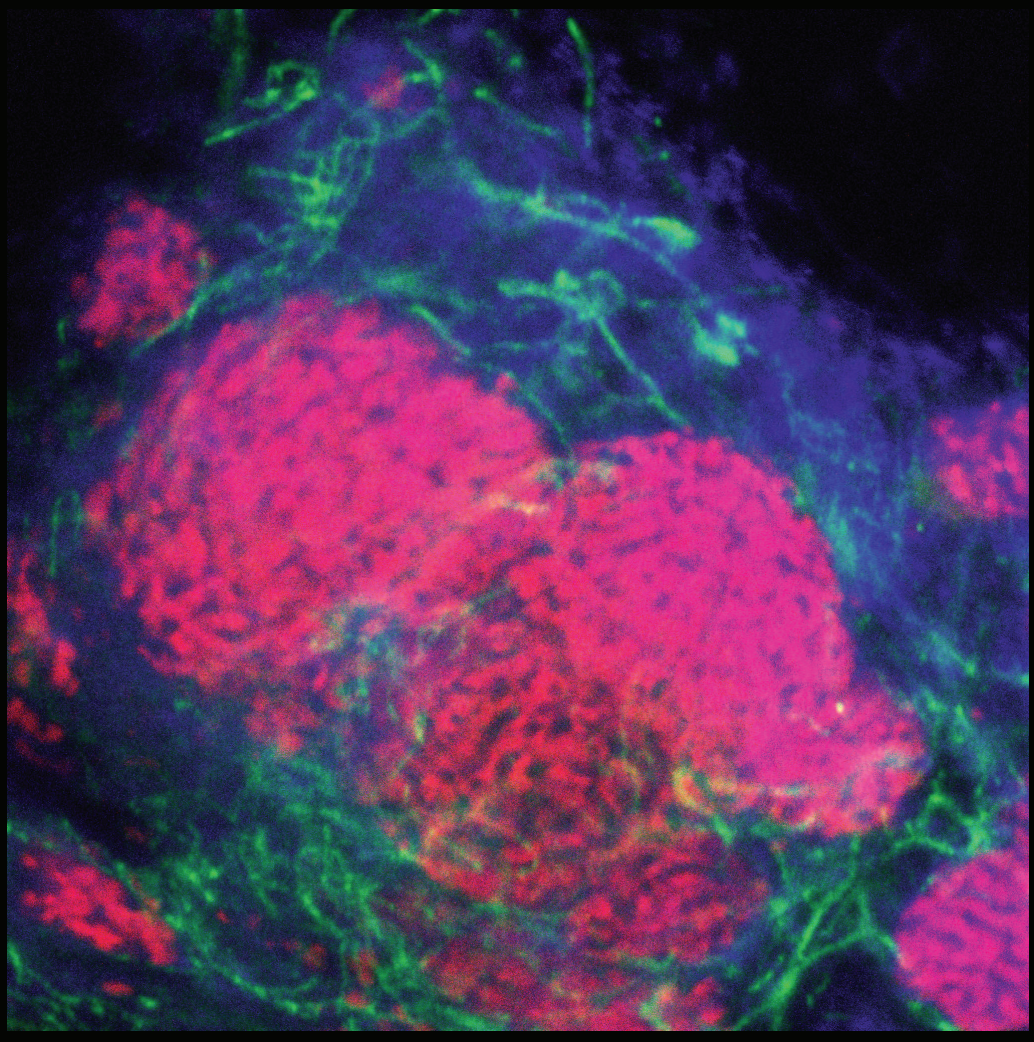
Molecular characterization of microbial populations in methane-rich marine habitats



Julia Arnds

Title page shows a laserscanning micrograph (picture taken by A. Ellrott, MPI Bremen) of Chloroflexi bacteria (probe GNSB-941, green) and ANME-2 archaea (probe ANME2-538, red) cells from an AOM bioreactor enrichment (Deusner et al., in prep) of a Black Sea microbial reef mat (P784). Other cells are DAPI stained (blue).

Molecular characterization of microbial populations in methane-rich marine habitats

Dissertation zur Erlangung eines Doktorgrades

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Abbreviations

AMO	aerobic methane oxidation
ANME	anaerobic methanotrophic archaea
AOM	anaerobic oxidation of methane
AAA	AOM-associated archaea
CARD	Catalyzed Amplified Reporter Deposition
Cfx	Chloroflexi
Ct	Cycle threshold
DAPI	4`, 6`-diamidino-2-phenylindole
DBB	*Desulfobulbus*
(c) DNA	(complementary) deoxyribonucleic acid
DOC	dissolved organic carbon
DSS	*Desulfosarcina/Desulfococcus*
FISH	fluorescence *in situ* hybridisation
FtsZ	filamenting temperature-sensitive mutant Z
GNSB	green non sulfur bacteria
HRP	horseradish peroxidase
IPL	intact polar lipids
mcr	methyl-coenzyme M reductase encoding gene
MCR	methyl-coenzyme M reductase
mCfx	marine Chloroflexi
NE	Northeast
PCR	polymerase chain reaction
(r) RNA	(ribosomal) ribonucleic acid
SOB	sulfur-oxidizing bacteria
SMTZ	sulfate-methane-transition zone
SR	sulfate reduction
SRB	sulfate-reducing bacteria
SW	Southwest
V and VER	Verrucomicrobia

Index of contents

Summary

In the ocean, a major methane sink is the anaerobic oxidation of methane (AOM) with sulfate, which is apparently catalyzed by syntrophic consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). An understanding of this globally relevant process is important for modeling the carbon cycle. The main objective of this thesis was to gain further insights into AOM habitats and communities. The particular focus was on the *in situ* abundance and distribution of microorganisms potentially involved in methane turnover and sulfur cycling.

There is evidence for a global distribution of ANME and associated SRB, but putting methane-rich sites and ANME habitats on the ocean map suggests that our picture is yet patchy. In this thesis, another spot was added on the map: ANME and SRB were found at recently discovered cold seeps from the Hikurangi margin (New Zealand), far away from all known ANME habitats. Here, high methane concentrations and oxidation rates were measured over relatively broad sediment horizons.

The most intensively studied ANME habitats are Black Sea microbial reefs fueled by AOM. In this thesis, sediment and various reef samples were investigated. Microbial communities at the surface sediment and reef top nodules were dominated by ANME-2 and DSS, whereas ANME-1 dominated the communities in the subsurface sediment and reefs` ex- and interior. Reefs varied little along the northwestern shelf indicating relatively stable environmental conditions in this area.

Besides ANME-1 and ANME-2, the reefs host diverse other microorganisms, which might either immigrate from surrounding sediments or settle from the seawater. Chloroflexi related cells were identified as additional key reef players accounting for up to 28% of total cells. Interestingly, they were often tightly associated with ANME-2/SRB consortia and might share some features with sludge granule forming Chloroflexi spp..

Some findings indicate that the reefs and other AOM habitats, and marine sediments in general harbor Verrucomicrobia, but they were not detected in the reefs analyzed and rare in various marine samples screened in this thesis. Subsequently, another aquatic habitat was analyzed: diverse and large populations of Verrucomicrobia making up to 19% of all cells were found in the humic lake Große Fuchskuhle (Germany), where their distribution varied only little between oxic and anoxic water, but

strongly with time and between a more and a less humic basin. This might explain why they were not detected more often by random screening of marine samples.

The quantification of ANME by fluorescence *in situ* hybridization is time-consuming. Therefore, another aim of this thesis was to establish a real time PCR assay allowing rapid quantification of ANME. Primer and probe sets were developed for all ANME groups. The ANME-3 set was further tested via TaqMan and SybrGreen technique revealing a poorly efficient amplification. This problem could not be solved during this thesis.

While insights into ANME-1 and ANME-2 genomes have been gained, nothing was so far known about the ANME-3 genome. An additional objective was to establish a protocol, which allows ANME-3 cell sorting by flow cytometry for subsequent genome analysis. Preliminary results indicate high purity of the so sorted cells. Preliminary pyrosequencing revealed genes assigned to ones of ANME-3 and *Methanococcoides burtonii*, the closest ANME-3 relative, for which genome data are available.

Zusammenfassung

Eine wesentliche Methansenke im Ozean ist die anaerobe Oxidation von Methan (AOM), die offenbar von syntrophen Konsortien anaerob methanotropher Archaeen (ANME) und Sulfat-reduzierender Bakterien (SRB) katalysiert wird. Ein Verständnis dieses global bedeutsamen Prozesses ist wichtig für Modellierungen des Kohlenstoffzyklus. Das Hauptziel dieser Arbeit war es, weitere Erkenntnisse zu AOM-Habitaten und zu den dort vorkommenden mikrobiellen Gemeinschaften zu gewinnen. Der Fokus war auf die *in situ* Abundanz und Verteilung von Mikroorganismen gerichtet, die potentiell am Umsatz von Methan und am Schwefelzyklus beteiligt sind.

Es gibt Hinweise darauf, dass ANME und SRB global verbreitet sind. Noch ist die Kartierung von methanreichen Standorten und ANME Habitaten aber unvollständig. In dieser Arbeit wurde ein weiterer Ort in die Karte eingetragen: ANME und SRB wurden an kürzlich entdeckten kalten, diffusiven Methanquellen im Sediment des Hikurangi-Kontinentalrands (Neuseeland) gefunden, weit weg von allen bisher bekannten ANME-Habitaten. Hier wurden hohe Methankonzentrationen und AOM-Raten über relativ breite Sedimenthorizonte gemessen.

Die am intensivsten studierten ANME-Habitate sind mikrobielle Riffe im Schwarzen Meer, die durch AOM gebildet werden. Mikrobielle Gemeinschaften an der Sedimentoberfläche und im oberen Riffbereich waren dominiert von ANME-2 und DSS, während Gemeinschaften in tieferen Sedimentschichten sowie äußere und innere Riffzonen von ANME-1 dominiert waren. Riffe entlang des Schelfs unterschieden sich geringfügig voneinander, was auf relativ stabile Umweltbedingungen in dieser Gegend hindeutet.

Außer ANME-1 und ANME-2 beherbergen die Riffe diverse andere Mikroorganismen, die entweder aus dem umliegenden Sediment immigrieren oder sich aus dem Seewasser absetzen. Chloroflexi verwandte Zellen wurden als wichtige Riffbewohner identifiziert, die bis zu 28% aller Zellen ausmachten. Interessanterweise waren sie oft eng mit ANME-2/DSS Konsortien assoziiert und teilen manche Eigenschaften mit Schlammflocken formenden Chloroflexi spp..

Einige Funde deuten darauf hin, dass die Riffe und andere AOM-Habitate, sowie marine Sedimente im Allgemeinen Verrucomicrobia beherbergen, aber sie wurden nicht

in den analysierten Riffen detektiert und waren rar in verschiedenen anderen marinen Proben, die in dieser Arbeit untersucht wurden. Daraufhin wurden Proben eines anderen aquatischen Habitats untersucht: diverse und grosse Population von Verrucomicrobia, die bis zu 19% aller Zellen ausmachten, wurden im huminstoffreichen See Große Fuchskuhle (Deutschland) gefunden, in dem ihre Verteilung wenig zwischem oxischen und anoxischen Wasser, aber zeitabhängig und zwischen einem mehr und einem weniger huminstoffreichen Becken variierte. Dies könnte erklären, warum sie nicht öfter in marinen Stichproben gefunden wurden.

Die Quantifizierung von ANME mittels Fluoreszenz *in situ* Hybridisierung ist zeitaufwendig. Daher war es ein weiteres Ziel dieser Arbeit, einen Echtzeit-PCR-Ansatz zu etablieren, der eine schnelle Quantifizierung von ANME ermöglicht. Primer und Sonden wurden für alle ANME Gruppen entwickelt und jene für ANME-3 mittels TaqMan- und SybrGreen-Technik getestet. Wie sich herausstellte, war die Effizienz der Echtzeit-PCR gering. Dieses Problem konnte in dieser Arbeit nicht behoben werden.

Während Einblicke in die ANME-1 and ANME-2 Genome bereits gewonnen wurden, war bisher nichts über das ANME-3 Genom bekannt. Ein weiteres Ziel war es, ein Protokoll zu etablieren, mit dem ANME-3 Zellen mittels Durchflusszytometrie für anschließende Genomanalysen sortieren werden können. Vorläufige Ergebnisse deuten auf eine hohe Reinheit der derart sortierten Zellen hin. Vorläufige Pyrosequenzanalysen zeigen, dass die meisten erhaltenen Gene denen von *Methanococcoides burtonii*, dem zu ANME-3 nächstverwandten kultivierten Organismus, von dem Genomdaten verfügbar sind, sehr ähnlich sind.

A. Introduction

1. Oceanic methane

Methane, the simplest hydrocarbon, is only a trace gas in today`s atmosphere (<1.8 ppmv). Nevertheless, it plays an important role in the radiative balance on Earth (Reeburgh 2003, 2007). Atmospheric methane concentrations varied strongly throughout the past 150.000 years correlating with temperature rises and falls (Houghton 1997). However, they increased continuously since pre-industrial times, stagnated in the last years, and increased again in 2007. If this rising trend is due to an increase in sources or decrease in sinks, is yet unknown (Rigby et al. 2008 and references therein). While atmospheric methane has been studied for many years, oceanic methane research began only about 50 years ago.

Quantification of oceanic methane is challenging, because it occurs in various forms, is “invisible” below the sediment surface, released variably in both time and space, rapidly diluted in seawater, and distributed by currents. However, methane concentrations decrease from millimolar in the seabed to nanomolar in seawater. Hence, the ocean contributes only minor amounts (2%) of methane to the global atmospheric budget, although the subsurface stores large amounts. Most oceanic methane is oxidized prior to emission (Judd et al. 2002, Reeburgh 2007).

At atmospheric pressure, methane is poorly water soluble and solubility decreases with rising salinity and temperature (Yamamoto et al. 1976). Oceanic methane occurs as dissolved gas, forms bubbles when its solubility is exceeded, or hydrates at low temperatures and under high pressure, e.g. in the deep seafloor (Kvenvolden 1993). Hydrates are crystalline clathrates composed of water cages enclosing single methane molecules (Figure 1). In the hydrate stability zone, hydrate coated methane bubbles have been recently observed (Sauter et al. 2006). Great effort has been devoted to the study of hydrates, because they may represent the greatest reservoir of methane carbon on earth (~7×10^5 – 7×10^7 Tg) providing an enormous exploitable energy source and their decomposition probably destabilizes the seafloor (Kvenvolden 1999, Judd et al. 2002).

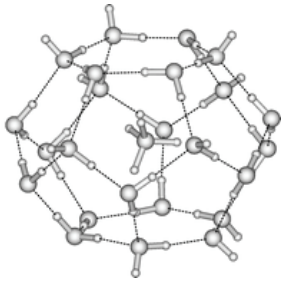


Figure 1: Water cage enclosing single methane molecule (model obtained from www.rsc.org).

1.1 Sources

Seawater methane concentrations are quite low, despite nearly ubiquitous methane maxima (~20 nM) in upper surface mixed layers. Here, flux to the atmosphere appears to be the main methane sink (Holmes et al. 2000). However, global oceanic methane emission values have probably been overestimated 10-fold (Rhee et al. 2009). Transport of methane emitted from seeps can yield further maxima in greater water depth (Sansone et al. 2001, Cynar and Yayanos 1993). Exceptional high seawater methane concentrations are only found in anoxic basins, e.g. the deep Black Sea (~11 µM; Reeburgh et al. 1991). Most oceanic methane is stored in the seabed.

Most organic matter settling from seawater to the seafloor is decomposed to carbon dioxide by aerobic microorganisms using oxygen as electron acceptor. Below the oxic surface buried compounds are degraded by anaerobic microorganisms utilizing other electron acceptors as e.g. nitrate, manganese- and iron-oxides, or sulfate. Where such electron acceptors are depleted, organic matter is degraded by microbial fermentation. The final step in fermentation is methanogenesis, catalyzed by Archaea.

Most methane on earth is produced microbially. Methane is further produced independently of microbial catalysis by thermogenic decomposition of organic matter in subsurface sites, where temperature and pressure are high, typically in depths of 1 km. Further, methane is formed independently of organic matter from hydrogen and carbon dioxide by rock/water interaction at temperatures >300 °C occurring at spreading centers (Tissot and Welte 1984, Horita and Berndt 1999, Boudreau and Jørgensen 2001, Reeburgh 2007, Judd et al. 2002 and references herein).

Some authors define methane as biotic when it has been formed from organic matter as opposed to abiotic methane derived from processes that do not involve organic matter (Welhan 1988 and references herein). Other authors distinguish between biotic methane that is microbially produced and abiotic methane that either results from

chemical transformation reactions of organic matter or from rock/water interaction (e.g. Widdel et al. 2006 and references herein). Some clues on the origin of methane can be obtained from stable isotope measurments (summarized by Op den Camp et al. 2009): microbially produced methane is very light ($\delta^{13}C$ CH_4 of -55‰ to -110‰), whereas thermogenic methane (-20‰ to -55‰) and abiogenic methane (-5‰ to -45‰) are typically heavier (Etiope and Klusman, 2002; Kvenvolden and Rogers, 2005), although there are exceptions (Horita and Berndt, 1999).

