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Characterizing lipid biomarkers in methanotrophic communities of gas hydrate-bearing sediments in the Sea of Okhotsk

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ABSTRACT

We studied specific lipid biomarkers of archaea and bacteria, that are associated with the anaerobic oxidation of methane (AOM) in a cold seep environment as well as the origin of sedimentary organic matter on the continental slope off NE Sakhalin in the Sea of Okhotsk. The organic geochemical parameters demonstrated that most of the sedimentary organic matter containing hydrate layers could be derived from marine phytoplankton and bacteria, except for a station (LV39-29H) which was remarkably affected by terrestrial vascular plant. Specific methanotrophic archaea biomarkers was vertically detected in hydrate-bearing cores (LV39-40H), coinciding with the negative excursion of the $\delta^{13}C_{org}$ at core depths of 90–100 cm below the seafloor. These results suggest that methane provided from gas hydrates are already available substrates for microbes thriving in this sediment depth. In addition, the stable isotope mass balance method revealed that approximately 2.77–3.41% of the total organic carbon (or 0.036–0.044% dry weight sediment) was generated by the activity of the AOM consortium in the corresponding depth of core LV39-40H. On the other hand, the heavier $\delta^{13}C$ values of archaeol in the gas hydrate stability zone may allow ongoing methanogenesis in deeper sediment depth.

1. Introduction

Large amounts of methane, which is approximately 20 times more powerful as a greenhouse gas than CO_2, are primarily stored in marine sediments as clathrate (Wuebbles and Hayhoe, 2002; Buffett and Archer, 2004). Despite high production rates and massive reservoirs of methane in marine sediments, the global contribution of methane from the oceans to atmospheric pools is estimated to be less than 3% due to the anaerobic oxidation of methane (AOM) with sulphate as the terminal electron acceptor (Barnes and Goldberg, 1976; Reeburgh, 1996; Hinrichs and Boetius, 2002).

Even though pure cultures of AOM communities are not yet available, many researchers have provided evidence that AOM is mediated by a consortium consisting of methanotrophic *Archaea* and sulphate reducing *Bacteria* (SRB) in marine environments using various techniques. Examples of these techniques include small-subunit ribosomal RNA (16S rRNA) gene sequencing (Hinrichs et al., 1999), fluorescence *in situ* hybridization with a secondary ion mass spectrometry technique (FISH-SIMS) (Orphan et al., 2001), an *in vitro* $^{13}CH_4$ labelling experiment (Blumenberg et al., 2005) and extremely ^{13}C-depleted carbon isotopic compositions of lipid biomarkers (Elvert et al., 1999; Hinrichs et al., 1999; Peckmann et al., 1999; Thiel et al., 1999). The strong ^{13}C-depletions of the biomarkers is a consequence of the biological isotope fractionation of the carbon substrate (methane), which is known to show extreme carbon isotope depletions (Valentine and Reeburgh, 2000; Hinrichs and Boetius, 2002; Niemann et al., 2006a,b).

The most reliable lipid biomarkers in methane-seepage sites indicative for AOM are PMI (2,6,10,15,19-pentamethylicosane), crocetane (2,6,11,15-tetramethylhexadecane), *sn*-2-hydroxyarchaeol (2-*O*-3-hydroxyphytanyl-3-*O*-phytanyl-*sn*-glycerol), and archaeol (2,3-di-*O*-phytanyl-*sn*-glycerol) for *Archaea*, as well as *iso*- and anteiso- $C_{15:0}$ and $C_{16:1\omega5}$ fatty acids and non-isoprenoidal diether lipids for SRB (Elvert et al., 1999; Hinrichs et al., 1999; Peckmann et al., 1999; Thiel et al., 1999; Pancost et al., 2001; Blumenberg et al., 2004).

