lipids (Hinrichs et al., 1999; Elvert et al., 2003; Rossel et al., 2011).

In the last decade, the improvement in deep-sea technologies such as remotely operated vehicles or submersibles enabled the scientific community to explore seep ecosystems in detail by performing focused sampling and in situ measurements. These in situ investigations have significantly increased our knowledge of the small-scale variability of biodiversity and of biogeochemical activities within and between seep ecosystems (Jørgensen & Boetius, 2007; Boetius & Wenzhöfer, 2013, and references therein). However, only a few studies exist in water depths >4000 m because it is a technological challenge to access these remote abyssal habitats for sampling and in situ measurements (Boetius & Wenzhöfer, 2013). It is known from the Nankai Trough or the Japan Trench that cold seeps occur frequently even down to water depths of at least 7500 m (Kobayashi, 2002; Arakawa et al., 2005, and reference therein). This tectonically active area hosts numerous seeps and the deepest known vesicomyid clam colonies at 6437 m (Sibuet et al., 1988; Ogawa et al., 1996; Fujikura et al., 1999). Japan Trench seeps offer a unique opportunity to study microbial community structure and biogeochemical processes at abyssal seep ecosystems as most seep studies have been conducted at shallower sites (Sibuet et al., 1988; Boetius & Wenzhöfer, 2013).

Although chemosynthetic clam colonies in the Japan Trench are known, detailed insights into the underlying biogeochemical processes and predominant microbial communities fueling these remote and high-biomass seep communities are sparse. Here, we combined analyses of sediment pore water chemistry, sediment–water interface exchange processes, as well as methane and sulfate turnover rate measurements with community analyses based on 16S rRNA genes and intact polar lipids (IPLs) to thoroughly investigate the biogeochemistry and microbial community. To our knowledge, this is the first and most comprehensive study on the functioning of an abyssal seep ecosystem using in situ activity measurements in the Japan Trench to date. Our main hypotheses were (i) the key biogeochemical processes in the sediment that fuel the spatially restricted clam colony are similar to those found at shallow seeps and (ii) the microbial community composition of this ecosystem differs from that of shallow seeps.

**MATERIALS AND METHODS**

**Seafloor observations and sampling**

During the cruise YK06-05 in 2006 with the RV *Yokouka* to the Japan Trench, we investigated a clam colony inhabited by *Abyssogena phascoliformis* (former known as *Calyptogena phascoliformis*) and *Isosevadon fosajaponicum* (former known as *Calyptogena fosajaponica*) at 5346 m water depth. The names of both species were adapted according to the most recent taxonomic studies of the family *Vesicomyidae* (Krylova & Sahling, 2010, and references therein) and the accepted nomenclature in the World Register of Marine Species ([http://www.marinespecies.org/](http://www.marinespecies.org/)). The targeted sampling and precise positioning of the in situ instruments were achieved with the manned research submersible *Shinkai 6500* (JAMSTEC, Nankoku, Kochi, Japan). Besides the well-defined vesicomyid clam
colonies present in this area of the Japan Trench, no other chemosynthetic communities, such as sulfide-oxidizing bacterial mats, were observed. The colonies, however, were associated with different groups of benthic organisms including actiniaria, holothurians, and tube-dwelling polychaetes. Typically, the clam patches were round with diameters ranging from a few decimeters to 2 m (Fig. 1B). Distances between the widespread colonies were a few tens of meters, and we observed several trails of moving clams during the dives.

One large vesicomyid clam colony (Fig. 1A; 39°6.3560′ N, 143°53.5619′ E) was studied in detail with microbiological and biogeochemical methods. In the following text, this particular Japan Trench clam colony is termed JTC colony. Sampling was first performed close to the rim of the JTC colony and then at the center (Fig. 1C). Immediately after sample recovery onboard, the sediment core was sub-sampled for *ex situ* rate measurements or preserved for later analyses.

**Geochemistry**

*Ex situ* pore water concentrations of sulfate and dissolved inorganic carbon (DIC) were measured, along with the concentrations and isotopic compositions of dissolved methane, total organic carbon (TOC) content, and turnover rates of sulfate as well as methane. In addition, *in situ* benthic oxygen uptake rates were determined with a microprofiler and a benthic chamber module. The data are available online via the Data Publisher for Earth & Environmental Science PANGAEA (doi:10.1594/PANGAEA.826602).

**Ex situ measurements**

To measure the concentrations of pore water constituents, push cores were sub-sampled in 1 cm intervals and pore water was extracted via sediment squeezing (Reeburgh, 1967; 0.45 μm Durapore Filter; Millipore, Bedford, MA, USA). For each sample depth, we obtained 1–5 mL pore water that was immediately preserved and stored at 4 °C until the measurements were taken in the home laboratory. To determine sulfate concentrations, 0.5–1 mL pore water was fixed in 1 mL 2% zinc acetate (ZnAc) solution. Samples were diluted and filtered before concentrations were determined by non-suppressed anion exchange chromatography (Waters IC-Pak anion exchange column, Waters 430 conductivity detector; Waters, Milford, MA, USA). For measuring DIC concentrations, the pore water was preserved with 20 μL saturated mercuric chloride (HgCl2) solution and stored headspace free. DIC content of the samples was measured by the flow injection method (detector VWR scientific model 1054) according to Hall & Aller (1992). Dissolved methane concentrations and isotopic compositions were determined with the headspace method according to Kvenvolden & McDonald (1986) and Ertefai et al. (2010) using gas chromatography and isotope ratio mass spectrometry, respectively. Carbon iso-}

© 2014 The Authors. Geobiology Published by John Wiley & Sons Ltd
standard deviations of δ^{13}C values were obtained from repeated measurements and were usually less than ±1.0‰.

Pyrite and carbonate content of the sediment was measured by X-ray refraction analysis as previously described (Ertefai et al., 2010). TOC contents were measured from dry and homogenized sediment samples using a Leco CS 200 analyzer (LECO, St. Joseph, MI, USA). Prior to the TOC analysis, the samples were treated with 12.5% hydrogen chloride (HCl) solution to remove any inorganic carbon.

Sulfate reduction and AOM were measured ex situ by the whole core injection method (Jørgensen, 1978). We incubated the samples at in situ temperature (1.5 °C) for 48 h with either ^14^CH_4 (dissolved in water, 2.5 kBq) or carrier-free ^35^SO_4 (dissolved in water, 50 kBq). Sediment was fixed in 25 mL sodium hydroxide (NaOH) solution (2.5%, w/v) or 20 mL ZnAc solution (20%, w/v) for AOM or SR, respectively. Turnover rates were measured as previously described (Treude et al., 2003; Kallmeyer et al., 2004).

In situ measurements
Total oxygen uptake (TOU) and diffusive oxygen uptake (DOU) were measured at the center and the rim of the JTC colony, respectively. The difference between TOU and DOU is commonly dedicated to faunal-mediated consumption, including bioirrigation and bioturbation as well as the animal respiration itself (Glud, 2008 and references therein). TOU of the JTC colony center was determined with a small cylindrical benthic chamber module, which enclosed a sediment area of 284 cm^2 (radius = 9.5 cm) together with 15 cm of overlying bottom water (equivalent to approximately 5 L). Two Clark-type minielectrodes continuously recorded the oxygen concentration of the enclosed water body during the incubation (Treude et al., 2009). Sensors were calibrated against bottom water oxygen concentration (determined from Winkler titration) and a zero reading recorded at in situ temperature on board. TOU (mmol m^{-2} day^{-1}) was calculated from the initial linear change in oxygen concentration vs. time (for more details see Wenzhöfer & Glud, 2002).