1.2 Sinks

The ocean can be seen as a large reactor that oxidizes methane from various marine sediment sources. Most methane is oxidized by anaerobic microorganisms populating anoxic sediment horizons and by aerobic microorganisms inhabiting the oxic benthic boundary layer or seawater (Reeburgh 2007).

1.2.1 Aerobic oxidation of methane

In contrast to terrestrial habitats, the aerobic oxidation of methane (AMO) appears to be only a minor biological sink of methane in the ocean. It sets where methane availability coincides with that of oxygen. Thus, AMO mediating microorganisms are restricted to oxygenated seawater and the oxic benthic boundary layer. Little is yet known about AMO in marine sediments. Studies on the Haakon Mosby Mud Volcano (HMMV) in the Barents Sea (Norway) revealed bacterial AMO as the main methane-consuming process in the active, oxic volcano center (Niemann et al. 2006, De Beer et al. 2006, Lösekann et al. 2007).

In the open ocean, methane concentrations and AMO rates are generally viewed as being quite low. It is suggested that methane is not oxidized below a threshold concentration, but it remains unknown which factors control this threshold (reviewed in Reeburgh 2007). Relatively high AMO rates have been observed in maxima with methane concentrations of ~20 nM. The highest rates (0.15 nM d^{-1}) were observed in deep-sea plumes generated by vents (de Angelis et al. 1993).

1.2.2 Anaerobic oxidation of methane

The major methane sink in the ocean is the anaerobic oxidation of methane (AOM) coupled to the reduction of sulfate (Eq. 1), which has a relatively low potential Gibbs free energy (ΔG).

$$(1)\ CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O,\ \Delta G = -21\ kJ\ mol^{-1}$$

The discovery of this process is based on observations of sulfate-methane transition zones (SMTZ) in a wide variety of environments during the late seventies and correlations between high AOM and SR rates determined by radiotracer and stable isotope measurements. In the last decade, new biomarker and culture-independent phylogenetic techniques were introduced to AOM research (reviewed in Reeburgh 2007).

Recent studies have revealed that AOM is coupled to a larger variety of oxidants than previously thought. Electron acceptor such as nitrite (Raghoebarsing et al. 2006, Ettwig et al. 2008), birnessite or ferrihydrite (Beal et al. 2009) are energetically more favorable than sulfate.

2. Methane-rich marine habitats

Various methane-rich marine habitats are known to date. Shelf and margin sediments, as well as the deep seafloor, are large methane sources accounting the high organic matter content and extensive size, respectively. In seawater, high methane concentrations are only found in permanently anoxic seawater (Reeburgh 2007). Hereafter, diffusive and advective systems are distinguished from permanently anoxic seawater with a strong focus on cold seep ecosystems, the study objects of this thesis.

2.1 Diffusive systems

From a global perspective, the bulk of AOM occurs in passive continental shelf sediments characterized by diffusion-controlled porewater transport of methane from below and sulfate from above. In diffusive systems, AOM sets in relatively broad SMTZs located between one and ten meters below the benthic boundary layer. Here, AOM rates are relatively low ranging from a few pmol cm^{-3} day^{-1} to tens of nmol cm^{-3} day^{-1} (e.g. Wellsbury et al. 2000). However, methane is completely oxidized within the SMTZs

by methanotrophic microorganisms forming efficient filters against methane emission to the hydrosphere (Dale et al. 2008, Wegener and Boetius 2008).

2.2 Advective systems

In advective systems, SMTZs are narrow and close to the benthic boundary layer, and characterized by high AOM rates and biomasses (Dale et al. 2008, Wegener and Boetius 2008). AOM rates reach up to hundreds of nmol cm^{-3} day^{-1} (e.g. Boetius and Suess 2004, Niemann et al. 2006). At flow velocities of >0.4 m yr^{-1}, AOM becomes sulfate-limited and the efficiency of the microbial filter shrinks (Niemann et al. 2006, Wegener and Boetius 2009). Flow velocities above 2.5 m yr^{-1} can even prevent sulfate penetration completely and inhibit AOM activity (De Beer et al. 2006).

High advective transport can cause methane escape into the hydrosphere. Methane seepage sets in various environments: nearshore, continental shelf to deep seafloor. The gas emits from shallow accumulations, deep ancient reservoirs, or dewatering hydrates (Judd et al. 2002). Cold seeps and hot vents host various escape routes for methane-rich gas bubbles and fluids transported by advective processes (Reeburgh 2007, Knittel and Boetius 2009, Levin 2005).

Cold seeps are defined as ecosystems, in which methane-rich gases and/or pore waters rise through the sediments forced by pressure gradients and emerge from the seafloor without substantial temperature increase (Levin 2005). They are characterized by the highest AOM rates ever measured sustained by strong upward advection of porewater flow. Numerous active and fossil cold seeps have been observed along continental margins around the globe. Seeps are often associated with carbonates forming crusts, chimney- or reef-like structures (Hovland and Judd 1988).

At cold seeps, AOM activity yields high sulfide concentrations, availability of hard carbonate substratum, and increased food supply providing the basis for complex chemosynthetic communities similar to ones found at hot vents. Cold seep sediments are often covered by white and yellow, or orange microbial mats, such as formed by sulfur- or iron-oxidizing bacteria, respectively. Mats can be formed by various sulfur-oxidizers: *Beggiatoa, Thioploca, Thiothrix,* or *Arcobacter* (Tryon and Brown 2001, Levin 2005). Giant, vacuolated filaments, such as *Beggiatoa* or *Thioploca*, as well as *Arcobacter* spp. have been suggested as key sulfur-oxidizers at AOM sites (Treude et

al. 2003, De Beer et al. 2006, Omoregie et al. 2008). Characteristic seep fauna is dominated by various bivalves, tube worms, sponges, gastropods and shrimp sometimes abundant (Levin 2005).

One of the best studied cold seep systems is located off the coast of Oregon at the Cascadia margin (Hydrate Ridge) with intense fluid flow and large-scale gas hydrate deposits. Here, active venting of fluids and gases, exposure of methane hydrates at the seafloor, composition and distribution of chemosynthetic communities, authigenic chemoherms forming carbonates, and gas plumes in the water column have been investigated by international and interdisciplinary research teams. They found some of the highest methane oxidation rates ever measured in the ocean system (Boetius and Suess 2004).

Unique cold seep structures are the Black Sea microbial reefs, up to 4 m high and 1 m in diameter, fueled by AOM (Michaelis et al. 2002). They grow along the deep permanently anoxic shelf west of the Crimea peninsula, where hundreds of active gas seeps occur between 35 and 800 m water depth (Ivanov et al. 1991). They are covered by up to 10 cm thick microbial mats internally stabilized by carbonate precipitates deriving from AOM. The mat forming microorganisms do not grow on preformed carbonates, but induce and shape their formation (Pimenov et al. 1997, Michaelis et al. 2002).

Other seep systems are mud volcanoes occuring in zones of compression or areas with high sediment deposition rates, formed by expulsion of water, gas, and mud from sediments (Milkov 2000). Other volcanoes erupt asphalt (MacDonald et al. 2004). Pockmarks, sediment depressions, are formed when pressurized methane is locally released from impermeable sediments (Hovland et al. 2002). Hot vents are found where hot basalts meet cold seawater producing methane by rock-water interactions. They have been found along all active mid-oceanic ridges and back-arc spreading centers (Van Dover 2000).

2.3 Permanently anoxic seawater

Despite the surface mixed layer maxima, suggestably produced by fermentation in digestive tracts (de Angelis and Lee 1994), fecal pellets, marine snow (Burke et al. 1983), and probably some algae (Scranton and Brewer 1977), methane-rich seawater is only found in nutrient-rich, poorly mixed anoxic water, e.g. in the deep Black Sea, which is nearly landlocked and thus relatively independent from the global ocean system (Reeburgh 2007).

3. Key microorganisms in methane-rich marine habitats

While methanogens are known since the mid-fifties (Barker 1956), aerobic and anaerobic methanotrophs have been first described only in the early eighties and late nineties, respectively (Whittenbury and Dalton 1981, Hinrichs et al. 1999).

3.1 Aerobic methanotrophs

For over three decades, only aerobic Proteobacteria of the alpha-, beta-, and gamma-class were known to utilize methane as sole carbon and energy source (reviewed in Hanson and Hanson 1996). In 2007, members of another bacterial phylum capable of AMO were isolated: methanotrophic Verrucomicrobia mediate the reaction even at pH 1 and 55 °C (Pol et al. 2007, Dunfield et al. 2007, Islam et al. 2008). AMO is initiated by methane-monooxygenases splitting dioxygen-bonds and forming methanol, which is further oxidized to CO_2 (Hanson and Hanson 1996). However, little is yet known about aerobic methanotrophs in the marine environment. At the HMMV, diverse Gammaproteobacteria were identified as key players in benthic AMO (Niemann et al. 2006, Lösekann et al. 2007).

3.2 Anaerobic methanotrophs

3.2.1 Phylogeny, lipid composition, and morphology

Phylogeny. Zehnder and Brock (1979) hypothesized that AOM is mediated by a methanogenic archaeon, operating its common metabolism in reverse, and a sulfate-reducing bacterium in a syntrophic relation. Hinrichs et al. (1999) found methane derived archaeal lipids and archaeal 16S rRNA gene sequences in microbial communities populating a methane seep in the Eel River Basin offshore California. Two sequence types were found. One clustered between the orders Methanomicrobiales and Methanosarcinales, the other was related to Methanosarcinales. They were designated anaerobic methanotrophic archaea (ANME), with the groups ANME-1 and ANME-2, respectively. Boetius et al. (2000) provided first pictorial evidence supporting the Zehnders` and Brocks` hypothesis. They visualized consortia of ANME-2 and sulfate-reducing bacteria (SRB) in methane-rich sediments from the Hydrate Ridge off the coast of Oregon by using fluorescence *in situ* hybridization (FISH; Amann et al. 1990).

To date, two subgroups of ANME-1 (ANME-1a and -1b; Knittel et al. 2005) and three ANME-2 subgroups (ANME-2a, -2b, and -2c) are distinguished belonging to different orders or families (Figure 2, Knittel and Boetius 2009). Another ANME group with relation to Methanococcoides*,* ANME-3, was discovered by Niemann et al. (2006) at the HMMV. Two novel clades related to ANME-2 have recently been identified. For the clades GoM Arc 1 (Lloyd et al. 2006) and AOM-associated archaea (AAA), involvement in AOM remains uncertain (reviewed by Knittel and Boetius 2009). Latter comprises, among uncultured ones, archaea that were enriched from anoxic freshwater sediment of a Dutch canal fed with methane, nitrate, and nitrite (Raghoebarsing et al. 2006).

While ANME-1 and ANME-2 are associated with SRB of the *Desulfosarcina/Desulfococcus* group (DSS, Boetius et al. 2000, Michaelis et al. 2002, Knittel et al. 2005), ANME-3 are associated with *Desulfobulbus* spp. (DBB, Lösekann et al. 2007). ANME-2c were also described to be associated with Alpha- and Betaproteobacteria related to *Sphingomonas* and *Burkholderia* spp., respectively (Pernthaler et al. 2008). Little is yet known about other microorganisms associated with AOM communities. To date, neither ANME nor associated SRB have been isolated, but studies on enrichment cultures, cultivation-independent microbial approaches, and biogeochemical analyses revealed many insights into their lifestyle and habitats.

Recently, Beal et al. (2009) claimed that manganese-dependent methane oxidation might be mediated either by ANME-1 and/or Methanococcoides/ANME-3 with a bacterial partner, or solely by Bacteria, such as Bacteroidetes, Proteobacteria, Acidobacteria*,* or Verrucomicrobia. Potential iron-oxidizers have not been identified in AOM incubation studies, but one *Desulfobulbus* species is capable of iron reduction (Holmes et al. 2004).

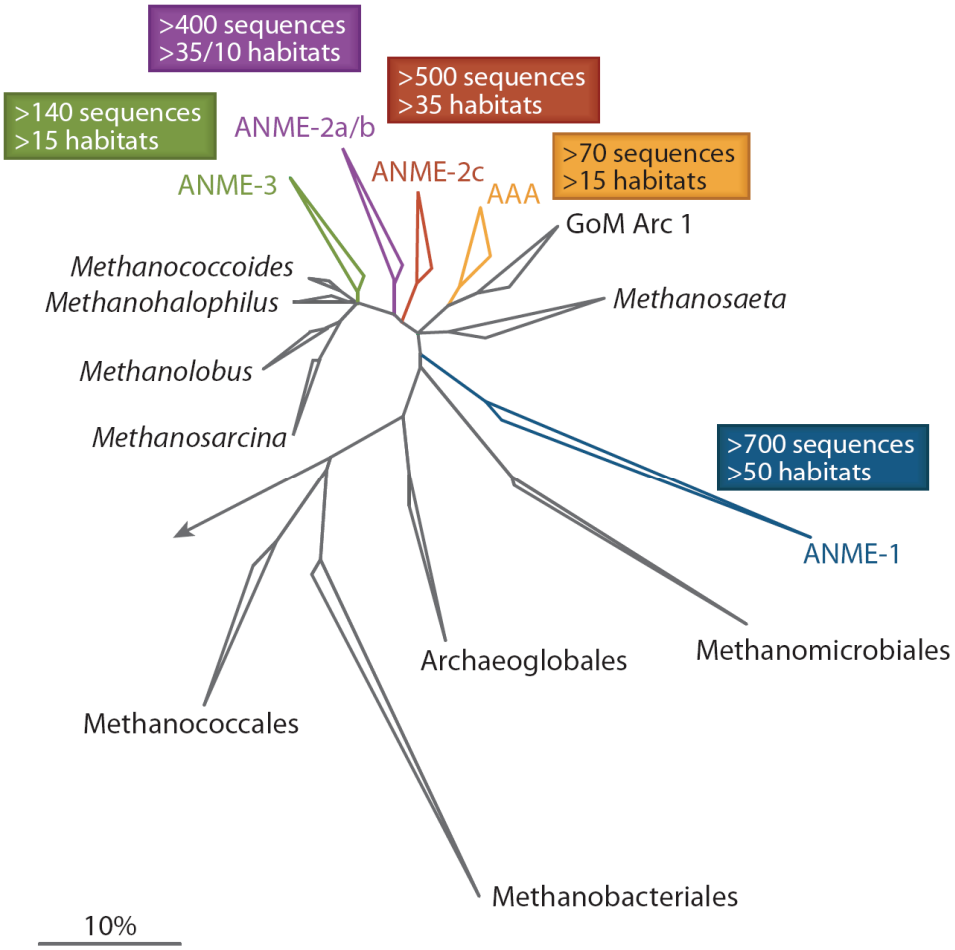


Figure 2: Phylogenetic tree showing the affiliations of ANME 16S rRNA gene sequences to selected reference sequences of the domain Archaea. Data in colored boxes give information about the distribution and abundance of sequence retrieval (Figure from Knittel and Boetius 2009).

Lipid composition. Microorganisms and their carbon fixation pathways can be identified by lipid biomarker and their stable carbon isotope signatures. Lipid biomarker analyses allow identification of fossil cells, but fossil and living cells can not be distinguished and the taxonomic resolution is low compared to 16S rRNA gene analysis. Intact polar lipids (IPL) are of higher taxonomic specificity (families to orders) and proper to select for living cells (reviewed in Niemann and Elvert 2008). ANME-1 dominated communities mainly comprise diglycosidic glycerol dialkyl glycerol tetraether derivatives and no or extremely few polar derivates of hydroxyarchaeol, whereas ANME-2 and ANME-3 dominated communities are characterized by high abundance of phosphate-based polar derivatives of archaeol and hydroxyarchaeol (Rossel et al. 2008).

Morphology. Most ANME are cocci (ANME-2: 0.5-1.4 µm, ANME-3: 0.7 µm in diameter), except for the rectangular-shaped ANME-1 (1.5-3 µm by 0.6 µm; Boetius et al. 2000, Knittel et al. 2005, Lösekann et al. 2007). Coccoid ANME are rarely found as single cells or in monospecific aggregates (e.g. Orphan et al. 2001, Treude et al. 2005[b], Lösekann et al. 2007). They mostly occur associated with SRB. ANME-1 can form chains of mostly two to four cells or multicellular chains longer than 100 µm (Reitner et al. 2005[a]). ANME-2c form shell-type (Figure 3a) or mixed-type aggregates with DSS (Figure 3b), ANME-2a and DSS also form mixed-type aggregates. Hereafter, aggregates are defined as ANME/DSS consortia. Also ANME-3 form aggregates, but they are, if at all, associated with only few DBB related species (Lösekann et al. 2007). Latter have been also found associated with ANME-2c (Pernthaler et al. 2008). DSS are coccoid- (0.3-1 µm in diameter) or rod-shaped (0.5 by 2.3 µm) or vibrioform, DBB are rod-shaped (1.1 by 0.5 µm; Boetius et al. 2000, Knittel et al. 2005, Lösekann et al. 2007, Reitner et al. 2005[b]).