Three different clades of anaerobic methanotrophs (ANME -1, -2, -3) are phylogenetically characterized by 16S rRNA gene sequences (Hinrichs et al., 1999; Orphan et al., 2002; Niemann et al., 2006a). ANME-1 and -2 archaea, which are distantly related to methanogens of the orders *Methanosarcinales* and *Methanomicrobiales*, are usually associated with the SRB of the *Desulfosarcina/Desulfococcus* branch (DSS) (Hinrichs et al., 1999; Boetius et al., 2000;

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Knittel et al., 2005). ANME-3 archaea, which are most closely related to the cultured *Methanococcoides* genera, serve as syntrophic partners of *Desulfobulbus* species (DBB) (Niemann et al., 2006a). Several recent studies have shown that membrane lipids from different clusters of ANMEs and SRBs are expressed by their abundance and composition of lipid biomarkers (Elvert et al., 2003, 2005; Blumenberg et al., 2004; Niemann et al., 2005). The literature database of diagnostic lipids has provided statistically relevant information, detailed information regarding the AOM consortium (Niemann and Elvert, 2008).

The Sea of Okhotsk is one of the largest marginal seas in the world and it is characterized by a region where the seasonal sea ice reaches the lowest latitudes (Kimura and Wakatsuchi, 2000). The Sea of Okhotsk is also one of the areas of the World Ocean that is highly biologically productive. In addition, it exhibits a large flux of organic carbon to the seafloor (Koblents-Mishke, 1967; Bogorov, 1974) and is characterized by high sedimentation rates (150–200 mm/ka) (Zonenshayn et al., 1987). In the present study, mud volcano and methane hydrate occurrences were associated with the active gas vents on the northeast Sakhalin Slope which were caused by high amounts of organic matter and the joint compression of the seafloor by the surrounding plates (Zonenshayn et al., 1987; Ginsburg et al., 1993; Soloviev et al., 1994; Greinert et al., 2002; Lüdmann and Wong, 2003; Shakirov et al., 2004; Shoji et al., 2005). For example, the bottom simulating reflector (BSR) from the geophysical investigation and the hydroacoustic anomalies in the water column were interpreted by examining the distribution of the methane hydrates and the signatures of the methane flumes that emanated from the seafloor, respectively (Zonenshayn et al., 1987; Ginsburg et al., 1993; Lüdmann and Wong, 2003; Jin et al., 2004; Luan et al., 2008). The methane concentrations in the water column and sediments have been measured in order to detect the locations of the gas seepage sites (Obzhirov, 1992, 1993; Obzhirov et al., 2002, 2004). Indirect indications of the presence of hydrate, such as visual observations, porewater chemistry data, and oxygen and hydrogen stable isotopic signatures ($\delta^{18}O$, δD) of the hydrate water, were also reported in the Sea of Okhotsk (Ginsburg et al., 1993; Matveeva et al., 2003). Most of the methane encaged in the hydrate and also migrating from the deep, buried reservoirs along the faulting zones in the Okhotsk Sea, was determined to be generated by microbial carbonate reduction rather than from thermogenic origin, by characterizing the composition of the hydrocarbon gases [$C_1/(C_2 + C_3)$] and the carbon and hydrogen stable isotope ratios ($\delta^{13}C$, δD) (Ginsburg et al., 1993; Matveeva et al., 2003; Mazurenko et al., 2005).

The presence of biogenic methane and AOM in the Sea of Okhotsk indicates that microbiological activities are important for the formation and dissociation of methane hydrate (Cragg et al., 1996; Wellsbury et al., 1997; Hinrichs et al., 1999; Dickens, 2003). Some previous investigation for understanding the process of AOM in this region have focused on the phylogenetic distribution of microbial communities (Inagaki et al., 2003), the symbiotic and benthic ecological aspects (Sahling et al., 2003; Pestrikova and Obzhirov, 2007), and the modelling approach for determining the organic matter flux (Wallmann et al., 2006). Much more studies also found evidence of AOM including lipid biomarkers (Peckmann et al., 1999; Thiel et al., 1999; Aloisi et al., 2004) as well as authigenic carbonate and barite precipitates with low $\delta^{13}C$ values (ca. $-40‰$) that were a result of the anaerobic oxidation of biogenic methane (Derkachev et al., 2000; Greinert et al., 2002; Aloisi et al., 2004; Lembke et al., 2007). The oldest record of AOM was as well found in seep-carbonates (Birgel et al., 2008). However, there have not been any studies conducted on the microbial lipid biomarkers in relation to the methane hydrate-bearing sediments in the well-known, cold seep environment (on the continental slope offshore of NE Sakhalin in the Sea of Okhotsk).