Oxygen penetration depth and DOU at the rim of the clam colony were measured with a small deep-sea microprofiler module (Treude et al., 2009), carrying three oxygen Clark-type microelectrodes (Revsbech et al., 1983) and one temperature sensor (Pt100; UST Umweltsensorentechnik GmbH, Geschwenda, Germany). High-resolution microprofiles across the sediment-water interface were measured with a vertical resolution of 100 μm on a total length of 15 cm. Oxygen electrodes had a linear response to the oxygen concentration in seawater and were calibrated in situ using constant readings in the bottom water (oxygen concentration determined by Winkler titration) and the anoxic parts of the sediment (Wenzhöfer et al., 2000; De Beer et al., 2006). DOU (mmol m^{-2} day^{-1}) was calculated from the measured microprofiles and Fick’s first law of diffusion with DOU = D_0 × (dC/dz), where D_0 (1.26 × 10^{-9} m^2 s^{-1}) is the molecular diffusion coefficient in water corrected for temperature and salinity (Li & Gregory, 1974), C (μM) is the solute concentration, and z (m) is the depth within the diffusive boundary layer (Rasmussen & Jørgensen, 1992).

Microbial community analysis
IPL analyses
Before intact and free cell membrane constituents were analyzed by liquid and gas chromatography, freeze dried sediment was spiked with internal standards and lipids were extracted using a modified Bligh and Dyer method (Sturt et al., 2004). The total lipid extract was separated chromatographically on a glass column using 3 g of silica gel (60 mesh) into three fractions: a non-polar fraction (dichloromethane), a glycolipid fraction (acetone), and a phospholipid fraction (methanol). The phospholipid fractions were analyzed for IPLs, which were analyzed by high performance liquid chromatography/electrospray ionization-multiple stage-mass spectrometry (HPLC/ESI-MS^n) as previously described (Sturt et al., 2004). The non-polar fractions were further separated for gas chromatography analyses, following standard protocols for separation, derivatization, and transesterification (Elvert et al., 2000, 2003) described in detail by Ertefai et al. (2008).

16S rRNA GENE ANALYSES
To analyze the microbial community composition, we constructed archael and bacterial 16S rRNA gene libraries of sediments from the center and the rim of the JTC colony. On board, sediment cores were sectioned into 1–5 cm intervals and frozen at 20 °C. Total community DNA was retrieved from 5 g of sediment (pooled from the 0 to 10 cm depth horizon) by chloroform extraction as described by Zhou et al. (1996) and purified using the Wizard DNA clean-up system (Promega, Madison, WI, USA). PCRs for 16S rRNA gene libraries were carried out using the Master Taq polymerase (Eppendorf, Hamburg, Germany), 26–30 cycles and the bacterial primers GM3/GM4 (Muyzer et al., 1995) or archael primers Arch20F/Uni1392R (Lane et al., 1985; Massana et al., 1997). Purification of PCR products, cloning reactions, and the sequencings of inserts were performed as previously described (Niemann et al., 2006a), and chimeric sequences were removed using Mallard (Ashelford et al., 2006). The 16S rRNA gene sequences were aligned with SILVA INcremental Aligner (SINA; Prüse et al., 2007) and manually optimized according to the secondary structure. Phylogenetic classification was carried out using the ARB
software package (Ludwig et al., 2004) based on the SILVA small subunit 16S rRNA reference sequence database (SSUREF v111; Quast et al., 2013). Phylogenetic trees were calculated with the maximum likelihood algorithm PHYLML (100 bootstraps) and a positional variability filter as described before (Ruff et al., 2013). Operational taxonomic units at 98% 16S rRNA gene identity (OTU0.02) and Chao1 richness estimates were calculated using the software MOTHUR v1.24 (Schloss et al., 2009). The nucleotide sequences reported in this paper have been archived in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers HG425384–HG425704.

RESULTS

Sediment solid phase

The recovered sediment cores were visually differentiated into upper (0–10 cm below seafloor – cmbsf), middle (10–25 cmbsf), and lower (>25 cmbsf) sections (Fig. 2A). The upper 10 cm showed a light brown color and were characterized by living vesicomyid clams being partly buried into the slightly sandy sediment. The middle section of the core was black with broken shells, and a sulfidic smell was noticed during subsampling. Below 25 cm depth, the sediment was of uniform gray color. The differentiation of the sediment into different horizons was also reflected in the pyrite, carbonate, and TOC contents of the sediment (Fig. 2B). In the upper sediment horizon (0–10 cm), pyrite (FeS₂) was absent and carbonate was low (5–7 wt-%). In the middle section, the amount of pyrite and carbonate increased to up to 8 and 32 wt-%, respectively. The carbonate content declined again in the lower section in contrast to pyrite, which reached values of up to 12 wt-%. TOC content in the sediment was constant in the upper 15 cm (approximately 1.7 wt-%), decreased in the middle section of the core (14–18 cmbsf), and remained constant again in the lower section (Fig. 2B).

Sediment geochemistry

Pore water geochemistry

In the center and at the rim of the JTC colony, sulfate, DIC, and dissolved methane concentrations as well as the methane isotopic composition were determined. In the center of the clam patch, sulfate concentration decreased to <1 mM at 12 cmbsf (Fig. 3A). The DIC concentration profile showed an opposite behavior to the sulfate profile, as it first increased with depth and then stayed nearly constant at more than 100 mM below 10 cmbsf. In contrast, at the JTC colony rim, sulfate penetrated deeper into the sediment (18 cmbsf) as compared to the center (Fig. 3), and the maximum DIC concentration (approximately 100 mM) was found at 31 cmbsf. Dissolved methane was analyzed in all three lithostratigraphic horizons (Fig. 3). Concentrations and isotopic compositions varied with sediment depth and differed between sampling spots. The center revealed higher dissolved methane concentrations than the rim, with a maximum between 25 and 33 cmbsf (Fig. 3A). At the center, values of dissolved methane ranged from −84 to −79‰. The highest δ¹³C values were found in the middle section of the core at 15–20 cmbsf. At the rim, the dissolved methane was less depleted in ¹³C (δ¹³C values of −72 to −66‰; Fig. 3B).

Methane oxidation and SR rates

Sulfate consumption was measured at the rim and at the center of the JTC colony, whereas methane turnover could only be quantified in the rim sediment (Fig. 3). At the center, SR values were scattered over the investigated depth horizon and ranged from 16 to 128 nmol mL⁻¹ day⁻¹. The averaged depth integrated SR rate (0–16 cm) was 6.3 mmol m⁻² day⁻¹. At the colony rim, sulfate turnover was lower (1.4–64 nmol mL⁻¹ day⁻¹) with a maximum at about 5 cm below seafloor and decreased with increasing sediment depth. Horizontal distribution of methane consumption at the rim was similar to SR rates with values ranging from 2 to 52 nmol mL⁻¹ day⁻¹. The average depth (0–16 cm below seafloor)-integrated turnover rates of methane and sulfate at the rim were in the same range with 2.4 (n = 3) and 2.1 (n = 3) mmol m⁻² day⁻¹, respectively.
In situ oxygen uptake measurements

The microprofiler module was placed at the sediment next to the JTC colony, because a direct placement of the fragile glass sensors in the JTC colony was not possible. Approximately 20 cm beside the colony rim, the average oxygen penetration depth was 1.64 cm (n = 3) with an average DOU of 1.9 mmol m⁻² day⁻¹ (Fig. 4). Temperature remained constant at about 1.3 °C for the entire profiling length, and thus no heat flow was observed. In contrast to the microprofiler, the benthic chamber was
placed directly on the clams, enclosing about 20 clams. A TOU of 21 mmol m$^{-2}$ day$^{-1}$ was measured, which is one order of magnitude higher than the DOU outside the colony. Assuming that the DOU represents the benthic oxygen consumption of the sediment in the Japan Trench at 5346 m, we calculated the oxygen consumption related to the benthic chemosynthetic community (CCOU) by subtracting DOU from TOU (CCOU = TOU − DOU). For our investigated JTC colony, this resulted in a community consumption of 19 mmol O$_2$ m$^{-2}$ day$^{-1}$. The chamber enclosed a sediment area of 0.0284 m$^2$, populated by approximately 20 clams, which resulted in a clam density of approximately 700 clams m$^{-2}$. Thus, one clam consumed about 27 µmol oxygen per day.