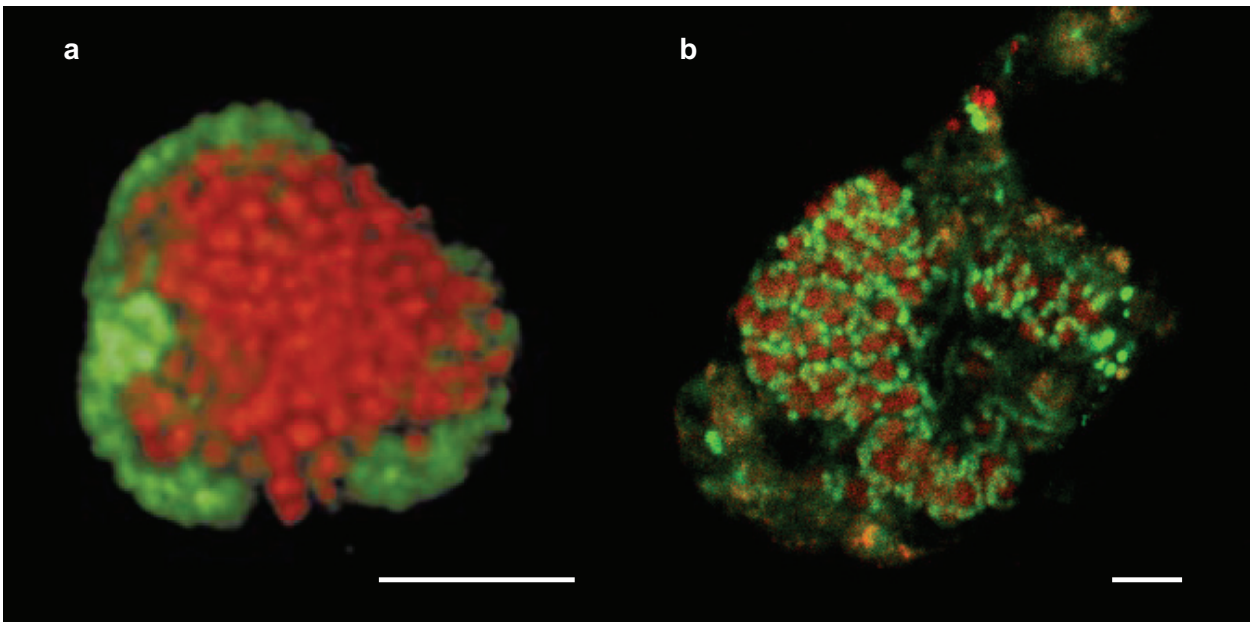


Figure 3: Laserscanning micrographs of (a) shell- and (b) mixed-type ANME-2/SRB consortium (Figure a from Boetius et al. 2000; Figure b from Knittel et al. 2005, scale bars 5 µm).

3.2.2 Global distribution in the ocean

ANME 16S rRNA genes are found in many methane-rich marine benthic habitats and ANME cells strongly dominate AOM communities (~90%; Boetius et al. 2000, Michaelis et al. 2002, Knittel et al. 2005, Lösekann et al. 2007). However, *in situ* studies are still rare. The largest ANME populations (10^{11} cells ml^{-1}) known to date inhabit cold seeps at the Hydrate Ridge (Boetius et al. 2000) and Black Sea microbial reefs (Michaelis et al. 2002).

In the Atlantic Ocean and connected basins, ANME genes and/or cells have been found in the Black and Mediterranean Sea, Gulf of Cadiz, off England, in the North Sea, North Sea-Baltic Sea transition zone, Baltic Sea, and Barents Sea, at the Juan de Fuca Ridge, in the Gulf of Mexico, and off Brazil. In the northeastern Pacific Ocean, they have been found off Oregon, California, and Mexico. In the northwestern Pacific Ocean, genes have been found off China and Japan. They were also found in the Arctic off Alaska and in the Antarctic (Figure 4) indicating their global distribution (reviewed in Knittel and Boetius 2009).

However, the mapping is yet spotty. The Indian Ocean is completely, and the central and southern Pacific is largely under-sampled. Assuming that ANME occur at methane-rich sites worldwide and based on available geophysical data, further ANME hot-spots may be located off the coasts of central and southern America, South Africa, India, Indonesia, and Russia, and probably many other sites (Figure 4).

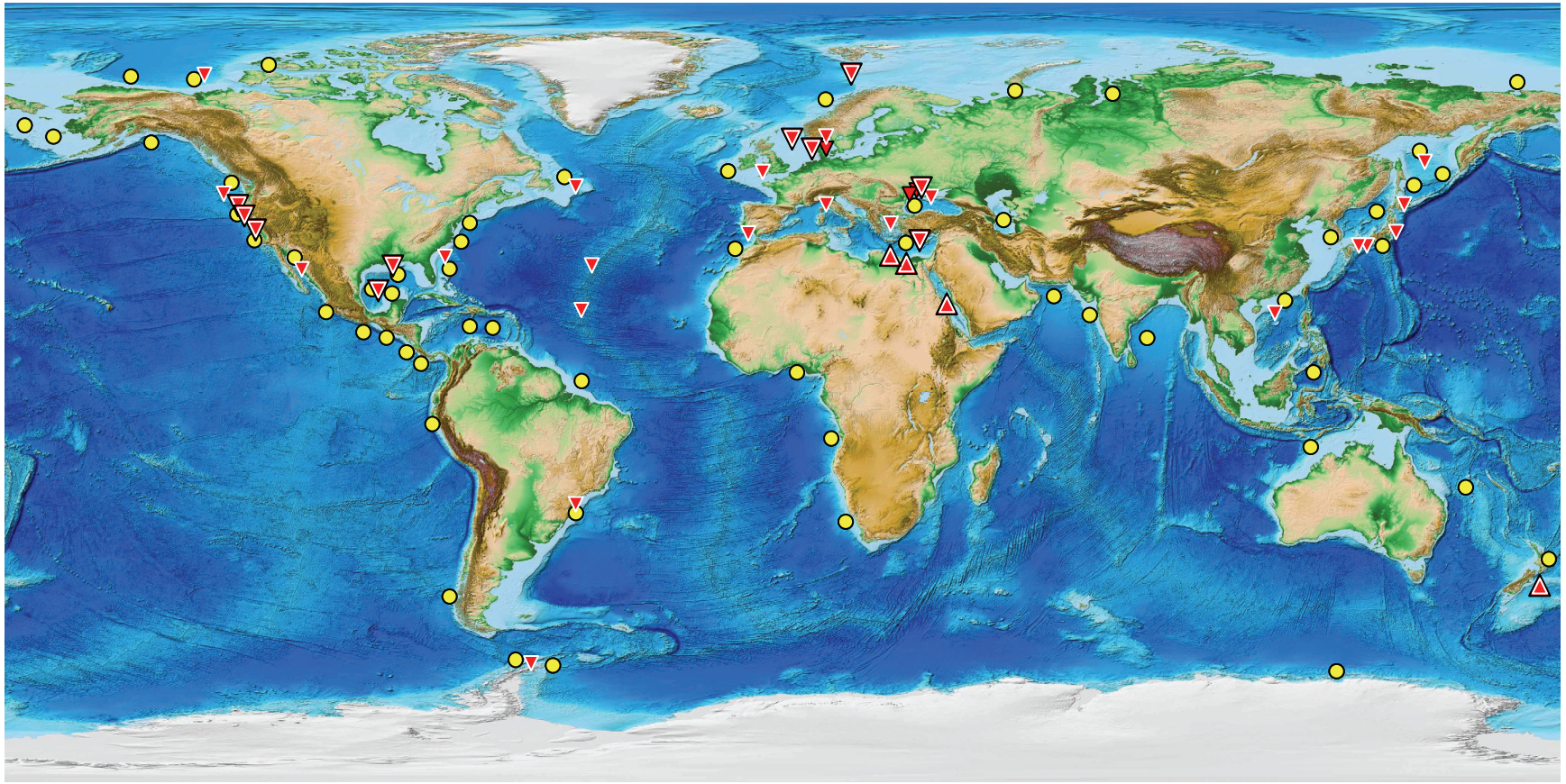


Figure 4: Global distribution of gas hydrates (yellow dots) and ANME (red triangles) in marine systems. Detection of ANME 16S rRNA genes (reviewed by Knittel and Boetius 2009), ANME cells *in situ*, or both is highlighted by white, black, and black-white surrounded triangles, respectively. Map obtained from NGDC, modified after Kvenvolden (USGS) and Greinert (IFM Geomar). Gas hydrates have been observed or indicated geophysically. FISH data on Spiekeroog`s intertidal flat sediment (German Wadden Sea) kindly provided by K. Bischof, MPI Bremen, unpublished data. Note that most molecular studies reporting on ANME detection have focused on the coasts off the USA, Europe, and Japan.

3.2.3 Physiology, genomics, and proteomics

Physiology. ANME and associated SRB grow together in anoxic enrichment cultures on methane and sulfate (Nauhaus et al. 2002, 2005, 2007), but neither of them has been isolated in pure culture. *In vitro*, AOM and SR rates show a 1:1 coupling. The process could not yet be uncoupled by feeding potential intermediates. Assuming a syntrophic relation, an energy transfer could either be mediated by an intermediate in form of a so far unknown substrate or by electrons. Or they are not in a syntrophic relation and AOM and SR occur in the same cell. Bacteria of the NC10 clade, enriched from anoxic freshwater sediment of the Dutch canal (Raghoebarsing et al. 2006), solely couple AOM to denitrification (Ettwig et al. 2008). Cultivation studies are time-consuming, because AOM mediators grow slowly. Doubling of ANME/SRB consortia takes months due to the low free energy yield of AOM with sulfate (Nauhaus et al. 2007). Under standard conditions, $\Delta G°$ is -21 kJ mol^{-1} (Thauer and Shima 2008). *In situ*, it ranges between approximately -10 to -40 kJ mol^{-1} (Nauhaus et al. 2007).

Genomics and Proteomics. Based on metagenomic analysis, Hallam et al. (2003, 2004) suggested the involvement of methyl-coenzyme M reductase (MCR) in AOM. The *mcr* retrieved from AOM communities are homolog to ones of methanogenic archaea and MCR can work reversible (reviewed by Chistoserdova et al. 2005) supporting the hypothesis that AOM is a reversed methanogenesis (Zehnder and Brock 1979). In mats of Black Sea microbial reefs fueled by AOM, MCR are expressed at a high level (Heller et al. 2008). From such mats, Krüger et al. (2003) extracted, purified, and identified two MCRs: nickel protein I and II. Recently, *mcr* encoding the nickel protein I and II were linked to the metagenomes of ANME-1 and ANME-2, respectively (Krüger et al. 2003, Meyerdierks et al., MPI Bremen, unpublished data). Metagenomic analyses further revealed the presence of nearly all genes typically associated with methanogenesis in ANME-1, and to a lesser extend in ANME-2 (Hallam et al. 2004). However, the biogeochemistry of AOM remains poorly understood (reviewed in Thauer and Shima 2008).

4. Identification and quantification of microorganisms by molecular techniques

Classical microbiology has shown that microorganisms are most versatile “chemists”. Molecular biology revealed them to be sophisticated and complex beyond our ability to describe (Shapiro and Dworkin 1997). Molecular techniques provide yet limited and biased insights into the microbial world, but they are the only tools to study the complex communities in nature. To date, several techniques are used to characterize environmental microorganisms (Figure 5).

Molecular identification of microorganisms from mixed communities is conventionally based on polymerase chain reaction (PCR) invented in 1985 to amplify specific segments from complex DNA mixtures (Saiki et al. 1985). For this method, Kary Mullis was awarded the 1993 year`s Nobel prize in Chemistry. The key phylogenetic marker gene is the one encoding 16S rRNA, which has both conserved and variable regions allowing the identification on both higher and lower taxonomic levels (Woese and Fox 1977). However, its taxonomic resolution is limited. Species with identical 16S rRNA, may differ significantly in morphology and/or physiology (e.g. Jaspers and Overmann 2004).

PCR amplified 16S rRNA genes can be separated by cloning, and identified by sequencing and subsequent phylogenetic analysis. The 16S rRNA databases are the most comprehensive collections of microbial genes and enlarge exponentially. Soon, the number of deposited 16S rRNA gene sequences will rise above 1 million (upcoming SILVA release 100 contains 995.747 aligned sequences, Prüsse et al. 2007). However, conventional PCR is not quantitative due to the inherent bias associated with endpoint PCR (Polz and Cavanaugh 1998).

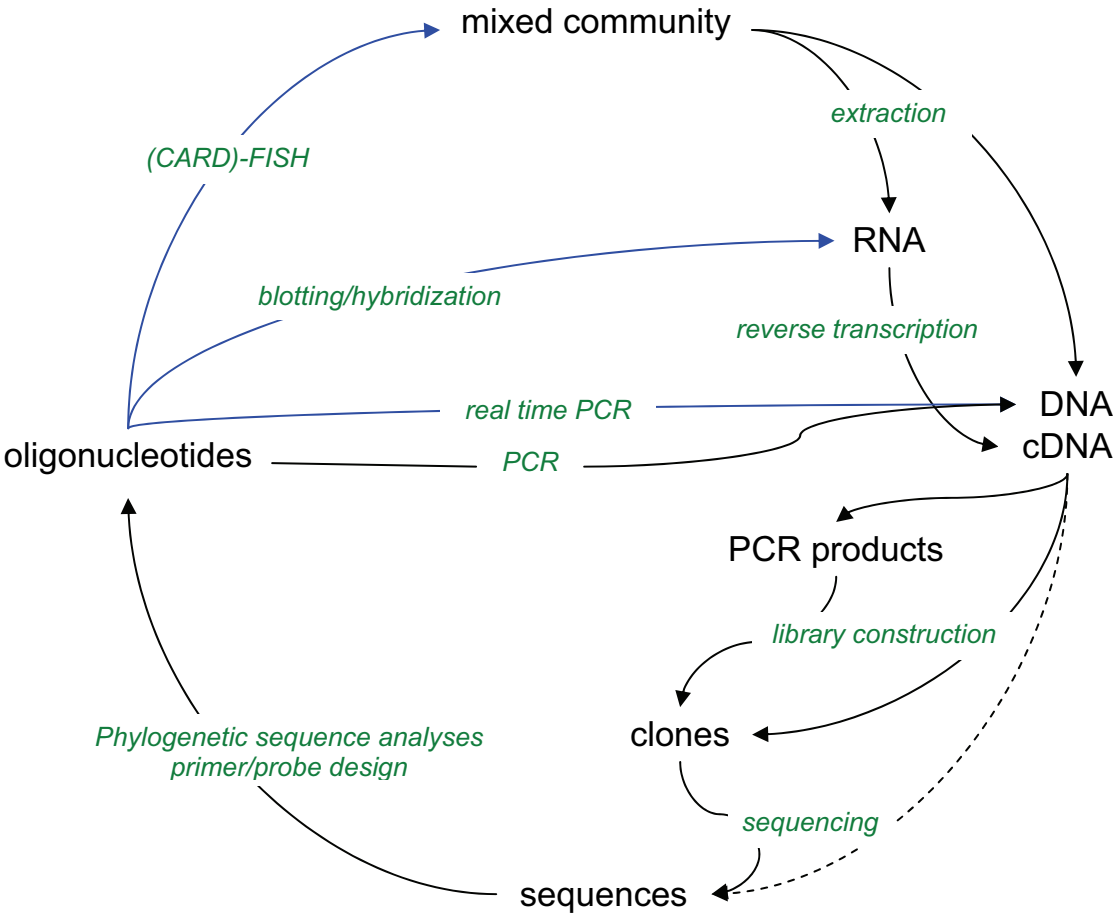


Figure 5: Starting material of molecular studies on a complex microbial community is commonly extracted DNA. RNA can also be extracted and transcribed into cDNA. Genes of interest, e.g. 16S rRNA genes, can be PCR amplified, cloned, and sequenced. (c)DNA can also be directly cloned and sequenced without prior amplification (bacterial artificial chromosome, cosmid, and fosmid library construction). New technologies (e.g. nanopore-sequencing) allow sequencing of (c)DNA without prior amplification and cloning, but are not yet standardized (dotted line). Targets of interest can be identified and quantified by hybridizing oligonucleotides against RNA/DNA in the cell (see also Figure 15) or extracted DNA/RNA. Techniques are highlighted in green. Blue arrows represent quantitative approaches (scheme modified after Amann et al. 1995).