The objective of this study is to identify the origin of sedimentary organic matter and to characterize the indigenous microbial community distribution under various sediment environments in the Sea of Okhotsk (i.e. large methane flux, presence and non-presence of gas hydrate, etc.).

2. Materials and methods

2.1. Study area and sample collection

Multidisciplinary field investigations were conducted in May 2006 at the Derugin Basin in the central portion of the Sea of Okhotsk using the R/V *Akademik M.A. Lavrentyev* (LV) from the Russian Academy of Sciences (RAS). These investigations were conducted within the framework of the CHAOS project (hydro-Carbon Hydrate Accumulations in the Okhotsk Sea), which was supported by China, Japan, Korea, and Russia. Nine sediment cores were obtained from various sites (KOPRI, CHAOS, POI, GIZELLA and new gas-venting sites) using a 5.5-m gravity corer with a 126-mm diameter, lengthwise-cut split plastic liner in order to collect gas hydrate-bearing sediment (Fig. 1, Table 1).

Gas hydrates were observed at the LV39-25H and LV39-40H cores (Table 1), and the gas hydrate content in the hydrate-bearing intervals of 60–245 cm and 165–265 cm below the seafloor (cmbsf) were visually estimated to be as high as 5–10% and 15–20% by sediment volume, respectively (Luan et al., 2008). The LV39-40H core was characterized by the presence of carbonate concretions that were up to 3 cm in diameter within the core interval of 90–95 cmbsf. In addition, gas-saturated characteristics including cheese-like structures created through degassing and a strong H_2S odour were also present above the lenticular-bedded sediments from a thickness of a few millimeters to 3 cm in the hydrate-bearing zone (Fig. 2). Sub-samples were sliced into depth intervals of 10 cm and were collected in 100-ml glass bottles for organic geochemical analysis. The sub-samples were immediately frozen at −20 °C and were kept frozen until the analysis was conducted. The LV39-30H and LV39-40H cores were analyzed vertically in order to compare the organic, geochemical distinction with the existence of the gas hydrate layers.

2.2. Extraction, chromatographic separation and derivatization

Lipids were extracted from approximately 4 g freeze-dried and grinded sub-samples via sonication (Branson 5510) for 15 min at 20 °C three times in a row in a solvent mixture (15 ml) of dichloromethane/methanol (99/1, v/v). Subsequently, the aliquots of the resulting extract were concentrated using a rotary evaporator (EYELA n-1000) and then evaporated to dryness under a nitrogen gas stream.

Neutral- and polar-lipids present in the aliquots were cleaved by saponification with methanolic KOH solution. After the extraction of the neutral lipid fraction from this mixture, the carboxylic acids were obtained by acidification (HCl, pH 1–2) of the residual reaction mixture and extraction with *n*-hexane instead of water. The hydrocarbons and alcohols in the neutral lipid fraction were further separated into hydrocarbons, ketones, and alcohols using an SPE silica glass cartridge (0.5 g packing, Agilent Technologies) as described by Niemann et al. (2005).

The alcohol fractions were transformed into trimethylsilyl-derivatives by heating them at 70 °C for 1 h after the addition of 100 μl pyridine and 50 μl BSTFA. After cooling at room temperature, the excess solvent was evaporated, and the remaining TMS adducts were re-suspended in 500 μl *n*-hexane. The carboxylic acids were methylated by heating them at 60 °C for 1 h in a boron trifluouride–methanol complex. The hydrocarbons, alcohols, and polar fractions of the samples were stored at −20 °C until they underwent gas chromatography (GC), gas chromatography-mass