**Microbial community**

**Biomarker analyses**

Analyses of microbial lipids as both intact polar membrane lipids and free lipids were performed using sediment from below the clams in the center of the JTC colony (Fig. 5). The HPLC-MS$^n$ analysis revealed phosphate-based IPLs in the form of hydroxyarchaeol (OH-Ar) with phosphatidylglycerol (PG) and phosphatidylserine (PS) as polar head- groups. Both IPL types increased with sediment depth from 0.1 to 2.8 µg g$^{-1}$ dry sediment and were most abundant in the sediment horizon between 12 and 18 cm (2.8 µg g$^{-1}$) before their concentration declined to 0.5 µg g$^{-1}$ with sediment depth (Fig. 5). Bacterial dietherglycerolipids (DEG), occurring as phosphatidylethanolamine (PE) and PS, were absent in the upper 6 cm, but increased with sediment depth and peaked in the sediment horizon at 12–18 cm (0.6 µg g$^{-1}$) and then declined to 0.1 µg g$^{-1}$ dry sediment. The free (not intact) lipids included OH-Ar and monoalkyl glycerol ethers (MAGE). The δ$^{13}$C values of free lipids varied with sediment depth, and the most strongly δ$^{13}$C-depleted lipids were present at 12–18 cm below seafloor (Fig. 5).

**Phylogenetic diversity**

Sequencing of selected clones from 16S rRNA gene libraries resulted in a total of 147 archaial and 173 bacterial sequences (Fig. 6) from the surface sediment (0–10 cm) of the JTC colony center and the rim.

The archaial community of the center seemed to be extremely low in diversity (Fig. 7A), because we only
### Fig. 6 Relative 16S rRNA clone frequencies.

Archaeal and bacterial diversity in the center and at the rim of the JTC colony. The scale bar represents relative clone frequencies in percent. The total number of clones per gene library is indicated below the respective column. DSHVG, Deep Sea Hydrothermal Vent Group, MBGB, Marine Benthic Group B, MG1, Marine Group 1.

#### Table: Bacterial and Archaeal Diversity

| Bacteria | Archaea |
|----------|---------|
| **Center** | **Rim** |
| Bacteria | Archaea |
| Desulfofotaberiales | Desulfarculales |
| Campylobacterales | Thiotrichales |
| Pseudomonadales | Clostridiales |
| Other Bacteria | **ANME-2c** |
| **ANME-2a-2b** | Methanococcusidaes |
| DSHVG 6 | MBGB |
| n = 84 | n = 68 |

| Bacteria | Archaea |
|----------|---------|
| **Center** | **Rim** |
| Bacteria | Archaea |
| JTC Colony Rim clone Arch 109, HG425390 | JTC Colony Rim clone Arch 92, HG425459 |
| JTC Colony Rim clone Arch 10, HG425426 | JTC Colony Rim clone Arch 26, HG425427 |
| JTC Colony Rim clone Arch 50, HG425448 | JTC Colony Rim clone Arch 160, HG425572 |
| JTC Colony Rim clone Arch 129, HG425406 | JTC Colony Rim clone Arch 34, HG425431 |
| JTC Colony Rim clone Arch 201, HG425561 | JTC Colony Rim clone Arch 202, HG425501 |
| Methanococcusidaes | JTC Colony Rim clone Arch 203, HG425502 |
| **ANME-2c** | **ANME-2a-2b** |
| **Methanococcoides** | **Methanococcoides** |
| **DSHVG 6** | MBGB |
| n = 89 | n = 79 |

### Fig. 7 Phylogenetic affiliation of Archaea (A), Alpha-, Gamma-, and Epsilonproteobacteria (B) and Deltaproteobacteria (C) of the JTC colony center (red) and colony rim (blue) sediments based on 16S rRNA gene sequences. The scale bars represent 10% estimated sequence divergence.

© 2014 The Authors. *Geobiology* Published by John Wiley & Sons Ltd
**Psychrobacter frigidicola**, AJ609556
JTC Colony Rim clone Bac 85, HG425541
JTC Colony Rim clone Bac 80, HG425536
**Psychrobacter articulus** 234-4, AY444822
JTC Colony Rim clone Bac 91, HG425545
**Psychrobacter cryohalolentis** K5, CP000323
**Psychrobacter nivimaris**, AJ313425
JTC Colony Rim clone Bac 27, HG425484
**Psychrobacter arcticus** 273-4, AY444822
JTC Colony Rim clone Bac 80, HG425536
**Psychrobacter celer**, AY842259
JTC Colony Rim clone Bac 100, HG425463

**Vibrionales**
**Methylococcales**
JTC Colony Rim clone Bac 72, HG425682
**Leucothrix mucor**, X87277
JTC Colony Rim clone Bac 50, HG425666
**Arctic sediment clone Sva0164**, AJ240988
JTC Colony Rim clone Bac 80, HG425467
**Hydrothermal sediment clone 285-48**, FN553626
JTC Colony Rim clone Bac 80, HG425666

**Thiomargarita**
**Roseobacter**
JTC Colony Rim clone Bac 91, HG425545
JTC Colony Rim clone Bac 92, HG425698

**Arcobacter**
95 – 100% bootstrap support
70 – 94% bootstrap support
Japan Trench Clam Colony Rim
Japan Trench Clam Colony Center

Vesicomyid clam colony in the Japan Trench
obtained four OTU0.02, too few to reasonably estimate Chao1 richness. The OTU0.02 belonged to the ANME-2c clade (88% of all clones), the ANME-2a clade (1%), and the genus Methanococoides (1%). The bacterial gene library of the center was more diverse (Chao1 = 41 OTU0.02) and was dominated by deltaproteobacterial SRB of the orders Desulfobacterales (26%) and Desulfarcitales (4%). The diversity of sulfate reducers was high, including members of the genera Desulfarculaceae, Desulfohabluca, Desulforhopalus, and Desulfofotobacterium (Fig. 7C). Interestingly, we did not detect sequences of the SEEP-SRB-1 clade, which is common at seep ecosystems, instead the SRB community seemed to be dominated by Desulfobacterales (20%), such as SEEP-SRB-3 and SEEP-SRB-4. The sulfur-oxidizing community seemed to be dominated by chemolithoautotrophic Sulfurovum species (14%) within the Epsilonproteobacteria, because sequences of Thiococcales (approximately 1%) were rare. Additional clades included Planctomycetes and the candidate divisions JS1, OD1, and Hyd24-12 (Fig. S2).

The archaeal community of the JTC colony rim appeared to be more diverse than that of the center (Chao1 = 12 OTU0.02). Most sequences belonged to the genus Methanococoides (63%), followed by ANME-2c archaea (18%). In addition, we found members of Marine Benthic Group B (14%), Deep-Sea Hydrothermal Vent Group 6 (4%), and Marine Group 1 (approximately 1%). The bacterial gene library of the rim sediment (Chao1 = 25 OTU0.02) was greatly dominated by Psychrobacter (51%) of the order Pseudomonadales, showing a high microdiversity (Fig. 7B) and also included many Clostridiales (Fig. S2) and several sequences of a SEEP-SRB-4 organism. The bacterial libraries of the rim and center contained distinct populations of Flavobacterales and other clades (Figs S1 and S2). Remarkably, the seep sediments seemed to lack clades that are common to many seep sites worldwide, such as SEEP-SRB-1, Methylococcales, ANME-1, and Thermoplasmatales.