Specific genes in an environmental sample can be quantified by the sensitive real time PCR, which allows to monitor the amplification process by labeling and detecting generated amplicons. Quantification is linked to standards with known DNA concentrations. Amplicons can be visualized by different labeling strategies: the SybrGreen technique (Wittwer et al. 1997) uses double-stranded DNA binding fluorescent dyes for labeling and is the simplest approach. The disadvantage of this technique is that unspecific PCR products and primer dimers are also detected. More specific is the TaqMan approach (Holland et al. 1991) using fluorescent probes as labels. The probes bind within the target region of the primers and their fluorescences are quenched unless they are degraded by the exonuclease activity of DNA polymerase during elongation (Cozzareli et al. 1969). This technique is currently the most frequently used method to study microbial abundance in the environment. The highly sensitive technique allows even the detection of very rare genes, but gene copy numbers do not represent microbial cell numbers.

The introduction of whole cell fluorescence *in situ* hybridisation (FISH) for the identification of microbial cells (DeLong et al. 1989) allowed the quantification of specific cells in mixed communities (Amann et al. 1990). However, cell detection by using fluorescent probes hybridized against their 16S rRNA complement is limited by the cellular ribosome content. To overcome this limit, horseradish peroxidase (HRP)-labeled probes were introduced and the HRP fed with fluorescein-tyramides. Thereby, signal intensities were enhanced significantly (Schönhuber et al. 1997) strongly enlarging detection rates (Pernthaler et al. 2002). The sensitivity of CARD-FISH is 26 to 41-fold higher than that of conventional FISH (Hoshino et al. 2008). FISH is to date the most specific approach to quantify microbial cells in mixed communities, but starving, dormant, or dead cells with very low or no rRNA content are not detectable with this method.

5. Thesis aims

In the last decade, many insights have been gained into AOM coupled to SR and the key players have been identified as ANME and associated SRB. However, many questions remain. The main objective of this thesis was to gain further insights into AOM communities in their natural habitats by using molecular tools.

Numerous studies indicate the global distribution of ANME and SRB. However, mapping methane-rich and ANME habitats suggests that our picture of the distribution is still patchy. One aim of this thesis was to investigate recently discovered cold seeps from the Hikurangi margin in the East of New Zealand`s North Island, far away from all known ANME habitats and populated by a new species of ampharetid polychaetes.

The most intensively studied ANME habitats are the Black Sea microbial reefs. It was shown that ANME and associated SRB strongly dominate the reef microbial communities. However, little is yet known about other reef microbes, how reefs develop, and whether they differ. In this thesis, various samples from different reefs along the northwestern Black Sea shelf, different reef zones, and different reef horizons were studied.

To date, quantification of ANME by FISH is a time-consuming task limiting the number of samples, which can be analyzed. Therefore, another aim of this thesis was to set up a real time PCR assay that allows rapid quantification of ANME 16S rRNA genes to detect hot-spots of ANME.

Insights into the genome of ANME have been obtained only from ANME-1 and ANME-2. Nothing is known about the ANME-3 genome. Therefore, one further aim of this thesis was to initiate genomic analysis on ANME-3.

B. Synthesis

1. Microbial communities in marine cold seeps

1.1 First insights into novel cold seeps around New Zealand (manuscript I)

AOM takes place in globally distributed areas along continental shelfs and margins and ANME 16S rRNA genes have been found at several of these sites. However, their *in situ* abundance was yet studied only off European and North American coasts. In this thesis, recently discovered cold seeps from the Hikurangi margin (New Zealand) were investigated providing first evidence for the occurrence of ANME-2 and ANME-3 in the South Pacific Ocean, far away from all known ANME habitats (Figure 4). This further supports the global distribution of ANME (Knittel and Boetius 2009), and the Baas-Becking theorem that everything is everywhere, but the environment selects ("Baas-Becking 1934"), at least in the ocean.

One of the seep sites investigated in this thesis was covered by white filamentous mats, probably formed by *Beggiatoa* sp. and/or other giant SOB, and *Arcobacter* sp., which are common in sulfidic habitats (Teske and Nelson 2006, Campbell et al. 2006). This site was referred to as SOB site. An adjacent seep was covered by a new species of ampharetid polychaetes. Ampharetidae have been found at other cold seeps and whale falls, but never constituted the key fauna (Sommer et al. 2008). Beneath the SOB mats, high AOM and SR activity (averaged 11 vs. 14 mol m^{-2} y^{-1}), ANME-2 (up to 15%) and DSS (up to 30%) were distributed over a broad sediment horizon, forming a filter against methane emission. The deep sulfate penetration at this site might be explained by convective influx of seawater due to focused fluid upflow. In comparison to other active seep sites, the ANME numbers were low, which might be related to higher cell-specific activities. At the Polychaete site, characterized by high seepage fluxes (Sommer et al. 2008), AOM and SR activity (averaged 6 vs. 2 mol m^{-2} y^{-1}), ANME-2 (up to 25%), and DSS (up to 12%) were restricted to subsurface sediment layers, likely due to deeper oxygen penetration by bioturbation. Rates as well as total ANME and DSS numbers were lower than at the SOB site. Here, deep sulfate penetration is also best explained by bioturbation. Giant filamentous bacteria were not detected, but several other potential SOB. The resident community was highly diverse. This site was hypothesized as an initial seep stage (Sommer et al. 2008 and Figure 6).

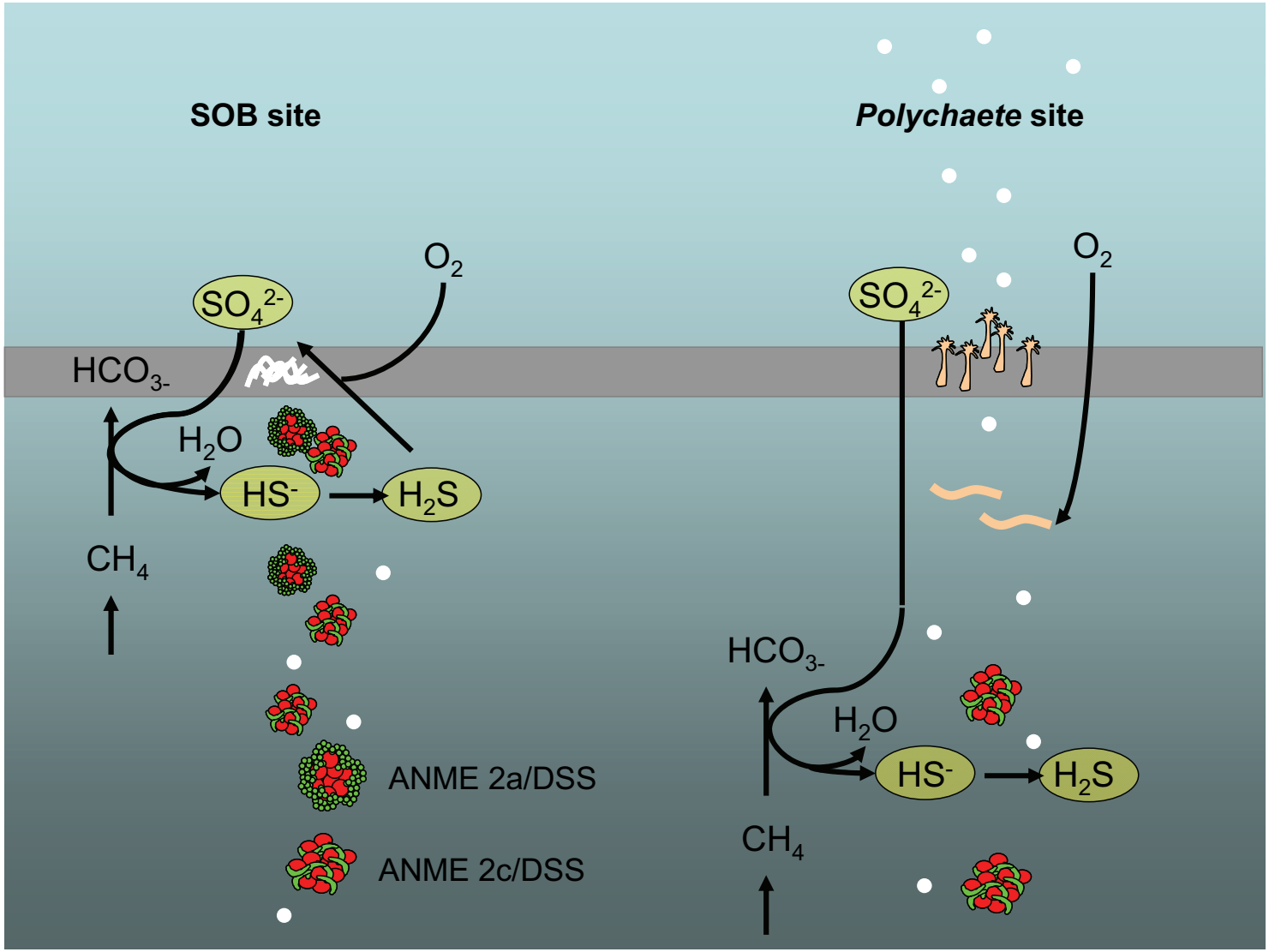


Figure 6: Scheme of hypothesized structures of cold seeps at the Hikurangi margin (white cycles: methane, white filaments: giant SOB, pink: worms with polychaetes at surface and nematodes in deeper layers). Distribution of AOM activity and ANME/DSS consortia (red: ANME, green: DSS) seems to depend on depth of oxygen penetration. The polychaetes might either initiate AOM (Sommer et al. 2008) by sulfate penetration or settle at SOB sites.

1.2 New insights into intensively studied Black Sea microbial reefs (manuscript II)

ANME populations shape Black Sea microbial reefs, the most intensively studied ANME habitats. However, little is known about other reef microbes, how reefs develop, and whether they differ. In this thesis, recently discovered reefs from the Northwest Shelf and the Danube Canyon were compared to ones from the well known Dnepr Area (Michaelis et al. 2002, Reitner et al. 2005[a,b], Treude et al. 2005[a], Knittel et al. 2005, Krüger et al. 2008). Furthermore, different reef zones (top, ex-, and interior) were investigated. From this reef, insights into microbial diversity were gained. Differently colored mat layers were additionally analyzed in small-scale, because it has been reported that they are dominated by either ANME-1 or ANME-2 (Reitner et al. 2005[b], Krüger et al. 2008). Methane seeping sediment was included in the analysis.

All reefs hosted ANME-1, ANME-2, DSS, and diverse other microbes, e.g. Chloroflexi, Epsilonproteobacteria, Planctomycetes, and Bacteroidetes. However, reef communities varied between distinct reef zones and mat layers, but little between individual reefs along the shelf. This suggests that environmental conditions change within the reefs, but little along the shelf. Differently colored mat layers hosted either ANME-1 or ANME-2, but the color was no reliable criterion to predict dominance of one or another.

The reefs` top nodules were characterized by high AOM activity and high numbers of ANME-2 and DSS. In the reefs` ex- and interior, AOM activity was lower and the ANME population, dominated by ANME-1, smaller. Interestingly, DSS were almost absent. While ANME-2 might benefit from low methane, but high sulfate fluxes on the reefs` top, ANME-1 seem to cope with both high methane and low sulfate fluxes in the interior, as well as with very low methane fluxes in the exterior. *In vitro*, high growth rates of ANME-1 and ANME-2 were found at high and low methane flow rates, respectively (Girguis et al. 2005). Microbial communities at the surface sediment and reef top nodules were dominated by ANME-2 and DSS, whereas ANME-1 dominated the communities in the subsurface sediment and reefs` ex- and interior. The interior zone is likely the most aged zone, but still provides a niche for ANME-1, which might be here limited by space (Figure 7).

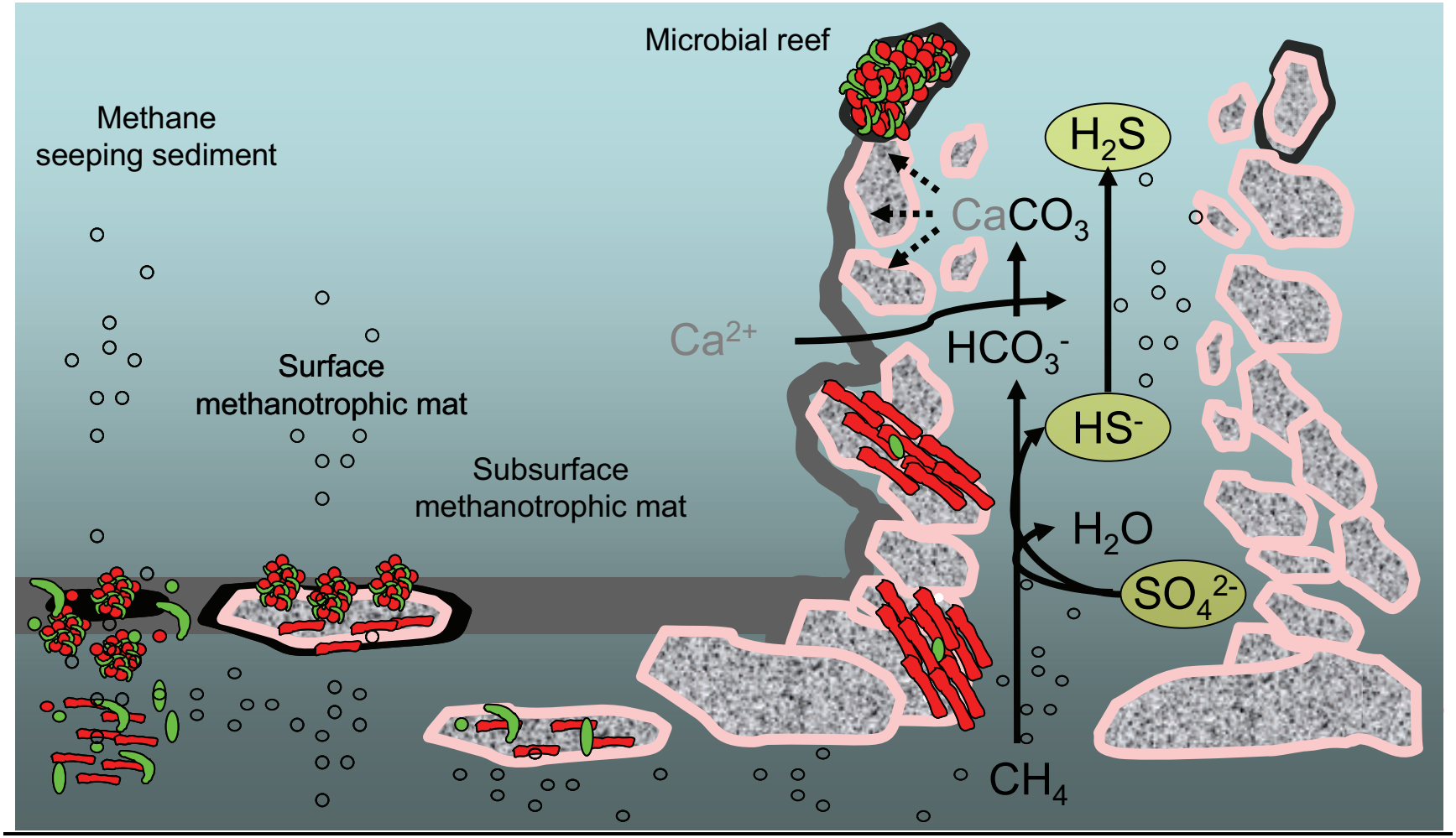


Figure 7: Scheme of hypothesized reef development fueled by AOM (black: FeS_2, dark grey: sediment surface, pink: methanotrophic mats, grained grey and brown: precipitated carbonates; modified after Widdel et al. 2006). ANME and DSS might be released from reefs by methane outbursts, settle nearby and thrive within methane seeping sediment forming initial reef stages on the sediment surface. Surface methanotrophic mats (Krüger et al. 2008) may develop from such initial reef stages. Methane may accumulate below subsurface methanotrophic mats (Treude et al. 2005[a]) providing hot-spots for initial reef stages or they represent subsurface parts of reefs. Rectangular and coccoid shaped ANME-1 and ANME-2a, respectively (red) and DSS (green). Note that only very few DSS are associated with ANME-1.

1.3 Morphology and structural diversity of ANME and associated SRB

ANME and associated SRB are found as single cells, but mostly occur together and form aggregates questioning the selective advantage of multicellularity. Interestingly, ANME are often surrounded by a matrix, probably a glycocalix, which might glue the cells together (Thauer and Shima 2008). Rectangular shaped ANME-1 can form chains. Chain formation might protect cells from grazing, chains might elongate towards favorable conditions, or nutrients are distributed along the chains (Shapiro and Dworkin 1997).