**DISCUSSION**

**Seepage intensity at a Japan Trench clam colony**

The distribution of clams at seeps is strongly controlled by the biogeochemical processes in underlying sediments, which are influenced by the supply of methane-rich fluids from the subsurface. Upward flow of hydrocarbon-rich fluids through thrust faults (Kobayashi, 2002) at fracture zones of the Japan Trench has been described at sites, where vesicomyid clam colonies with sharp boundaries have been detected (Juniper & Sibuet, 1987; Sibuet et al., 1988; Ogawa et al., 1996). Oxygen and temperature profiles obtained in this study point also to a locally focused release of seep fluids at the investigated JTC colony. The measured oxygen penetration depth of 1.6 cm (Fig. 4) and the corresponding low benthic oxygen consumption rate indicated that sediments a few centimeters away from the colony rim were similar to non-seep influenced sediments (Wenzhöfer & Glud, 2002; De Beer et al., 2006). At typical cold seep habitats, oxygen penetration into the sediment is usually limited to the top few millimeters (Lichtschlag et al., 2010; Felden et al., 2013), and is reduced even at vesicomyid clam sites that exhibit bioturbating activity (Levin et al., 2003). Moreover, a straight temperature profile (Fig. 4) indicated that fluid flow from the deep subsurface was not detectable next to the colony rim. At active seep sediments, upward fluid flow is indicated by increasing temperatures with increasing depths (Feseker et al., 2008). Such temperature gradients have been recorded for numerous clam colonies, for example at the Nankai Trough (Kobayashi, 2002) and at the Peruvian margin (Olu et al., 1996). Unfortunately, we could not measure sediment temperature profiles directly below the clam patch. However, the concave-shaped sulfate concentration profile and the methane concentrations measured at the colony center (Fig. 3) indicated a low seepage activity nourishing the clam community. Methane δ13C values of <-80‰ at the JTC colony center (Fig. 3) suggested biogenic methane formation from CO2 and H2 rather than a deep subsurface thermogenic origin (Whiticar, 1999).

Alternatively, the JTC colony could have also developed, because methane-rich and sulfate-free deep sediment layers were suddenly exposed to oxygenated and sulfate-rich bottom waters as proposed for the Monterey Canyon seep ecosystems (Paull et al., 2005). There, chemosynthetic benthic communities are most common on steep slopes where seafloor erosion occurs and tectonically driven fluid flow is lacking (Paull et al., 2005). Erosion of the sediment in the Japan Trench might have happened when the upper sediment layer was removed during one of the regularly occurring earthquakes (Kobayashi, 2002; Kawagucci et al., 2012), however, our data and dive observations do not indicate such sediment instabilities for the investigated site.

**Biogeochemical processes in sediments of the Japan Trench clam colony**

To investigate whether there are also similarities to shallow seeps concerning the underlying biogeochemical processes, we analyzed methane and sulfate consumption rates. Fluid flow and associated methane availability at the JTC colony were rather low, but sufficient to maintain a dense seep community of living clams. The depth integrated rates of AOM and SR have a ratio close to one, indicating a close coupling of methane consumption and sulfide production (Treude et al., 2003; Niemann et al., 2006b), which constantly nourished the clams and their chemosynthetic symbionts.
Sulfate reduction rates of clam patches at different seep ecosystems cover a wide range of turnover rates (Treude et al., 2003; Boetius & Suess, 2004; Pop Ristova et al., 2012), and seem to correlate with the methane availability in the sediment. Rates measured at the JTC colony (maximum 64 nmol mL$^{-1}$ day$^{-1}$) are in the same range as those of a clam colony at the REGAB pockmarks (maximum 154 nmol mL$^{-1}$ day$^{-1}$; Pop Ristova et al., 2012). But these values are nearly two orders of magnitude lower compared to Hydrate Ridge off Oregon, where SR rates of up to 3000 nmol mL$^{-1}$ day$^{-1}$ in combination with methane concentration of up to 10 mM have been found (Torres et al., 2002; Boetius & Suess, 2004). In fact, even lower methane concentrations sustain clam habitats (Barry et al., 1997; Wallmann et al., 1997; Cambon-Bonavita et al., 2009), which underlines the capability of Vesicomyidae clams to adapt to different environmental conditions. Low methane concentrations are not only an indicator for low seepage rates, they also result in lower sulfide availability within the sediment. Vesicomyidae clams are able to inhabit sites with low sulfide concentrations simply due to their ability to enrich sulfide in their body fluids above ambient concentrations (Childress et al., 1993; Barry et al., 1997; Barry & Kochevar, 1998). It also has been proposed that a continuous supply of sulfide is more important for these animals than the absolute concentration (Dubilier et al., 2008). Furthermore, clams could influence benthic biogeochemical processes similar to vestimentiferan tubeworms, which supply the microbial community close to their roots with sulfate and thus enhance locally the microbial sulfide production (Cordes et al., 2005). Furthermore, clams can move to sediments with higher sulfide concentrations as soon as sulfide is depleted at one location (Sibuet et al., 1988; Olu et al., 1996; Levin, 2005). In fact, such single moving clams were observed during our exploration. However, because the majority of clams were associated in patches (Fig. 1B), methane seepage and subsequent sulfide availability seemed to be sufficient to maintain the colonies.

At the JTC colony, the low methane concentrations in the surface sediments might not only be due to low seepage activity but could have also resulted from efficient methane consumption by the benthic filter. Indeed, we measured SR rates of 6.3 nmol m$^{-2}$ day$^{-1}$ in the sediment below the clam colony. If we assume that oxygen was used as the terminal electron acceptor for sulfate oxidation, which in turn is mainly produced by SR coupled to AOM, then TOU can be used to estimate the in situ methane consumption within the sediment. An oxygen uptake of 21 nmol m$^{-2}$ day$^{-1}$ would correspond to a methane consumption rate of 10.5 mmol m$^{-2}$ day$^{-1}$ based on the stoichiometric ratios of methane to sulfide (1:1) and of sulfide to oxygen (1:2). Methane efflux measurements at other clam habitats indicated that the uprising methane is completely oxidized in the sediment (Sommer et al., 2006; Pop Ristova et al., 2012). Therefore, the methane flux from the deep subsurface for the entire JTC colony (diameter 1.8 m$^2$) would have been 19 mmol day$^{-1}$, which corroborated that seepage was relatively low compared to other clam habitats (Torres et al., 2002; Boetius & Suess, 2004; Sommer et al., 2006; Pop Ristova et al., 2012). This could be either a temporal effect because fluid flow may slightly vary over time at seeps (Olu et al., 1996) or the seep community of the JTC colony is well adapted to efficiently use a low, but constant methane supply to build up the observed high biomasses.

**Chemosynthetic seep community at the Japan Trench clam colony**

**Clams as bioengineers**

Vesicomyid clams rely on the biogeochemical processes in the sediment for their sulfide supply and at the same time strongly influence the benthic biogeochemical regime by bioirrigation and bioturbation (Wallmann et al., 1997; Fischer et al., 2012). Geochemical gradients (DIC, pyrite, calcium carbonate content) and turnover rates at the JTC colony showed an active community performing SR and AOM in the upper 10–15 cm of the sediment. However, we did not find a distinct production zone at the JTC colony, which is usually present at seep habitats of other associated organisms (Treude et al., 2003; Felden et al., 2010, 2013). The activity was rather spread throughout the sediment, within a depth range that was affected by the clams, which had an average body length of 15–17 cm and were buried up to four-fifths in the sediment. Clams and other seep-associated fauna, such as polychaete tubeworms, are known to enhance the availability of electron acceptors in deeper sediment horizons by bioirrigation, which results in a lowering of the sulfate methane transition zone (Wallmann et al., 1997; Levin et al., 2003; Treude et al., 2003; Fischer et al., 2012; Ruff et al., 2013). By this mechanism, competing chemosynthetic surface organisms are separated from their energy source over time (Fischer et al., 2012; Pop Ristova et al., 2012). At the JTC colony, the clams seemed to have successfully altered the sulfide availability within the sediment and thus other common members of cold seeps such as thiotrophic bacterial mats were not observed (Treude et al., 2003; Felden et al., 2010, 2013; Lichtschlag et al., 2010).

Using average growth rates of other vesicomyid clam species (Barry & Kochevar, 1998) and the measured shell sizes, we estimated an average age of 10–15 years for the living clams at the JTC colony. The mixture of living clams and empty shells suggested that the clam colony existed for more than 15 years; consequently, methane seepage has likely influenced this site at least for several decades. The reduced faunal diversity at the JTC colony dominated
by only two clam species indicated relatively stable spatial
and temporal environmental conditions (e.g., fluid flow),
because it was proposed that the reduction in ecological
iches and thus diversity results from habitat stability (Sib-
uet & Olu-Le Roy, 2002; Cordes et al., 2010). Thus, the reduced JTC colony diver-
sity as compared to other seep ecosystems is likely due to
the low fluid flow and not an effect of water depth. Con-
trastingly, the faunal abundance and diversity in non-seep
sediments decrease with depth (Rex et al., 2006; Wei et
al., 2010) as they rely on the organic carbon from the
photic zone (Smith et al., 2008). Deep cold seep com-
unities (e.g., this study, Kobayashi, 2002; Arakawa
et al., 2010) as they rely on the organic carbon input from
sediments decrease with depth (Rex et al., 2006; Wei et
al., 2010). Thus, the reduced JTC colony diver-
sity as compared to other seep ecosystems is likely due to
the low fluid flow and not an effect of water depth. Con-
trastingly, the faunal abundance and diversity in non-seep
sediments decrease with depth (Rex et al., 2006; Wei et
al., 2010) as they rely on the organic carbon from the
photic zone (Smith et al., 2008). Deep cold seep com-
unities (e.g., this study, Kobayashi, 2002; Arakawa
et al., 2010) as they rely on the organic carbon input from
sediments decrease with depth (Rex et al., 2006; Wei et
al., 2010) as they rely on the organic carbon from the
photic zone (Smith et al., 2008). Deep cold seep com-

Benthic microbial community in a low seepage, abyssal
clam habitat
We demonstrated the presence of an active microbial com-

munity at the JTC colony that couples SR to AOM by bio-
geochemoical measurements, lipid analyses, and 16S rRNA
gene analyses. In the sediment below the clams, the lipid
analyses revealed diagnostic biomarkers for AOM-specific
archaeal and bacterial groups. The depth trend of IPL con-
centrations indicated an increase of prokaryotic cell abund-
cances at the depth of the geochemical reaction zone, where
sulfate and methane were metabolized (Fig. 5). Archaeal-based IPLs such as PI-
and PG-OH-AR and high ratios of OH-Ar vs. Ar lipids strongly suggested a predom-
nance of ANME-2 archaea in the center of the JTC col-
yon (Niemann & Elvert, 2008; Rossel et al., 2008, 2011).

The gene library results indicated that the dominant clade
for anaerobic methane oxidation was ANME-2c, which is
supported by previous findings (Vossmeyer et al., 2012).
ANME-2c seems to preferentially occur in bioirrigated
sediments by clams, for example at Hydrate Ridge in the
Northeast Pacific and the REGAB pockmark in the Kongo
Basin (Elvert et al., 2005; Knittel et al., 2005; Pop Ristova
et al., 2012), and in low fluid flux regimes (Elvert et al.,
2005; Wegener et al., 2008). The ANME-2c organisms
that we found were closely related to those of other seeps
worldwide, indicating a global distribution. Hence, the
environmental niche of ANME-2c seemed to be deter-
mined by bioturbation and low methane seepage, rather
than water depth and geographic location.

In contrast to other studies (Knittel et al., 2003; Cam-
bon-Bonavita et al., 2009), we did not detect ANME-1 or
ANME-3 in sediments below vesicomyids (Fig. 7A). The
absence of ANME-1 at the JTC colony was previously
observed (Vossmeyer et al., 2012) and might be due to
the environmental requirements of this organism. The
upper sediment at the JTC colony was light brown (Fig. 2)
and pyrite was absent, suggesting oxygenation due to fau-
nal activity. ANME-1 seemed to be oxygen sensitive,
because they were absent at bioirrigated seeps of Hikurangi
margin (Ruff et al., 2013) and increased with sediment
depth and decreasing sediment irrigation at clam colonies
(Knittel et al., 2005; Rossel et al., 2011). Additionally,
ANME-1 appeared to be more sensitive to cold tempera-
tures than ANME-2 (Rossel et al., 2011). In contrast to
our findings, the presence of ANME-3 in the JTC sedi-
ments was reported previously based on T-RFLP using
*HhaI* (Vossmeyer et al., 2012). However, ANME-3 and
*Methanovuvido* spp. can not be distinguished with this
method because they are closely related and include organ-
isms that have the same restriction site for this enzyme
(not shown).

The presence of sulfate-reducing *Deltaproteobacteria*,
which include the partner SRB of ANME, was shown by the
¹³C-depleted IPL-derived bacterial lipids and the high
amounts of MAGE (Hinrichs et al., 2000; Teske et al.,
2002; Niemann & Elvert, 2008) in the center of the JTC
colony. Remarkably, we did not detect sequences of the
SEEP-SRB1 or SEEP-SRB-2 clades (Fig. 7C), which are
typically the syntrophic partner SRB of ANME-2 archaea
(Schreiber et al., 2010; Kleindienst et al., 2012). Instead,
we found many sequences of the clades SEEP-SRB-3 and
SEEP-SRB-4 within the *Desulfobulbaceae* (Fig. 7C). These
clades occurred as single cells in surface sediments of bacte-
rial mat- or clam-covered seeps (Knittel et al., 2003; Kle-
indienst et al., 2012) and decreased with increasing se-
sediment depth (Knittel et al., 2003). SEEP-SRB-3 were
also found at a clam colony in the Nankai trough (Li et al.,
1999a) and both clades occurred in bioirrigated seeps at
Hikurangi margin (Ruff et al., 2013). Hence, SEEP-SRB-3
and SEEP-SRB-4 might have an advantage over other SRB
clades in bioturbated and thus oxygenated sediments. The
occurrence of ANME-2c without their partner SRB indi-
cated that ANME-2c organisms were either associated to
other SRBs, or occurred as aggregates or single cells with-
out direct contact to SRBs (Knittel & Boetius, 2009), or
performed AOM without a partner SRB (Milucka et al.,
2012).

Unexpectedly, aerobic methylotrophic bacteria were not
detected in the sediment of the JTC colony, although they
are widespread in methane-rich ecosystems, especially in
disturbed or bioirrigated cold seep sediments (Inagaki
et al., 2004b; Lösckann et al., 2007; Tavormina et al.,
2008; Ruff et al., 2013) and contribute significantly to the
benthic methane and oxygen consumption (Felden et al.,
2010, 2013; Boetius & Wenzhöfer, 2013). Benthic oxygen
consumption at the JTC colony was in the same range as
sulfide production, which also indicated that aerobic methane oxidation was low or absent. In contrast, at the REGAB clam colonies benthic oxygen consumption was up to three orders of magnitude higher than sulfide production rates (Decker et al., 2012; Pop Ristova et al., 2012), which was assigned to aerobic methanotrophy (Pop Ristova et al., 2012).

Sulfide oxidation at the JTC colony seemed to be performed not only by the chemosynthetic vesicomyids, but also by *Sulfurovum* spp., which are sulfur oxidizers that were first isolated from hydrothermal vent sediments of the mid-Okinawa Trough (Inagaki et al., 2004a). Although we cannot exclude that elemental sulfur was present in the JTC colony sediment, their occurrence indicated that *Sulfurovum* organisms are also able to oxidize sulfide (Li et al., 1999a,b; Inagaki et al., 2002; Fang et al., 2006). *Thiotrichales* and *Arcobacter* spp., which are sulfur-oxidizing bacteria commonly detected at cold seeps (Omorgie et al., 2008; Grüne et al., 2011, 2012), did not seem to be important at the JTC colony.