In this thesis, mixed consortia of ANME-2a with vibrioform DSS were found at both AOM study sites, the cold seeps at New Zealand`s continental margin (Figure 6 shows the dominant consortia only) and microbial reefs along the Black Sea shelf. In the New Zealand samples, also ANME-2c associated with vibrioform DSS were found. Some DSS vibrios almost completely surrounded single ANME cells, but they also occurred as single cells (Figure 8). So far, vibrioform DSS were only found in Black Sea reefs (Knittel et al. 2005, Reitner et al. 2005[b]). ANME associated DSS from other study sites are coccoid or rod-shaped suggesting various subpopulations or environmental conditions. However, the mixed-type association provides an optimal pattern for an exchange of substrates. Aggregated cells are probably protected from grazing, but nutrient availability of individual cells is likely reduced compared to single cells. The same might be true for shell-type consortia of ANME-2c and DSS (Boetius et al. 2000). However, it remains unknown how substrates are exchanged without close cell contact. ANME-3 aggregates are, if at all, associated with only few DBB (Lösekann et al. 2007).

It remains unknown why ANME and associated DSS mostly form consortia. Assuming a syntrophic relation, tightly associated cells might benefit from a fast exchange of intermediates. Another explanation might be that cells rather have evolved mechanisms to protect themselves from grazing by forming aggregates than to take up the highest possible amount of nutrients. Cells feeding on methane as main carbon source might be susceptible to predation, because they form a large biomass providing favorable carbon sources others than methane for non-methanotrophic organisms.

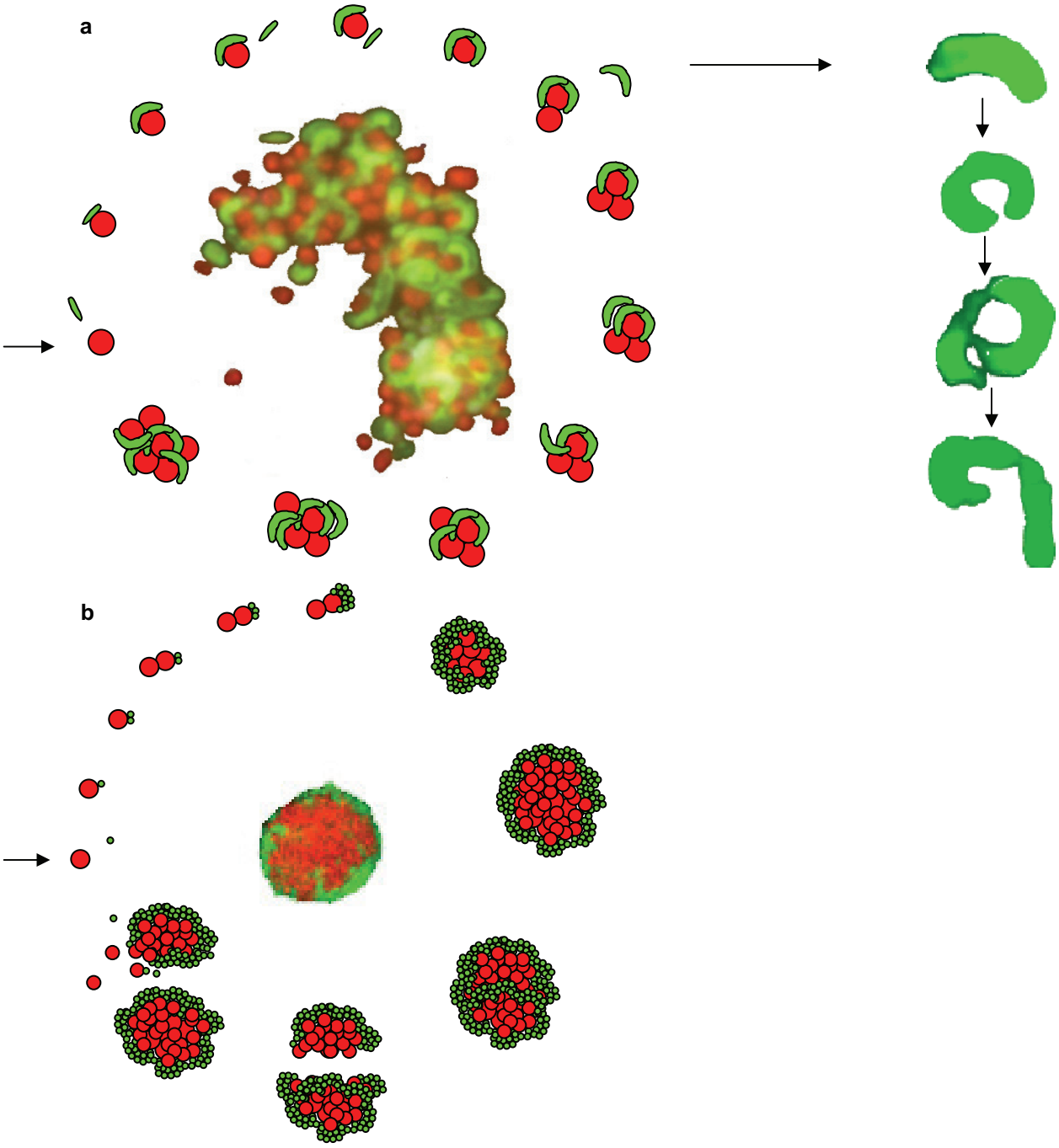


Figure 8: Scheme of hypothesized development of multicellular consortia based on microscopic observations (red: ANME, green: DSS). (a) Mixed-type ANME-2a/DSS and close-up of single and dividing vibrioform DSS, epifluorescence micrographs, scale bars 1 µm. (b) shell-type ANME-2c/DSS, Laserscanning micrograph (K. Knittel). Note that some single ANME cells are almost completely surrounded by single vibrioform or many small coccoid DSS. Arrows indicate initial stages of aggregate formation. Aggregates may start to form wherever an ANME and a DSS cell meet in their preferred environment. A similar aggregate formation pattern has been proposed by Nauhaus et al. (2007).

1.4 Contrasting SOB communities at different AOM sites

AOM-derived sulfide fluxes support growth of giant filamentous SOB, such as *Beggiatoa* or *Thioploca* –both Gammaproteobacteria- (Treude et al. 2003, De Beer et al. 2006), and *Arcobacter* spp., which belong to the Epsilonproteobacteria (Omoregie et al. 2008). Also at hydrothermal vents, Gamma- and Epsilonproteobacteria are the key players involved in sulfur-oxidiation (López-García 2003, Takai et al. 2006).

The SOB site at the Hikurangi margin was covered by a filamentous white mat indicating accumulation of sulfur and, indirectly, sulfidic conditions. Filamentous structures can be formed by giant SOB filaments, which internally store sulfur, such as *Beggiatoa* and *Thioploca* (Teske and Nelson 2006), or *Arcobacter* sp., which excrete filamentous sulfur (Wirsen et al. 2002). While three morphotypes of big bacterial filaments were detected at the sediment surface, filaments were absent at the adjacent Polychaete site. The least abundant type at the SOB site looked like *Beggiatoa* sp. and only one related 16S rRNA gene was identified in the constructed gene libraries. Instead, genes of various other free-living and symbiotic potential sulfur-oxidizing Gammaproteobacteria (e.g. *Methylophaga*, *Thiothrix*, *Maorithyas* thioautotrophic symbiont) and Epsilonproteobacteria (*Arcobacter* sp.) were recovered.

As revealed by CARD-FISH, sulfur-oxidizing Gammaproteobacteria (probe Gam660; Ravenschlag et al. 2001) and Epsilonproteobacteria (probe EPSI-682, Moussard et al. 2006) were equally abundant (~3% of all cells). However, the diversity of SOB indicated by 16S rRNA gene library analysis was not covered by the probes used. The major fraction of Gammaproteobacteria (4% of total cells) was not identified further.

In contrast, Black Sea microbial reefs hosted only few cells of Gammaproteobacteria (<1% of all cells). From the reefs, neither 16S rRNA genes of Alpha-, Beta-, Gamma-, nor Epsilonproteobacteria were obtained, but sulfur-oxidizing Epsilonproteobacteria were found *in situ* (up to 11% of total cells). Notably, they were most abundant in ANME-1 dominated reef samples. In sulfidic waters of the Black and Baltic Sea, both Gamma- and Epsilonproteobacteria are abundant *in situ* (Vetriani et al. 2003, Lin et al. 2006, Grote et al. 2007, 2008).

Likely, the filamentous sulfur-oxidizing Gammaproteobacteria commonly covering cold seep sediments are absent in the microbial reefs, because they depend on oxygen or nitrate, which are permanently absent in the deep Black Sea. The absence of other anaerobic Gammaproteobacteria might be related to the very high sulfide concentrations in the reefs (3 mM in reef porewater). However, many sulfur-oxidizing Gammaproteobacteria tolerate high sulfide concentrations (e.g. *Alkalispirillum* spp., ~5 mM, Sorokin et al. 2006). Another explanation for their low abundance might be that they lacked a useable carbon source (Kuenen et al. 1982, Jannasch 1985).

The rareness of Gammaproteobacteria in the reefs might have favored growth of sulfide-oxidizing Epsilonproteobacteria, which often live in anoxic, sulfidic habitats (Campbell et al. 2006, Omoregie et al. 2008). In the hydrothermal sediment studied by López-García et al. (2003), Epsilonproteobacteria were probably not abundant, but the authors showed that they are pioneer colonizers of various virgin surfaces exposed to fluid-seawater interphases. Epsilonproteobacteria were abundant only in few of the reef samples analyzed in this study suggesting that either yet unknown SOB oxidize sulfide or only minor amounts are oxidized within the Black Sea microbial mats.

1.5 Chloroflexi as newly identified key players in microbial reefs

In the Black Sea microbial mats, filamentous bacteria were detected in high numbers (up to 10% of total cells) using probe EUB338 III, which was designed by Daims et al. (1999) to cover among other Bacteria the phylum Verrucomicrobia. However, no 16S rRNA genes of Verrucomicrobia were amplified with general bacterial primers. Therefore, EUB338 III and a newly developed reverse primer (Appendix 1) were used to specifically amplify verrucomicrobial genes from reef material.

Unexpectedly, the majority of amplified 16S rRNA genes clustered with these of diverse Chloroflexi (subphylum I and II), most of them with *Bellilinea* sp. (86% to 92% similarity) and fewer with *Caldilinea* sp. (79% similarity). One 16S rRNA gene sequence affiliated with *Dehalococcoides/Dehalobium* related sequences (87/88% similarity). All sequences had highest similarities to ones of uncultured Chloroflexi from various marine benthic habitats including various seep sites (Figure 9). Probe match revealed that the Chloroflexi-like sequences obtained from the Black Sea microbial reefs are also

targeted by EUB338 III. GNSB-941, a general Chloroflexi probe (Gich et al. 2001), was further used in combination with a specific probe (mCfx665) newly designed to target the largest reef cluster (14 of 25 sequences with 99% similarity). Approximately 90% of all GNSB-941 positive cells were hybridized with mCfx665. They accounted for 0.4% to 28% of all cells in the reef samples (Table 1).

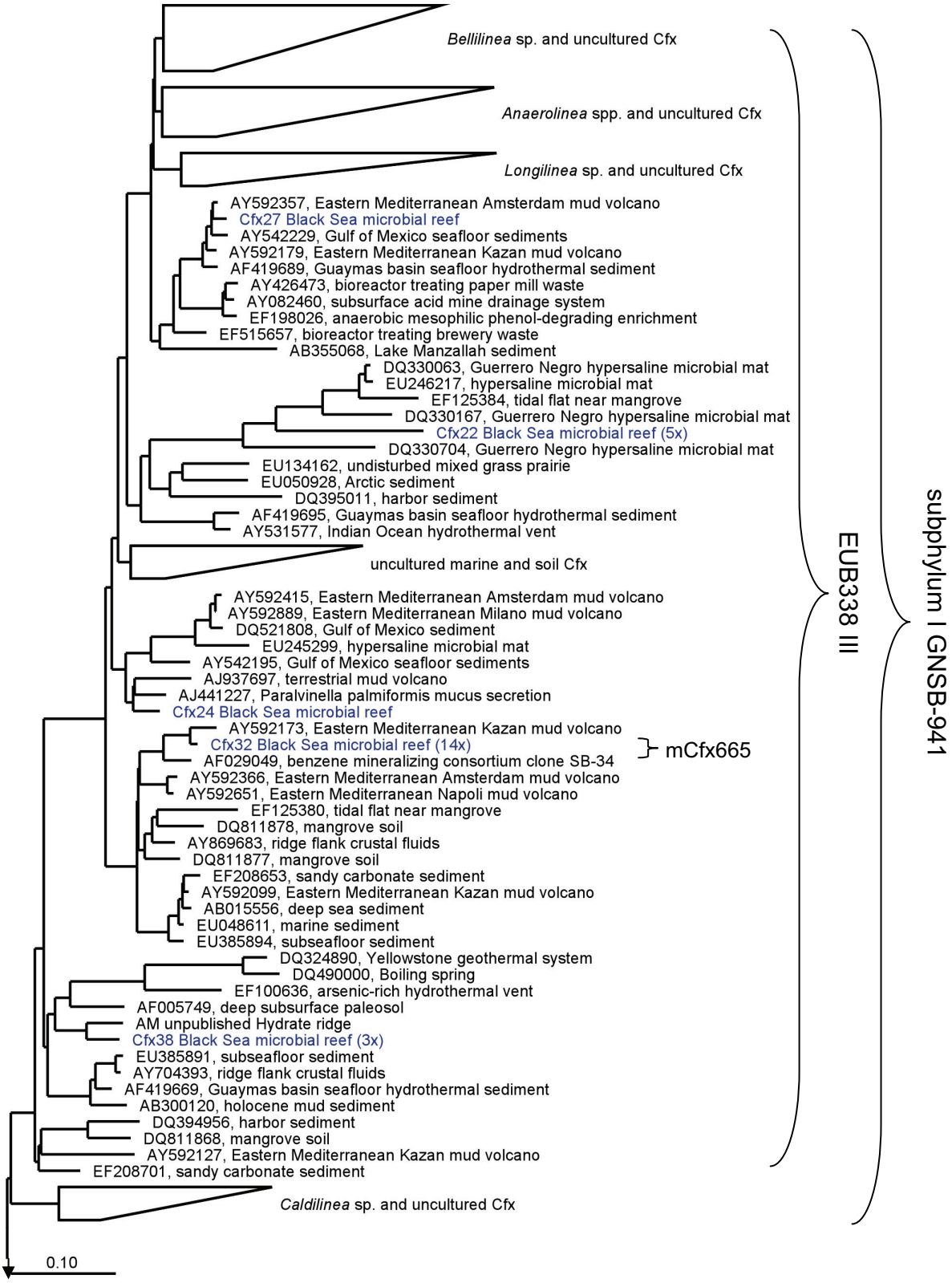
Table 1: Relative abundance of Chloroflexi-like (GNSB-941-positive) cells as revealed by CARD-FISH in different reefs from the northwestern Black Sea

reef	subsample	GNSB-941 relative abundance [%]
P822*	top	9.9
	exterior	9.1
	interior	8.5
P795*	out	n.a.
	mid	n.a.
	center	0.4
P787*	pink	4.7
P784*	brown	6.4
P780*	out	23.2
	mid	27.8
	center	4.5

*: for a detailed description of the sampling site see manuscript II
n.a.: not analyzed (the focus was on the center mat layer forming most of the mats biomass)

Interestingly, they were still found in an AOM bioreactor enrichment, supplied with methane and sulfate, inoculated with Black Sea microbial reef material (reef P784), and operated under high pressure. They were abundant, but inactive in the reactor after a starvation period of two months and numerically abundant, but active after re-supply of methane and sulfate (Deusner et al., in prep). Here, cells were <1 µm in width and up to 100 µM in length (Figure 10a). Long, but not that long filaments were also found in the original reef samples. Most of those might have been destroyed by the harsh homogenization conducted prior to quantification.

In both original complex and enrichment, Chloroflexi often grew thoroughly mixed with ANME-2 (Figures 10b, 11). In ANME-1 dominated samples, Chloroflexi were rare, with one exception (Manuscript II). No signals were detected with a new specific probe (Dhc1252) for reef *Dehalococcoides/Dehalobium*, which might either be explained by a very low abundance or an absence of these cells, or methodological artifacts such as suboptimal permeabilization of their cell walls or target inaccessibility.