The microbial community at the rim of the JTC colony differed greatly from the one at the center, despite a distance of only 30 cm and mirrored the sharp biogeochemical gradients and defined ecosystem boundaries. The sediment at the rim of the colony was dominated by psychrophilic *Gammaproteobacteria* and comprised clades that are common to deep-sea sediments, such as *Thaueraarchaeota* (Durbin & Teske, 2011). However, sequences of the Marine Benthic Group B, *Desulfobaterales*, ANME-2c, and *Methanoococoides*, which occur in methane-rich subsurface sediments (Biddle et al., 2006; Inagaki et al., 2006), were also found indicating that methane was at least occasionally present. Nevertheless, there was little community overlap between the sediments on species-level (98% 16S rRNA gene identity; Fig. 7A–C; Figs S1 and S2), corroborating distinct differences between these seafloor habitats.

CONCLUSION

In contrast to non-seep systems, a correlation of biodiversity and biomass with water depth was, so far, not found for methane seeps. However, piezophilic adaptations of the methane-oxidizing microbial community remain speculative, as only a few studies have been conducted below 5000 m water depth. Our investigation includes the first in-depth analysis of the microbial community structure and activity at an abyssal seep site. We could show that an abyssal clam colony in the Japan Trench was similar to the ones found at shallower depths, concerning the predominant biogeochemical processes, such as AOM, SR, and benthic oxygen consumption. The tight coupling of AOM and SR rates indicated that abyssal benthic methane filters are as efficient as those of shallow seeps. Our findings suggested that the environmental niche of the dominant ANME archaea and sulfate reducers may be determined by bioturbation of the clams and low methane seepage rather than by pressure or geographic location. However, other key functional populations, such as thiotrophs, differed from those found at shallow clam seeps, or seemed to be absent, indicating environmental filtering due to the extreme environment or dispersal limitation. We show that advances in deep-sea technology (Boetius & Wenzhöfer, 2013 and references therein) finally enable us to improve our limited knowledge about the biogeochemistry and microbiology of abyssal methane seeps, which occur frequently along deep-sea trenches and faults with active fluid flow (Sibuet & Olu, 1998; Tyler et al., 2002; Judd, 2003) and could be significant for the marine methane budget.

ACKNOWLEDGMENTS

We thank the members of the shipboard crew of the RV Yokosuka, the *Shinkai* 6500 team, and the shipboard scientific party for the excellent support during cruise YK06-05. We are grateful for the technical support from Martina Alisch, Viola Beier, Xavier Prieto Mollar, Gabriele Schüßler, and especially Gabriele Eickert. Special thanks to the ‘SeaTechs’ Axel Nordhausen, Marc Viehweger, Patrick Meyer, and Volker Asendorf for building and maintaining the *in situ* instruments. We thank Antje Boetius and Katrin Knittel for very helpful discussions during the preparation of the manuscript. This work was performed in the framework of the GEOTECHNOLOGIEN project MUMM II (03G0608C) funded by the German Ministry of Education and Research (BMBF) and German Research Foundation (DFG) as well as the Max Planck Society. The work of S.E. Ruff was supported by the Leibniz program of the DFG to Antje Boetius.

REFERENCES

Arakawa S, Mori M, Nogi Y, Sato T, Yoshida Y, Usami R, Kato C (2005) Cold-seep microbial communities are more abundant at deeper depths in the Japan Trench land slope. *Journal of Japanese Society for Extremophiles* 4, 50–55.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environment Microbiology* 72, 5734–5741.

Barry JP, Kochevar RE (1998) A tale of two clams: differing chemosynthetic life styles among vesicomyids in Monterey Bay cold seeps. *Cahiers de Biologie Marine* 39, 329–331.

Barry JP, Kochevar RE, Baxter CH (1997) The influence of pore-water chemistry and physiology on the distribution of vesicomyid clams at cold seeps in Monterey Bay: implications for patterns of chemosynthetic community organization. *Limnology and Oceanography* 42, 318–328.

Biddle JF, Lipps JS, Lever MA, Lloyd KG, Sorensen KB, Anderson R, Fredricks HF, Elvert M, Kelly TJ, Schrag DP, Sogin ML, Brenchley JE, Teske A, House CH, Hinrichs KU (2006)
Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3846–3851.

Boetius A, Suess E (2004) Hydrate Ridge: a natural laboratory for the study of microbial life fueled by methane from near-surface gas hydrates. *Chemical Geology* **205**, 291–310.

Boetius A, Wenzhöfer F (2013) Seafloor oxygen consumption fueled by methane from cold seeps. *Nature Geoscience* **6**, 725–734.

Boetius A, Ravensschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jørgensen BB, Witte U, Planckkuche O (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**, 623–626.

Boetius A, Holler T, Knittel K, Felsden J, Wenzhöfer F (2009) The seabed as natural laboratory: lessons from uncultivated methanotrophs. In *Uncultivated Microorganisms* (ed. Epstein S). Springer, Berlin/Heidelberg, pp. 59–82.

Cambon-Bonavita MA, Nadalig T, Roussel E, Delage E, Duperron S, Caprais JC, Boetius A, Sibuet M (2009) Diversity and distribution of methane-oxidizing microbial communities associated with different faunal assemblages in ingnant patchmark of the Gabon continental margin. *Deep-Sea Research Part II* **56**, 2248–2258.

Childress JJ, Fisher CR, Favuzza JA, Arp AJ, Oros DR (1993) The role of a zinc-based, serum-borne sulfide-binding component in the uptake and transport of dissolved sulfide by the chemosymbiotic sibutae containing clam *Calyptogena Clongata*. *Journal of Experimental Biology* **179**, 131–158.

Cordes EE, Arthur MA, Shea K, Arvidson RS, Fisher CR (2005) Modeling the mutualistic interactions between tubeworms and microbial consortia. *PLoS Biology* **3**, 497–506.

Cordes EE, Cunha MR, Galeron J, Mora C, Ou-Lee Roy K, Sibuet M, Van Gaever S, Vanreusel A, Levin LA (2010) The influence of geological, geochemical, and biogenic habitat heterogeneity on seep biodiversity. *Marine Ecology** **31**, 51–65.

De Beer D, Sauter E, Niemann H, Kaul N, Foucher JP, Witte U, Schlüter M, Boetius A (2006) In situ fluxes and zonation of microbial activity in surface sediments of the Håkon Mosby mud volcano. *Limnology and Oceanography* **51**, 1315–1331.

Decker C, Caprais JC, Kripouonoff A, Ou L (2012) First respiration estimates of cold-seep vesicomyid bivalves from in situ total oxygen uptake measurements. *Comptes Rendus Biologies* **335**, 261–270.

Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology* **6**, 725–740.

Durbin AM, Teske A (2011) Microbial diversity and stratification of South Pacific abyssal marine sediments. *Environmental Microbiology* **13**, 3219–3234.

Elvert M, Suess E, Greinert J, Whiticar MJ (2000) Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone. *Organic Geochemistry* **31**, 1175–1187.

Elvert M, Boetius A, Knittel K, Jørgensen BB (2003) Characterization of specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. *Geomicrobiology Journal* **20**, 403–419.

Elvert M, Hopmans EC, Treude T, Boetius A, Suess E (2005) Spatial variations of methanotrophic consortia at cold methane seeps: implications from a high-resolution molecular and isotopic approach. *Geobiology* **3**, 195–209.

Ertefai TF, Fisher MC, Fredricks HF, Lipp JS, Pearson A, Birgel D, Udert KM, Cavanaugh CM, Gschwend PM, Hinrichs KU (2008) Vertical distribution of microbial lipids and functional genes in chemically distinct layers of a highly polluted meromictic lake. *Organic Geochemistry* **39**, 1572–1588.

Ertefai TF, Heuer VB, Prieto-Mollor X, Vogt C, Sylva SP, Seewald J, Hinrichs KU (2010) The biogeochemistry of sorbed methane in marine sediments. *Geochimica et Cosmochimica Acta* **74**, 6023–6048.