-to be continued-

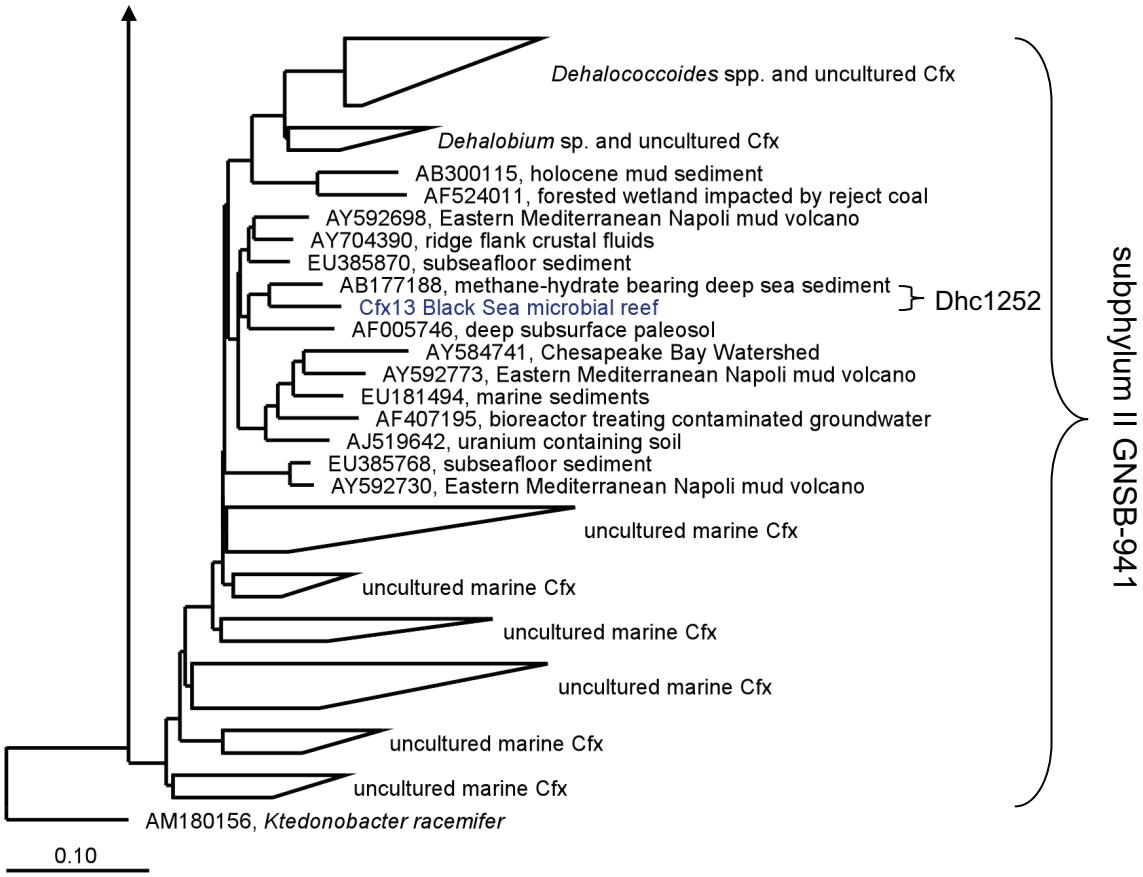


Figure 9: Phylogenetic tree showing affiliations of Black Sea microbial reef Chloroflexi-like 16S rRNA gene sequences (highlighted in blue) to selected reference sequences of Chloroflexi. Number of similar clones (99% sequence similarity) are given in brackets.

Database analyses for Chloroflexi 16S rRNA genes implicate that members of this phylum are globally distributed in various ecosystems such as freshwater and marine sediments including the subsurface, hot springs, sludge, and microbial mats (Björnsson et al. 2002, Hugenholtz et al. 1998, Juretschko et al. 2002, Sekiguchi et al. 1999). Members of the subphylum I are genetically and physiologically diverse (Hugenholtz and Stackebrandt 2004), but they all form long filaments with varying thickness (Sekiguchi et al. 1999, 2001, 2003, Björnsson et al. 2002, Juretschko et al. 2002). They are numerically abundant and important players in various types of anoxic waste water sludge (Björnsson et al. 2002, Juretschko et al. 2002, Yamada et al. 2005). Thermophilic sludge granules that settle well are entirely covered by members of this subphylum suggesting that they play an important role in granule formation (Sekiguchi et al. 1998, 1999, 2001, Yamaguchi et al. 2001). In contrast, mesophilic granules are not always surrounded by filaments. The granule forming filaments heterotrophically

metabolize either primary substrates in the wastewater or cellular matter (Yamada et al. 2005).

Bellilinea caldifistulae, isolated from sludge, is a strictly anaerobic, multicellular filamentous thermophile and can utilize numerous carbohydrates fermentatively (Yamada et al. 2007). *Caldilinea* sp. was isolated from a hot spring sulfur-turf in Japan and shares many features with *B. caldifistulae*, but is facultative aerobic (Sekiguchi et al. 2003). Their growth stagnates when hydrogen accumulates. Co-cultivation with methanogenic hydrogenotrophs promotes growth (Yamada et al. 2007). Growth of *B. caldifistulae* and some *Anaerolinea* spp. is enhanced in co-cultivation with *Methanothermobacter* sp.. *Longilinea* sp. benefits from co-cultivation with *Methanospirillum* (Sekiguchi et al. 2001, Yamada et al. 2007). Sludge granules are formed by consortia of methanogens and Chloroflexi. Interestingly, some aged granules solely host Chloroflexi (Sekiguchi et al. 2001).

This raises the question whether the reef Chloroflexi co-occurring with ANME-2/DSS consortia also produce hydrogen and if yes, whether hydrogen is consumed and by which organism. Methanogens and sulfate-reducers compete for hydrogen in anoxic sediments (Nedwell and Banat 1981, Lovley et al. 1982). Hydrogen has been frequently suggested to act as an electron shuttle in AOM, but this could not be confirmed (Nauhaus et al. 2005). However, the Black Sea consortia might share some features with those forming wastewater sludge granules.

The only cultivated organisms within the subphylum II are *Dehalococcoides* and *Dehalobium*. Isolates are capable of metabolizing chlorinated hydrocarbons, including tetrachloroethene and trichloroethene, which are used as solvents and are major contaminants of soil and groundwater (Galperin 2007). Some *Dehalococcoides* strains can be used for complete detoxification of these compounds (He et al. 2003).

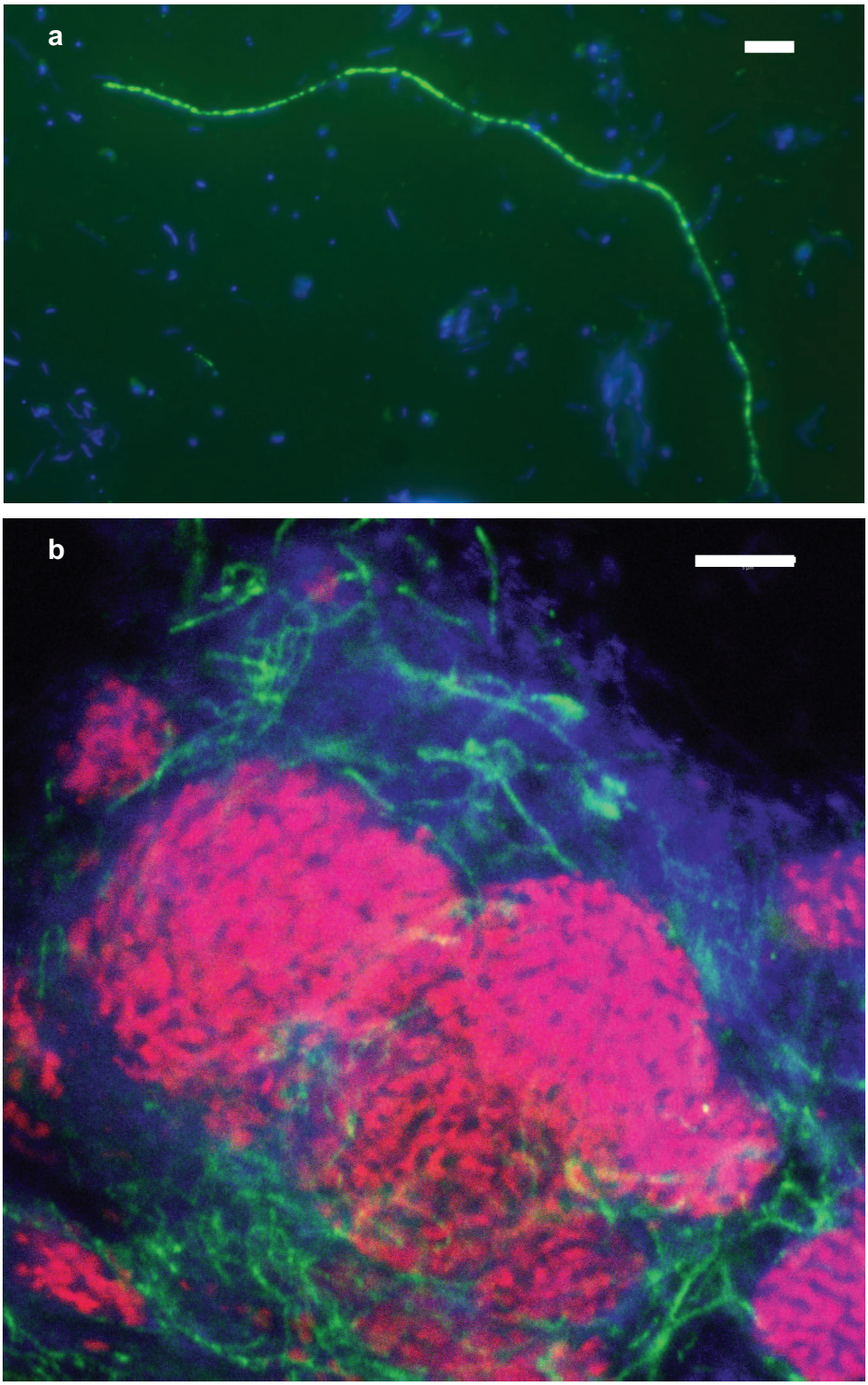


Figure 10: (a) Epifluorescence micrograph and (b) laserscanning micrograph (picture taken by A. Ellrott, MPI Bremen) of Chloroflexi (probe GNSB-941, green) and ANME-2 (probe ANME2-538, red) cells from an AOM bioreactor enrichment of a Black Sea microbial reef mat (P784). All cells are DAPI stained (blue). Scale bars 5 µm.

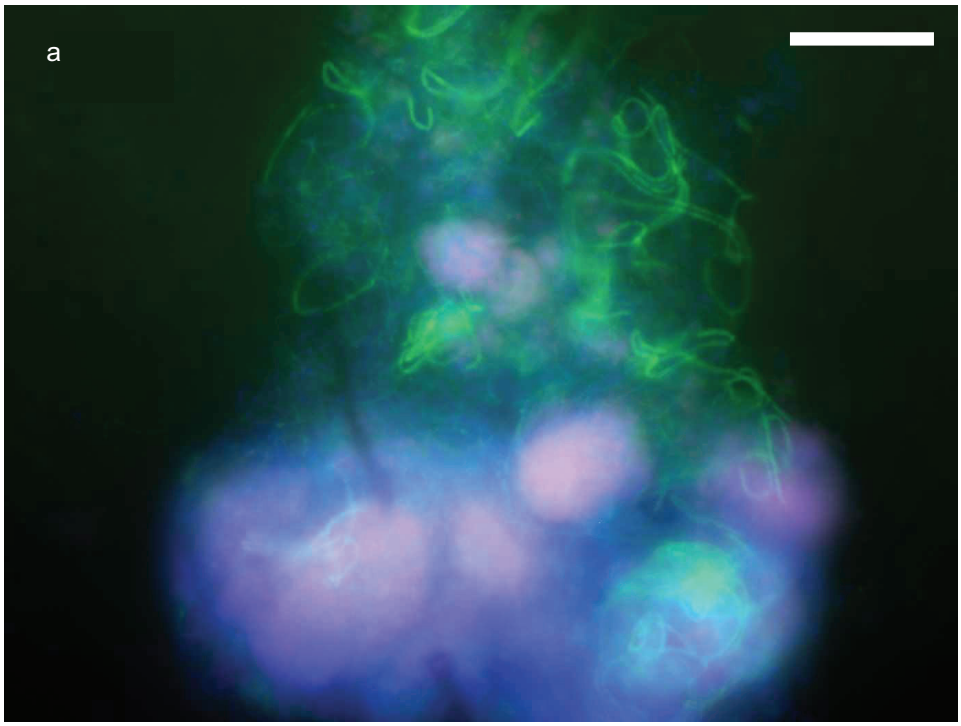


Figure 11: Epifluorescence micrograph of CARD-FISH stained Chloroflexi cells (probe GNSB-941, green) and ANME-2 (probe ANME2-538, red) from an AOM bioreactor enrichment of a Black Sea microbial reef mat (P784). Scale bar 10 µm.

2. Verrucomicrobia – overlooked players in aquatic habitats (manuscript III)

The reef filaments detected with the general verrucomicrobial probe EUB338 III were ultimately shown to be Chloroflexi. However, verrucomicrobial 16S rRNA genes were found in enrichment cultures inoculated with mat material from a Black Sea microbial reef (mat 322/152, obtained during a Meteor cruise M72/2 in 2007), supplied with sugar polymers, and incubated under anoxic conditions at 12 °C (T. Holler and A. Meyerdierks, MPI Bremen, unpublished data), and in several other marine AOM habitats. In a methane-hydrate bearing sediment core of the Mid-Chilean margin, most 16S rRNA genes affiliated with ones of Verrucomicrobia (Hamdan et al. 2008). Recently, Beal et al. (2009) observed an increase of verrucomicrobial 16S rRNA genes in an AOM enrichment culture. Further, it was shown recently that some Verrucomicrobia mediate the AMO (Pol et al. 2007, Dunfield et al. 2007, Islam et al. 2008). These organisms have been intensively studied in soils, but little is known about aquatic Verrucomicrobia, and FISH studies on Verrucomicrobia in nature are rare.

Intrigued by these reasons, published verrucomicrobial 16S rRNA gene sequences were collected, group-specific probes evaluated, and subgroup-specific probes designed to target major subgroups of Verrucomicrobia. In this and previous studies (Wagner and Horn 2006, Schlesner et al. 2006), in which sequence analysis has been conducted, Verrucomicrobia have been implicated to be globally distributed in terrestrial and aquatic habitats. Interestingly, sequences were found at many anthropogenically contaminated sites (Schlesner et al. 2006) suggesting that some members of this group might have potential for bioremediation. Furthermore, Verrucomicrobia might be ecto- and endosymbionts (reviewed by Schlesner et al. 2006), and *Akkermansia* sp. even inhabit human guts (Derrien et al. 2004). Specific hybridization conditions were tested for many of the probes, which target aquatic Verrucomicrobia (Manuscript III, Table 1).

Some of the new probes were tested on environmental samples. In screens of various marine water and sediment samples, Verrucomicrobia were detected only occasionally or often not found at all. Therefore, some of the new probes were tested on water samples of the humic lake Große Fuchskuhle near Berlin, because two studies had indicated that they are abundant in humic lakes (Lindström et al. 2004, Haukka et

al. 2005, 2006, Newton et al. 2006). It was shown that this lake hosts *Spartobacteria*, *Opitutus*, and *Prosthecobacter* spp. accounting for up to 19%, 7%, and 1% of all cells, respectively. The morphotypes detected differed little within the groups, but varied markedly between the three groups. Their abundance varied strongly with the season, as in soils (Buckley and Schmidt 2001). Obviously, they cope with a wide temperature range. The temporal variability might explain why they were rarely detected in random screens of marine samples.

Abundance varied little between oxic and anoxic waters suggesting that all resident Verrucomicrobia are facultative anaerobes or oxygen tolerant obligate anaerobes, as some of their relatives (Schlesner 1987, Janssen et al. 1997). No anaerobic *Prosthecobacter* sp. has yet been described. Verrucomicrobia were more abundant in the less humic basin suggesting that they are not directly involved in humic acid degradation or tolerate, but not prefer the low pH in the southwestern basin. Nevertheless, relatively small populations of *Opitutus* spp. were present, but only during summer, long after the large input of allochthonous matter. Some *Opitutus* sp. utilize pectin (Janssen et al. 1997), an abundant component of humic acids. Lake Große Fuchskuhle sediment seasonally seeps methane (Casper et al. 2003), but the similar distribution of resident Verrucomicrobia in oxic and seasonally anoxic (methane-rich) water suggests that they are not involved in methane degradation. Probably, they feed on polysaccharides. It has been shown for isolates of Verrucomicrobia that they use various polysaccharides (Schlesner et al. 2006). The results suggest that Verrucomicrobia are important components of the microbiota in Lake Große Fuchskuhle, and probably in other aquatic habitats.

3. Methodological aspects

3.1 Coverage of 16S rRNA diversity in marine sediments

For more than two decades, the PCR-based, cultivation-independent comparative 16S rRNA gene analysis has provided molecular ecologists with many insights into the diversity of natural microbial communities (Ward et al. 1990). In this time, a lot was also learnt on the high diversity of marine benthic microbial communities. In total, bacterial genes of more than 30 phyla and 10 candidate phyla were identified by conventional Sanger-sequencing analyses in marine sediments (Figure 12). However, a single pyrosequencing analysis revealed 21 bacterial phyla and 37 candidate phyla in a tidal flat nearby Korea (Kim et al. 2008). Nevertheless, a complete census of all 16S rRNA genes is still not possible (Schloss and Handelsman 2005).

Actinobacteria, Gamma-, and Deltaproteobacteria are found in most 16S rRNA gene libraries constructed with DNA from marine sediment. Also Alphaproteobacteria, Chloroflexi, Bacteroidetes, and Planctomycetes are frequently found. Other groups such as Beta- and Epsilonproteobacteria, Verrucomicrobia, Nitrospirae and Spirochaetes, as well as members of the clusters OP9, OP8, and OP11 are less often identified. Further phyla and candidate phyla have been already detected in low frequency, e.g. Thermoanaerobacter, Deinococcales, Deferribacterales, Treponema, Chlorobia, Fusabacteria, Fusobacteria and many others (Figure 12). The same groups are found in methane-rich marine sediments (e.g. Orphan et al. 2001, Mills et al. 2003, Inagaki et al. 2006, Lloyd et al. 2006, Lösekann et al. 2007, Wegener et al. 2008, Beal et al. 2009). Archaea seem to be on the phylum level less diverse than Bacteria. On the other hand, the archaeal diversity may be yet under-sampled due to primer biases, at least in deep marine subsurface sediments (Teske and Sørensen 2008).

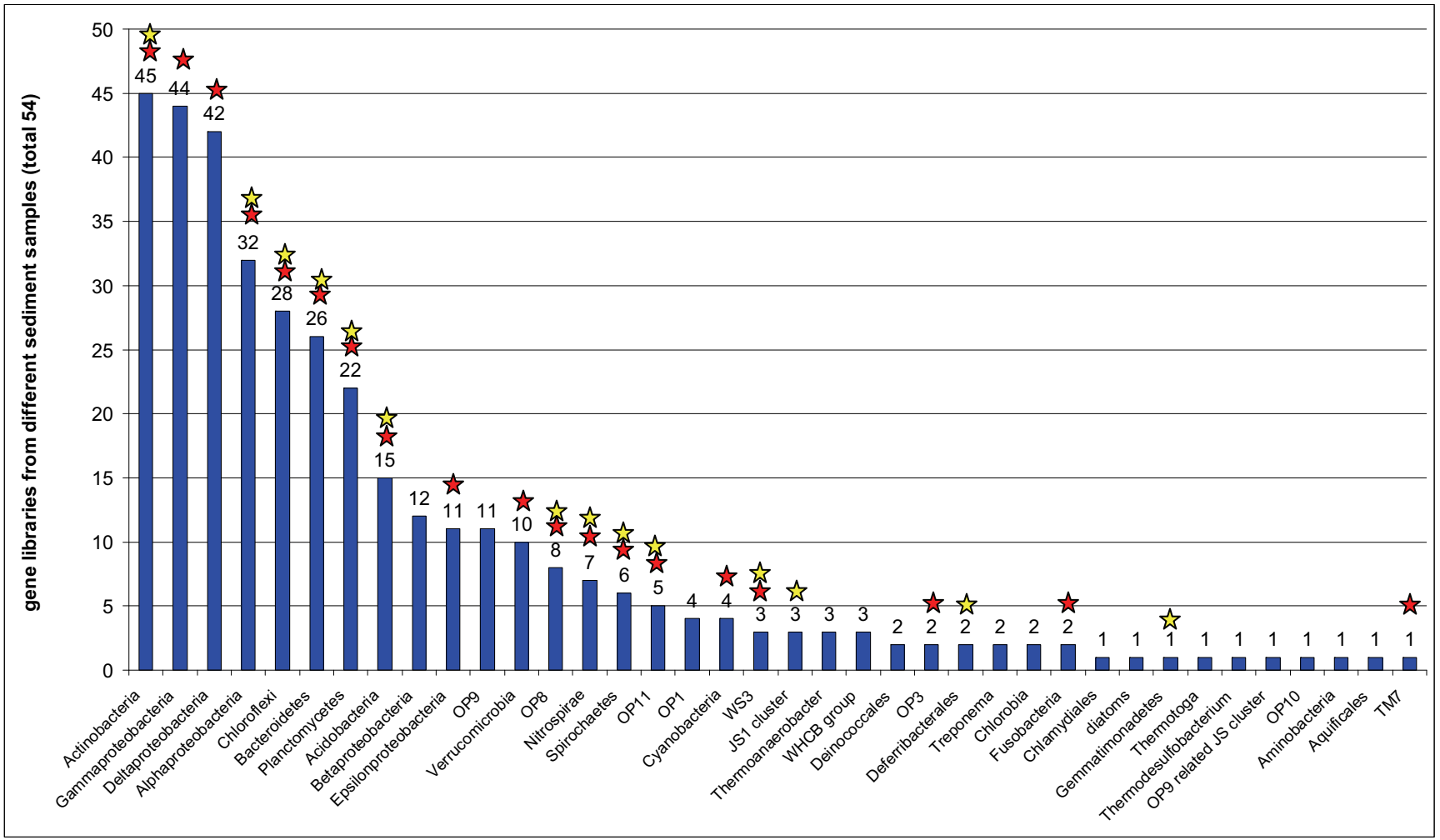


Figure 12: Presence of bacterial (candidate) divisions in 16S rRNA gene libraries from diversity studies on marine sediments. (Bowman and MacCuaig 2003, Musat et al. 2006, Gillan and Pernet 2007, Gray and Herwig 1996, Hunter et al. 2006, Hamdan et al. 2008, Ravenschlag et al. 1999, Lösekann et al. 2007, Kormas et al. 2008, Heijs et al. 2006 (carbonate crusts), Kim et al. 2008, Liang et al. 2006, Asami et al. 2005, Urakawa et al. 1999, 2000, Zhang et al. 2008, Reed et al. 2002, Rochelle et al. 1994, Inagaki et al. 2003, Li et al. 1999, Newberry et al. 2004, Hongchen et al. 2007, Marchesi et al. 2001, Tanner et al. 2000, Teske et al. 2002, Bidle et al. 1999, Mason et al. 2007 (basalts), Santelli et al. 2008). The summary includes studies with a focus on microbial diversity in marine sediments. Only studies with a detailed description of all phyla present in a single library were included in the analysis. Red and yellow stars highlight phyla found at the Hikurangi margin and in Black Sea microbial reefs.

In this thesis, conventional 16S rRNA gene libraries from Black Sea microbial reef mats and cold seeps at the Hikurangi margin (New Zealand) were constructed. The recovered diversity of Bacteria was high compared to other studies based on conventional Sanger-sequencing (up to 19 versus averaged 10 phyla per library). However, all phyla have been formerly found in marine sediments (Figure 12). The high diversity observed could be habitat specific, but was more likely due to the method used. DNA was extracted following protocols of Lueders et al. (2004) and Zhou et al. (1996), appropriate for DNA extraction from microbial mats and sediments, respectively. DNA was amplified in twenty replicates and only 15 PCR cycles, which likely reduced the PCR bias and increased the diversity.

To date, clone-library based microbial diversity studies are hardly comparable, because samples are (i) differently sampled, and stored, (ii) DNA is differently extracted, purified, amplified, cloned, and sequenced, (iii) sequences are analysed and data presented differently. Sediment descriptions should include at least coordinates, water and sediment depths of the sampling site and sampling time. Sediments are mostly frozen at -20 °C, ideally, at -80 °C until further processing. DNA from marine sediments is mostly extracted according to the method described by Zhou et al. (1996). Luna et al. (2006) recommend combined use of different DNA extraction methods with various principles of cell lysis for diversity studies. Various DNA purification kits are used, but few are made for soil/sediment samples. The most frequently used bacterial primer pair is 27F/1492R (Lane et al. 1985, Lane 1991/Stackebrandt and Liesack 1993). However, novel polymerases allow to use shorter primers broadening the range of PCR detectable sequences (Isenbarger et al. 2008). Acinas et al. (2005) showed that with more PCR replicates and reduced PCR cycle numbers, the PCR bias can be reduced. Nevertheless, genes from marine sediments are to date still often amplified with 30 or more cycles. In many habitats, species diversity can unlikely be covered by less than 400 sequences (Curtis et al. 2002, Gans et al. 2005, Torsvik et al. 1990). Still, on average only 60 sequences are analysed from different samples of marine sediments. New sequencing technologies will offer more opportunities for microbiologists, but also bring new challenges in data storing and mining (Mac Lean et al. 2009).

3.2 Quantification of marine benthic microorganisms

3.2.1 Set-up of a real time PCR assay

Real time PCR is a frequently used method for high-speed and high-resolution quantification of specific genes. It is often also used to quantify specific cell numbers. One aim of this thesis was to set up a real time PCR assay for a rapid quantification of the 16S rRNA genes of the various ANME groups. Primer sets and TaqMan probes are listed in Appendix Table 2. Probe target sites are almost identical to those of commonly used FISH probes.

Cloned 16S rRNA genes of ANME subgroups were used as standards, because no pure cultures are yet available. Full complementary and mismatch clones were selected for all primers and probes (Appendix Tables 3, 4). Fragments of the expected length were amplified with all primer pairs (Appendix Table 2). Temperature gradients were conducted to determine the optimal annealing temperatures for the primers and revealed that discrimination against one mismatch was impossible without accepting a significant loss of product.

The primer/probe set for ANME-3 was tested in detail with the TaqMan approach. After initial optimization, the amplification could be monitored (Figure 13). The detection showed two problems: nonlinearity in threshold cycle (Ct) values of the dilution series and a late Ct value. This indicates poor PCR efficiency, which can have several reasons: (i) the PCR annealing/extension time might have been too short, (ii) the PCR annealing/extension temperature too high, (iii) PCR annealing/extension temperature too low, (iv) primer concentrations or ratio suboptimal, (v) primers or probe degraded or probe bleached, (vi) primer-probe ratio suboptimal, (vii) template degraded, (viii) PCR product too long (ix) $MgCl_2$ concentration suboptimal, (x) pipetting errors, or (xi) the primer and probe design might have been suboptimal.

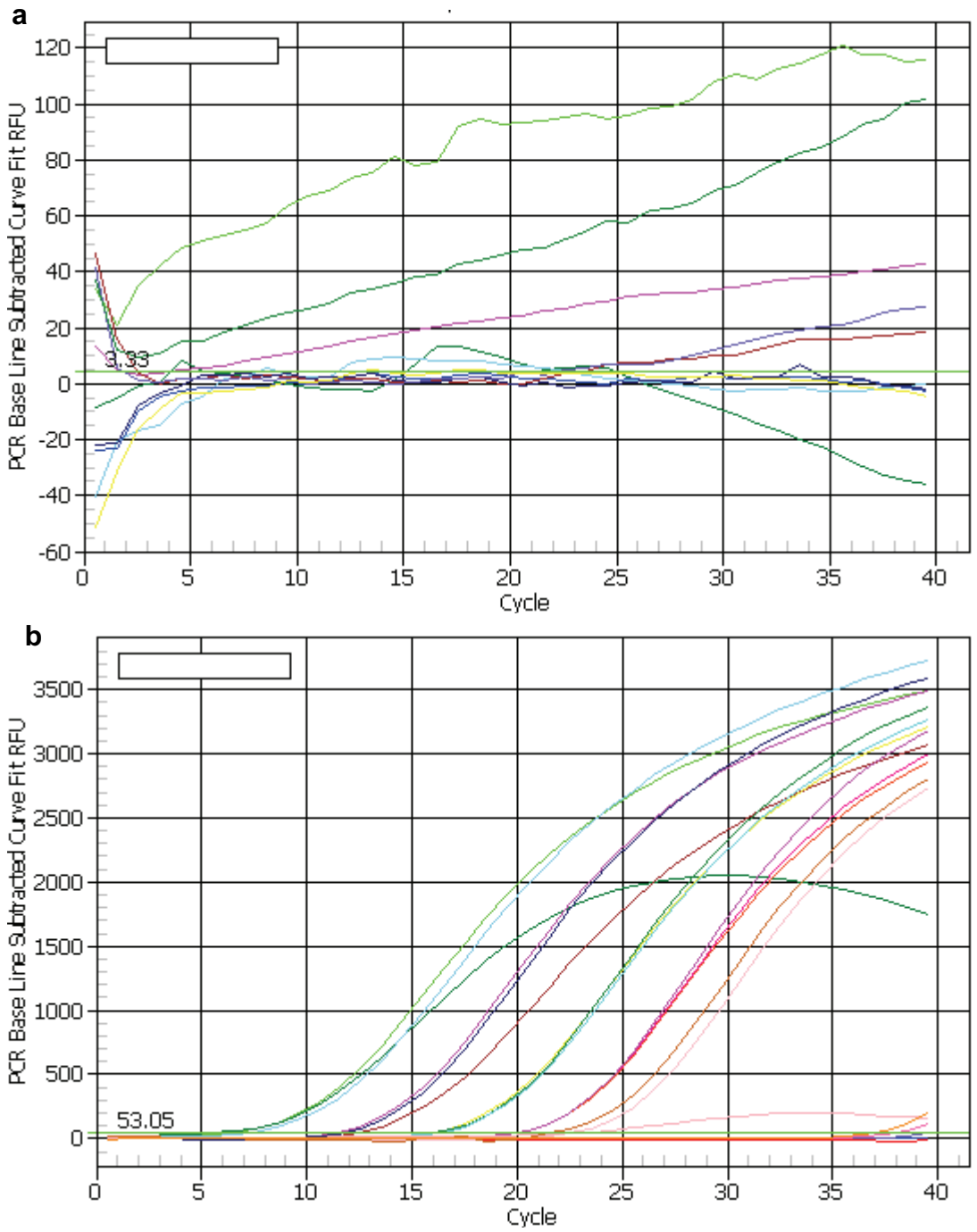


Figure 13: Amplification charts of the (a) first and (b) last real time PCR run using ANME-3 specific primers to amplify cloned 16S rRNA genes of ANME-3.

Different annealing/extension time (i) and temperatures (ii, iii) were tested in two and three step protocols. Short fragments (<300 base pairs) are commonly elongated in less than 1 min (Newton and Graham 1994). However, best results were obtained with an elongation time of 6 min according to Takai and Horikoshi (2000). (iv) Different primer ratios were tested following manufactures` recommendations resulting in major differences. The ratio resulting in the highest efficiency (50 nM reverse and 300 nM forward primer) was chosen for further runs. (vii) The use of different templates (plasmid and amplicon) made little difference. (viii) Real time PCR targets are ideally 80 to 150 base pairs long, but with adjusted reaction times up to 500 base pairs can be efficiently amplified. The ANME-3 amplicon was ~300 base pairs long. (ix) Addition of $MgCl_2$ was tried as well, although it is known that specificity is reduced. Efficiency was not improved. (x) Triplicates were mostly comparable and R^2 values of the standard curves were always >0.9 excluding pipetting errors.

In the TaqMan approach, nonlinearity in Ct values of the dilution series can be caused by probes that form secondary structures. However, these were not predicted by bioinformatic probe analysis (see Appendix 2). Probes have to bind to their target before the primer elongates the strand or the abundance will be underestimated. Probes were designed to have melting temperatures of only 3 to 5 °C instead of 10 °C higher as recommended to avoid unspecific bindings. To simplify trouble-shooting and to exclude possible problems with the probe the SybrGreen approach was further used testing the primers only. The efficiency was still poor.

Instead of further testing the new primers, the set up was tested by using a general archaeal primer/probe set developed by Takai and Horikoshi (2000). With this set, the amplification was almost linear, but the Ct value was still late, which might be caused by PCR inhibitors. The purified plasmid and PCR products used as templates were assumed to be of high quality. Nevertheless, different DNA purification procedures were tested improving efficiency only little. The DNA purity is a crucial factor in real time PCR, but the purification method of choice should yield as little DNA loss as possible. Another critical factor for real time PCR is the DNA extraction step. Different methods result in distinct diversity patterns and thus gene number variability (de Lipthay et al. 2004, Smith et al. 2006). The ANME-3 primer pair developed in this thesis was probably

suboptimally designed. A second primer pair was not yet tested. The reasons for the failure of a quantitative PCR for ANME-3 could not be found in this thesis. A real time PCR assay for ANME detection was recently published (Miyashita et al. 2009).

Very few studies have compared cell enumeration by FISH and real time PCR (e.g. Suzuki et al. 2000, Wuchter et al. 2006). However, cell numbers obtained by real time PCR should theoretically be larger than ones determined by FISH. First, cells with few or no ribosomes and missed by FISH should also be detected. Second, two to several genomes are found in dividing cells and only few studies compare cell and genome numbers (Becker et al. 2002). Third, the amount of DNA released from cells can be significant in the marine system (Paul et al. 1987). On the other hand, DNA is lost during extraction and purification prior to real time PCR. DNA loss can be estimated (Dionisi et al. 2003), but estimates are rarely included in the analyses. Cell numbers determined by DNA-staining techniques and real time PCR with universal or bacterial and archaeal primers should be better comparable, but allow no high taxonomic resolution.