Fang JS, Shizuka A, Katc C, Schouten S (2006) Microbial diversity of cold-seep sediments in Sagami Bay, Japan, as determined by 16S rRNA gene and lipid analyses. *FEMS Microbiology Ecology* **57**, 429–441.

Felden J, Wenzhöfer F, Feseker T, Boetius A (2010) Transport and consumption of oxygen and methane in different habitats of the Håkon Mosby Mud Volcano (HMMV). *Limnology and Oceanography* **55**, 2366–2380.

Felden J, Lichtschlag A, Wenzhöfer F, De Beer D, Feseker T, Ristova PP, De Lange G, Boetius A (2013) Limitations of microbial hydrocarbon degradation at the Nile mud volcano (Nile deep-sea fan). *Biogeosciences* **10**, 3269–3283.

Feseker T, Foucher JP, Harmegnies F (2008) Fluid flow or mud eruptions? Sediment temperature distributions on Håkon Mosby mud volcano, SW Barents Sea slope. *Marine Geology* **247**, 194–207.

Fischer D, Sahling H, Nothen K, Bohrmann G, Zabel M, Kasten S (2012) Interaction between hydrocarbon seepage, chemosynthetic communities, and bottom water redox at cold seeps of the Makran accretionary prism: insights from habitat-specific pore water sampling and modeling. *Biogeosciences* **9**, 2013–2031.

Fujikura K, Kojima S, Tamaki K, Maki Y, Hunt J, Okutani T (1999) The deepest chemosynthesis-based community yet discovered from the hadal zone, 7326 m deep, in the Japan Trench. *Marine Ecology Progress Series* **190**, 17–26.

Glud RN (2008) Oxygen dynamics of marine sediments. *Marine Biology Research* **4**, 243–289.

Golffredi SK, Barry JP (2002) Species-specific variation in sulfide physiology by closely related Vesicomyid clams. *Marine Ecology Progress Series* **225**, 227–238.

Grönke S, Felsden J, Lichtschlag A, Ginrth AC, De Beer D, Wenzhöfer F, Boetius A (2011) Niche differentiation among mat-forming, sulfide-oxidizing bacteria at cold seeps of the Nile Deep Seab (Eastern Mediterranean Sea). *Geobiology* **9**, 330–348.

Grönke S, Lichtschlag A, De Beer D, Felsden J, Salvan V, Rametec A, Schulz-Vogt HN, Boetius A (2012) Mats of psychrophilic thiotrophic bacteria associated with cold seeps of the Barents Sea. *Biogeosciences* **9**, 2947–2960.

Hall PO, Aller RC (1992) Rapid, small-volume, flow-Injection analysis for Sigma CO2 and NHa in marine and fresh-waters. *Limnology and Oceanography* **37**, 1113–1119.

Haroon MF, Hu SH, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan ZG, Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archael lineage. *Nature* **500**, 567–570.

Hinrichs KU, Hayes JM, Sylva SP, Brewer PG, Delong EF (1999) Methane-consuming archaeabacteria in marine sediments. *Nature* **398**, 802–805.

Hinrichs KU, Summons RE, Orphan V, Sylva SP, Hayes JM (2000) Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. *Organic Geochemistry* **31**, 1685–1701.

Holler T, Widdel F, Knittel K, Amann R, Kellermann MY, Hinrichs K-U, Teske A, Boetius A, Wegener G (2011) Thermophilic anaerobic oxidation of methane by marine microbial consortiant. *ISME Journal* **5**, 1946–1956.
Vesicomyid clam colony in the Japan Trench

Inagaki F, Sakihama Y, Inoue A, Kato C, Horikoshi K (2002) Molecular phylogenetic analyses of reverse-transcribed bacterial rRNA obtained from deep-sea cold seep sediments. *Environmental Microbiology* **4**, 277–286.

Inagaki F, Takai K, Nealson KH, Horikoshi K (2004a) *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the epsilon-Proteobacteria isolated from Okinawa Trough hydrothermal sediments. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1477–1482.

Inagaki F, Tsunogai U, Suzuki M, Kosaka A, Machiya H, Takai K, Nunoura T, Nealson KH, Horikoshi K (2004b) Characterization of C1-metabolizing prokaryotic communities in methane seep habitats at the Kuroshio Knoll, Southern Ryukyu Arc, by analyzing pmoA, mmoX, mxaF, mcrA, and 16S rRNA genes. *Applied and Environmental Microbiology* **70**, 7445–7455.

Inagaki F, Nunoura T, Nakagawa S, Teske A, Lever M, Lauer A, Suzuki M, Takai K, Delwiche M, Colwell FS, Nealson KH, Horikoshi K, D’hondt S, Jørgensen BB (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments, on the Pacific Ocean Margin. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2815–2820.

Jørgensen BB (1978) A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments I. Measurements with radiotracer techniques. *Geomicrobiology Journal* **1**, 11–27.

Jørgensen BB, Boetius A (2007) Feast and famine – microbial life in the deep-sea bed. *Nature Reviews Microbiology* **5**, 770–781.

Judd AG (2003) The global importance and context of methane escape from the seafloor. *Geo-Marine Letters* **23**, 147–154.

Juniper SK, Sibuet M (1987) Cold seep benthic communities in Japan subduction zones – spatial-organization, trophic strategies and evidence for temporal evolution. *Marine Ecology Progress Series* **40**, 115–126.

Kallmeyer J, Federman TG, Weber A, Fossing H, Jørgensen BB (2004) A cold chromium distillation procedure for radiolabeled sulfide applied to sulfate reduction measurements. *Limnology and Oceanography, Methods* **2**, 171–180.

Kawagucci S, Yoshida TT, Noguchi T, Honda MC, Uchida H, Ishibashi H, Nakagawa F, Tsunogai U, Okamura K, Takaki Y, Ishibashi H, Nakagawa F, Tsunogai U, Okamura K, Takaki Y, Hasegawa K, Nunoura T, Miyazaki J, Hirai M, Lin WR, Kitazato H, Takai K (2012) Disturbance of deep-sea environments induced by the mud volcano, Musashidake, in the Japan Trench. *Geo-Marine Letters* **32**, 271–278.

Kleindienst S, Ramette A, Kort R, Amann R (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments of the eastern Nankai accretionary wedge – an outcome of the 15 years’ KAIKO projects. *Marine Geology* **187**, 3–30.

Kaylova EM, Sahling H (2010) Vesicomyidae (Bivalvia): current taxonomy and distribution. *PlaSe One*, **5**, 107–132.

Kvenvolden KA, McDonald TJ (1986) Organic geochemistry on the JOIDES Resolution – an essay. *ODP Technology Note* **6**.

Lane DJ, Pace B, Ohen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955–6959.

Levin LA (2005) Ecology of cold seep sediments: interactions of fauna with flow, chemistry and microbes. In *Oceanography and Marine Biology – An Annual Review* (eds Gibson RN, Atkinson RJA, Gordon JDM). Taylor & Francis Group, London, UK, pp. 1–46.

Levin LA, Ziebis W, Mendoza GF, Growney VA, Tryon MD, Brown KM, Mahn C, Gieskes JM, Rathburn AE (2003) Spatial heterogeneity of macrofauna at northern California methane seeps: influence of sulfide concentration and fluid flow. *Marine Ecology Progress Series* **265**, 123–139.

Li YH, Gregory S (1974) Diffusion of ions in sea-water and in deep-sea sediments. *Geobiologia et Cosmochimica Acta* **38**, 703–714.

Li L, Guenzennec J, Nichols P, Henry P, Yanagibayashi M, Kato C (1999a) Microbial Diversity in Nankai Trough Sediments at a Depth of 3,843 m. *Journal of Oceanography* **55**, 635–642.

Li L, Kato C, Horikoshi K (1999b) Microbial diversity in sediments collected from the deepest cold-seep area, the Japan Trench. *Marine Biotecnology* **1**, 391–400.

Lichtschlag A, Felden J, Brichert V, Boetius A, De Beer D (2010) Geochemoical processes and chemosynthetic primary production in different thiotrophic mats of the Håkon Mosby Mud Volcano (Barents Sea). *Limnology and Oceanography* **55**, 931–949.

Lösekann T, Knittel K, Nadalig T, Fuchs B, Niemann H, Boetius A, Amann R (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Håkon Mosby mud volcano, Barents Sea. *Applied and Environment Microbiology* **73**, 3348–3362.

Ludwig W, Strunk O, Westram R, Richter I, Meier H, Yadukumar Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**, 1363–1371.

Massara R, Murray AE, Preston CM, Delong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Applied and Environment Microbiology* **63**, 50–56.

Milucka J, Federman TG, Polerecky I, Franzek D, Wegener G, Schmid M, Lieberwirth I, Wagner M, Widdel F, Kuyper MMM (2012) Zero-valent sulphur is a key intermediate in marine authotrophic ecosystems. *Nature** **491**, 541–546.

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic-relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel-electrophoresis of 16S rRNA fragments. *Archives of Microbiology* **164**, 165–172.

Niemann H, Elvert M (2008) Diagnostic lipid biomarker and stable carbon isotope signatures of microbial communities mediating the anaerobic oxidation of methane with sulphate. *Organic Geochemistry* **39**, 1668–1677.

Niemann H, Duarte J, Hensen C, Omoregie E, Magalhaes VH, Elvert M, Pinheiro LM, Kopf A, Boetius A (2006a) Microbial
methane turnover at mud volcanoes of the Gulf of Cadiz. *Geochimica et Cosmochimica Acta* **70**, 5336–5355.

Niemann H, Lösekann T, De Beer D, Elvert M, Nadalig T, Knittel K, Amann R, Sauter EJ, Schlüter M, Klages M, Foucher JP, Boetius A (2006b) Novel microbial communities of the Håkon Mosby mud volcano and their role as a methane sink. *Nature* **443**, 854–858.

Ogawa Y, Fujioka K, Fujikura K, Iwabuchi Y (1996) En echelon patterns of Calyptogena colonies in the Japan trench. *Geology* **24**, 807–810.

Olu K, Duperret A, Sibuet M, Foucher JP, Fialammedioti A (1996) Structure and distribution of cold seep communities along the Peruvian active margin: relationship to geological and fluid patterns. *Marine Ecology Progress Series* **132**, 109–125.

Omoregie EO, Mastalerz V, De Lange G, Staub KL, Kappler A, Roy H, Stadthitskaia A, Foucher JP, Boetius A (2008) Biogeochemistry and community composition of iron- and sulfur-precipitating microbial mats at the Chelten mud volcano (Nile Deep Sea fan, Eastern Mediterranean). *Applied and Environmental Microbiology* **74**, 3198–3215.

Orphan VJ, Hinrichs KU, Ussler W, Paull CK, Taylor LT, Sylva SP, Hayes JM, Delong EF (2001) Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Applied and Environmental Microbiology* **67**, 1922–1934.

Paull CK, Schlining B, Ussler W, Paduan JB, Caress D, Greene HG (2005) Distribution of chemosynthetic biological communities in Monterey Bay, California. *Geology* **33**, 85–88.

Perthaler A, Dekas AE, Brown CT, Goffredi SK, Embaye T, Olu K (1998) Microbial communities in methane- and hydrogen-oxidizing microbial mats from the DJF (Deep Sea) and TURC (Trench Ultima) hydrothermal vents (Gulf of Cadiz, Atlantic Ocean). *Applied and Environmental Microbiology* **64**, 5301–5308.

Prüse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Pfeffer J, Göckler FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* **35**, 7188–7196.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Golライブ M, Sibuet M, Olu-Le Roy K (2002) Cold-seep benthic communities in the Japan subduction zones – geological control of community-development. *Journal of Marine Research* **60**, 333–348.

Smith CR, De Leo FC, Bernardino AF, Sweetman AK, Arbizu PM (2008) Abyssal food limitation, ecosystem structure and climate change. *Trends in Ecology & Evolution* **23**, 518–528.

Sommer S, Pfannkuche O, Linke P, Luff R, Greinert J, Drews M, Gubisch S, Pieper M, Pester M, Viergutz T (2006) Efficiency of the benthic filter: biological control of the emission of dissolved methane from sediments containing shallow gas hydrates at Hydrate Ridge. *Global Biogeochemical Cycles* **20**, GB2019.

Stur HF, Summons RE, Smith CR, Deleo FC, Bernardino AF, Sweetman AK, Arbizu PM (2003) Methane oxidation above gas hydrates at a cold seep in the Gulf of Cadiz. *Science* **299**, 1928–1931.

Teske A, Hinrichs KU, Edgcomb V, Gomez AD, Kysela D, Sylva SP, Sogin ML, Jannasch HW (2002) Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. *Science* **298**, 1928–1931.
Hydrate Ridge, NE Pacific Ocean. *Marine Ecology Progress Series* **264**, 1–14.

Treude T, Smith CR, Wenzhöfer F, Carney E, Bernardino AF, Hannides AK, Krüger M, Boetius A (2009) Biogeochemistry of a deep-sea whale fall: sulfate reduction, sulfide efflux and methanogenesis. *Marine Ecology Progress Series* **382**, 1–21.

Tyler PA, German CR, Ramirez-Llodra E, Van Dover CL (2002) Understanding the biogeography of chemosynthetic ecosystems. *Oceanologica Acta* **25**, 227–241.

Vossmeier A, Deusner C, Kato C, Inagaki F, Ferdelman T (2012) Substrate-specific pressure dependence of microbial sulfate reduction in deep-sea cold seep sediments of the Japan Trench. *Frontiers in Microbiology* **3**, 1–12.

Wallmann K, Linke P, Suess E, Bohrmann G, Sahling H, Schlüter M, Dahlmann A, Lammers S, Greinert J, Von Mirbach N (1997) Quantifying fluid flow, solute mixing, and biogeochemical turnover at cold vents of the eastern Aleutian subduction zone. *Geochimica et Cosmochimica Acta* **61**, 5209–5219.

Wegener G, Niemann H, Elvert M, Hinrichs K-U, Boetius A (2008) Assimilation of methane and inorganic carbon by microbial communities mediating the anaerobic oxidation of methane. *Environmental Microbiology* **10**, 2287–2298.

Wei CL, Rowe GT, Escobar-Briones E, Boetius A, Soltwedel T, Caley MJ, Soliman Y, Huettmann F, Qu FY, Yu ZS, Pitcher CR, Haedrich RL, Wicksten MK, Rex MA, Baguley JG, Sharma J, Danovaro R, Macdonald IR, Nunnally CC, Deming JW, Montagna P, Levesque M, Woslavski JM, Wlodarska-Kowalczyk M, Ingole BS, Bett BJ, Billert DSM, Yool A, Bluhm BA, Iken K, Narayanaswamy BE (2010) Global patterns and predictions of seafloor biomass using random forests. *PLoS One*, **5**, e15323.

Wenzhöfer F, Glud RN (2002) Benthic carbon mineralization in the Atlantic: a synthesis based on in situ data from the last decade. *Deep-Sea Research Part I* **49**, 1255–1279.

Wenzhöfer F, Holby O, Glud RN, Nielsen HK, Gundersen JK (2000) In situ microsensor studies of a shallow water hydrothermal vent at Milos, Greece. *Marine Chemistry* **69**, 43–54.

Whiticar MJ (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chemical Geology* **161**, 291–314.

Zhou JZ, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. *Applied and Environment Microbiology* **62**, 316–322.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Phylogenetic affiliation of Bacteroidetes in JTC colony center (red) and colony rim (blue) sediments based on 16S rRNA gene sequences. The scale bar represents 10% estimated sequence divergence.

**Fig. S2** Phylogenetic affiliation of all other bacterial sequences retrieved from JTC colony center (red) and colony rim (blue) sediments based on 16S rRNA gene sequences. The scale bar represents 10% estimated sequence divergence.