Further biases are introduced by oligonucleotide sensitivity and specificity (Sipos et al. 2007, Bru and Philippot 2008). Moreover, cell enumeration via real time PCR requires knowledge on the gene copies encoded in the genomes of the target cells (for rRNA most Bacteria have between 1 and 12 genes; Fogel et al. 1999). For complex communities, a prediction seems impossible.

Therefore, all attempts for quantification of absolute gene numbers in environmental DNA and even more of absolute cell numbers need to be treated cautiously (Chandler 1998, Raeymaekers 2000, Smith et al. 2006, Bustin 2002). Nevertheless, real time PCR is invaluable for environmental microbiology, because it is yet the most appropriate tool to quantify genes in complex DNA mixtures and permits studies on rare genes undetectable with other methods.

3.2.2 Potential and limits of CARD-FISH

CARD-FISH is a valuable tool to study benthic microbes, because it allows detection of cells containing low numbers of ribosomes, such as starving or dormant cells, which are abundant in aquatic habitats (Morita 1997). In this thesis, microbial abundance was studied by epifluorescence microscopy after CARD-FISH on samples from Black Sea microbial reef mats, Hikurangi margin sediments, and Lake Große Fuchskuhle waters. Total microbial abundance was averaged 10^{10} cells ml^{-1} in the mats, 10^{9} cells ml^{-1} in the seep sediments, and 10^{6} cells ml^{-1} in the waters, which is in the common range for these habitats (Whitman et al. 1998). In the mats, CARD-FISH detection efficiencies with a probe set covering all domains ranged from 50 to 100%. In water samples, they were around 90%. The remaining DAPI-stained cells were either dead, or undetectable by CARD-FISH due to too low rRNA contents, target site inaccessibility or suboptimal cell wall permeabilization.

In theory, cell numbers determined by CARD-FISH should always be lower than those determined by DAPI staining, because cells that contain ribosomes should also have DNA. In the Hikurangi margin sediments studied in this thesis, numbers determined by CARD-FISH were higher, because DAPI signals were frequently too weak to distinguish cells from background. This might be due to the limitation of the DAPI dye or high sensitivity of CARD-FISH. To test this hypothesis and make more cells visible, cells were first hybridized with archaeal and bacterial HRP-probes and the HRP fed with Alexa350 tyramides (fluoresces like DAPI) before probing the cells of interest, but CARD-FISH detection rates still exceeded 100% suggesting that the limiting factor is the DAPI/Alexa350 dye. Consequently, CARD-FISH counting was uncoupled from DAPI counting. Total numbers were compared to cell numbers separately determined by acridine-orange-staining. The comparison revealed that some cells invisible by DAPI staining can still be stained with acridine-orange, but some cells are only stainable by the highly sensitive CARD-FISH.

However, detection is limited by too low rRNA contents and low cell numbers. The detection limit is further related to the dyes` signal intensities. More intense dyes or sensitive detection could help to better distinguish cells from background. Alternatively, background could be removed, e.g. by hydrofluoric acid treatment (Morono et al. 2009).

The comparability of CARD-FISH studies on marine benthic microbes is limited because cells are differently fixed, detached from particles, permeabilized, hybridized, and counted. The optimal fixation varies for different samples and cells. Sediment samples are mostly sonicated to detach cells from particles (Epstein and Rossel 1995, Epstein et al. 1997). Sonication can result in a loss of cells. CARD-FISH protocols include chemical or enzymatical permeabilization of the cell walls to open cells for the uptake of HRP-labeled probes. Also the sonication might already permeabilize the cells. Optimal permeabilization differs for distinct target groups due to the various structures of microbial cells. Cell walls can be composed of peptidoglycan, pseudopeptidoglycan, polysaccharides, glycoproteins, or protein. Some Bacteria cells have no cell wall at all (Madigan and Martinko 2003). The permeabilization of broad groups such as all Archaea or Bacteria is difficult, and the optimal protocol is the one permeabilizing most cells.

The exact quantification of microbial cells remains a challenging task (Amann and Fuchs 2008). However, quite often the microscopic analysis of (CARD)-FISH stained cells provides the least biased method to quantify specific microorganisms, and it provides furthermore insights into cell morphologies and sizes, as well as exact localizations to other cells and particles.

3.3 Construction of clone-FISH references by cloning probe targets

Melting temperatures for FISH probes are determined by hybridizing the probes against their 16S rRNA complements. For many probes, cells with the target RNA are not available in pure culture. Alternatively, probes can be evaluated by clone-FISH (Schramm et al. 2002). With this technique, probes are hybridized against 16S rRNA transcribed by *Escherichia coli* hosts from genes carried in plasmids. Unfortunately, plasmids carrying the 16S rRNA gene of interest are also not always available. Here, we tested a protocol to clone short *in vitro* synthesized 16S rRNA gene fragments for subsequent clone-FISH. The advantage of this method is that probes can be hybridized against their rRNA target. The disadvantage is that the target has not its original secondary structure.

As starting material, we used a plasmid carrying the 16S rRNA gene of *Congregibacter litoralis* (strain KT-71). This gene was chosen, because KT-71 is a *Gammaproteobacterium* as the clone-FISH host *E. coli*. Its genome has been completed (Fuchs et al. 2007). The plasmid was enzymatically linearized at a unique restriction site, located between the T7-RNA polymerase binding site and the 16S rRNA gene (Appendix B3.3). Subsequently, a DNA oligonucleotide encoding the probe target site was ordered and hybridized with a complementary DNA oligonucleotide. The hybrid was designed to have bases at both ends (linker bases), which are complementary to the sticky ends of the linearized plasmid.

GCATGCGTGGAGGATAAGGTCTTCGCATGCTCCC
CGTACGCACCTCCTATTCCAGAAGCGTACGAGGG
(black: sticky ends of linearized vector, green: hybrid, red: linker bases)

The hybrid was ligated into the linearized plasmid, and the plasmid transformed and amplified in *E. coli* hosts. Only 2% of all clones (in total 50) carried the modified plasmid. Efficiency might be optimized by higher insert to plasmid ratios, higher salt concentrations within the ligation buffer, cooling of the linarized plasmid before adding the insert etc..

Chimeric sequence:

…T7 binding site-
GGGCGATTGGGCCCGACGTCGCATGCGTGGAGGATAAGGTCTTCGCATGCTCCCGGCCGCCATGG
CGGCCGCGGGAATTCGATTAGAGTTTGATCATGGCTCAGATTGAACGCTGGCAGGCCTAACACATG
CAAGTCGAGCGCGAAAGTACTTCGGTATGAGTAGAGCGGCGGACGGGTGAGTAACGCGTAGGAAT
CTATCCAGTAGTGGGGGACAACTCGGGGAAACTCGAGCTAATACCGCATACGTCCTAGGGGAGAAA
GCGGGGGATCTTCGGACCTCGCGCTATTGGAGGAGCCTGCGTTGGATTAGCTAGTTGGTGGGGTAA
AGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAGACA
CGGCCCGGACTCCTACGGGAGGCAGCAG…

(black: vector sequence, green: desired oligonucleotide, red: linker bases, blue: GM3 sequence, grey: 16S rRNA gene sequence of strain KT71)

The cells were used for clone-FISH as described previously (Schramm et al. 2002) with minor modifications: NovaBlue (DE3) cells were used following manufacturers` recommendations. Melting curves conducted for a newly designed probe (VP403) with the constructed clone and a pure culture as targets were highly comparable (Figure 14). The hybrid was ligated close to the cloned 16S rRNA gene of KT71 to obtain *in vitro* transcripts with the typical 16S rRNA length.

Any desired oligonucleotide may be ordered with the linker bases on both ends, hybridized against its complement, and ligated into linearized plasmids of KT-71 to evaluate probes, for which original clones are not available.

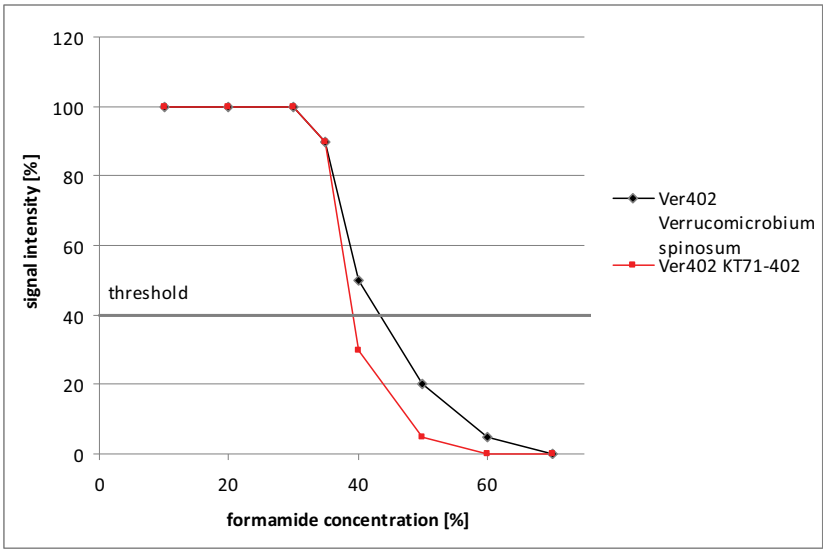


Figure 14: FISH melting curves of probe VP403. Black curve: *Verrucomicrobium spinosum* pure culture. Red curve: chimeric construct KT71-VP403 transcribed in *E. coli* host (graph from M. Winkel, “Projektarbeit” for the University of Bremen).

3.4 Flow sorting of ANME for genomic analysis

Many insights have been gained into the (meta-) genomes of ANME-1 and ANME-2 (Hallam et al. 2003, 2004, Krüger et al. 2003, Meyerdierks et al. 2005, Pernthaler et al. 2008), but so far nothing was known about the ANME-3 genome. Therefore, the aim of this study was to sort ANME-3 cells by flow cytometry for subsequent genome analysis.

An enrichment culture of ANME-3 cells from the Haakon Mosby mud volcano (Barents Sea) was provided by T. Holler (MPI Bremen). Sampling site and culture conditions are in detail described by Niemann et al. (2006) and Nauhaus et al. (2002), respectively. Previous experiments showed that DNA can be efficiently recovered from ethanol-fixed cells of *Methanosarcina mazei*, which are closely related to ANME-3 (K. Knittel, unpublished data). Consequently, material fixed with ethanol was used. Fixed cells were detached from residual sediment particles by sonication, identified by CARD-FISH in liquid, and sorted by flow cytometry (B. Fuchs, MPI Bremen).

Signals of FISH-stained ANME-3 cells were weak, likely because the enriched cells were inactive. In contrast to the original complex, in which most ANME-3 cells were aggregated, only single cells were detected. Therefore, CARD-FISH was used for the identification of the cells (Appendix B3.4). With this technique, cells were successfully sorted. Over 75% of sorted cells were microscopically identified as coccoid ANME-3. Around 6% were rods without a probe signal. The remaining 20% of DAPI-stained signals were coccoid and small (~0.1 µm in diameter). They might derive from Bacteria, viruses, or autofluorescent particles.

A purity check by 16S rRNA gene sequencing of the sorted cells revealed only genes similar to 16S rRNA genes of ANME-3 (accession number AJ704650) and DSS (accession number AJ704697) from the Haakon Mosby mud volcano (Lösekann et al. 2007). The DSS genes probably derived from the sorted rods. No bacterial gene other than that of the DSS was amplified (96 clones sequenced).

Sorted cells were sent to the Center for Genomic Sciences, Allegheny Singer Research Institute (Pittsburgh, USA) for multiple displacement amplification and 454-pyrosequencing. In total, 3.6 Mbp were sequenced. A first assembly of the sequence data resulted in a high number of contigs (1511) with relatively short contig sizes

(average 1867 bases). Approximately 5000 open reading frames were predicted in the dataset (K. Knittel and A. Meyerdierks, MPI Bremen, personal communication).

Initial sequence analysis identified genes almost identical to 16S/23S rRNA and *mcr* genes of ANME-3 from the Haakon Mosby mud volcano (Lösekann et al. 2007), several genes involved in methanogenesis, and many genes with highest similarities to those of *Methanococcoides burtonii*, the most closely related cultured organism, of which the genome has been fully sequenced (K. Knittel, A. Meyerdierks, and H. Teeling, MPI Bremen, unpublished data). No bacterial 16S rRNA genes were found in the dataset. These results confirmed the high purity of sorted ANME-3 and suggest that most of the obtained data derived from ANME-3. The sequence assembly is currently optimized based on the assumption that different ANME-3 populations were sorted and to exclude chimeric sequences, which are present in the actual dataset.

4. Outlook

New Zealand. The comparison of two novel cold seeps sites, namely the SOB and Polychaete site, from the southern end of the Hikurangi margin revealed differences in the distribution of AOM hot-spots and in the composition of microorganisms.

Investigations of further SOB and Polychaete sites located in the mid and at the northern end of the Hikurangi margin would provide insights into the stability of these systems and biogeographical distribution of the seep communities. Comparisons of total (including fossil) and intact ANME lipids would be interesting and might reveal insights into the "activity" of ANME at different sites. It remains speculative if the Polychaete site represents an early seep stage as hypothesized by Sommer et al. (2008). Repetitive *in situ* monitoring of habitats would reveal whether SOB settle at Polychaete sites, vice versa, or if at all. The polychaetes survived recovery of cores on board and might also be studied *in vitro* on a long-term basis. Many questions remain, e.g. are the worms detritus feeder, how do they influence the microbial community, and are both directly interacting?

Chloroflexi. Filamentous Chloroflexi related species were identified as new key players in Black Sea microbial reefs. They are probably associated with ANME-2/DSS consortia. 16S rRNA sequence analyses reveal that they might also be important in other AOM habitats and marine sediments in general.

Newly developed probes can now be tested for a global survey of these organisms to identify further hot-spots. Additional specific probes should be designed to target broad marine Chloroflexi subgroups. The reef sample, in which Chloroflexi accounted for almost 30% of all cells should be used for further analyses. This project will be continued by K. Kubo (MPI Bremen), who has recently constructed a metagenomic clone library of this sample. Cells from this sample will be isolated by flow sorting for genomic analysis. Another aim should be the isolation/enrichment of the filamentous Chloroflexi. The presence of a pure culture would allow to prove hypotheses suggested by the genome data.

Verrucomicrobia. It was shown in this work that diverse Verrucomicrobia are abundant in a humic lake. These results are of interest for limnologists. It would be interesting to investigate whether non-humic lakes also host these organisms and to continue the screening of freshwater systems.

The seasonal variability of Verrucomicrobia might complicate the determination of hot-spots. Real time PCR and slot blot hybridization are used to detect soil Verrucomicrobia (Lee et al. 1996, Buckley and Schmidt 2001) and could probably be used also to determine hot-spots of aquatic Verrucomicrobia. Genomes of several Verrucomicrobia isolates have recently been fully sequenced providing a useful base for further genomic analyses and to generate hypotheses regarding potential functionality, which could be further tested in laboratory and field experiments.

Methodological aspects. Quantification of microorganisms in nature, especially in sediments, remains a challenging and time-consuming task. Selection of hot-spots for in-depth studies benefits from high-speed screening tools, which are commonly based on DNA/RNA analyses. To provide a first impression of such sites, these tools do not need to be numerically precise, though they must provide unbiased outcomes. One must be cautious in assuming that samples are truly comparative with respect to the quality of DNA/RNA extract and purity. Nucleic acids obtained from sediments are of poor quality requiring improved protocols which enhance high quality amounts of nucleic acids. It is important to ensure a common sample quality “starting point”, before proceeding with downstream applications (e.g. considering inhibitors in DNA/RNA based screening tools). Without such caution, one might miss the true hot-spots. Addressing multiple primer/probe binding sites for one target and several targets would confirm specificity of molecular screening tools.

Numerically abundant populations have commonly been assumed to play important roles in the environment, but they are not necessarily active at the time of sampling. Moreover, recent studies showed that also small populations can solely drive entire processes (Kuypers et al. 2003, Musat et al. 2008). In the future, more studies will determine the activity of cells in relation to their environmental control.

A protocol to localize methane incorporation of ANME *in situ* by NanoSIMS technology is currently optimized (G. Wegener, MPI Bremen, unpublished data).

Metabolic activity of specific cells can also be studied by combining FISH and microautoradiography (MAR-FISH, e.g. Lee et al. 1999). Experiments to simultaneously detect ANME 16S rRNA and some of their functional genes by gene-FISH have been initiated (C. Moraru and A. Meyerdierks, MPI Bremen, unpublished data). First attempts to detect *mcr*-transcripts by mRNA-FISH after the method described by Pernthaler and Amann (2004) have failed, probably, because the probes used were to long (T. Lösekann 2006). J. Milucka has recently developed a method to combine FISH of ANME 16S rRNA with immuno-staining of MCR proteins.

Information on the genome, transcriptome, and proteome levels linked to phylogenetic information and environmental parameters would allow an *in situ* study of a cell's physiological potential, and its immediate and medium-term activity, respectively (Figure 15).

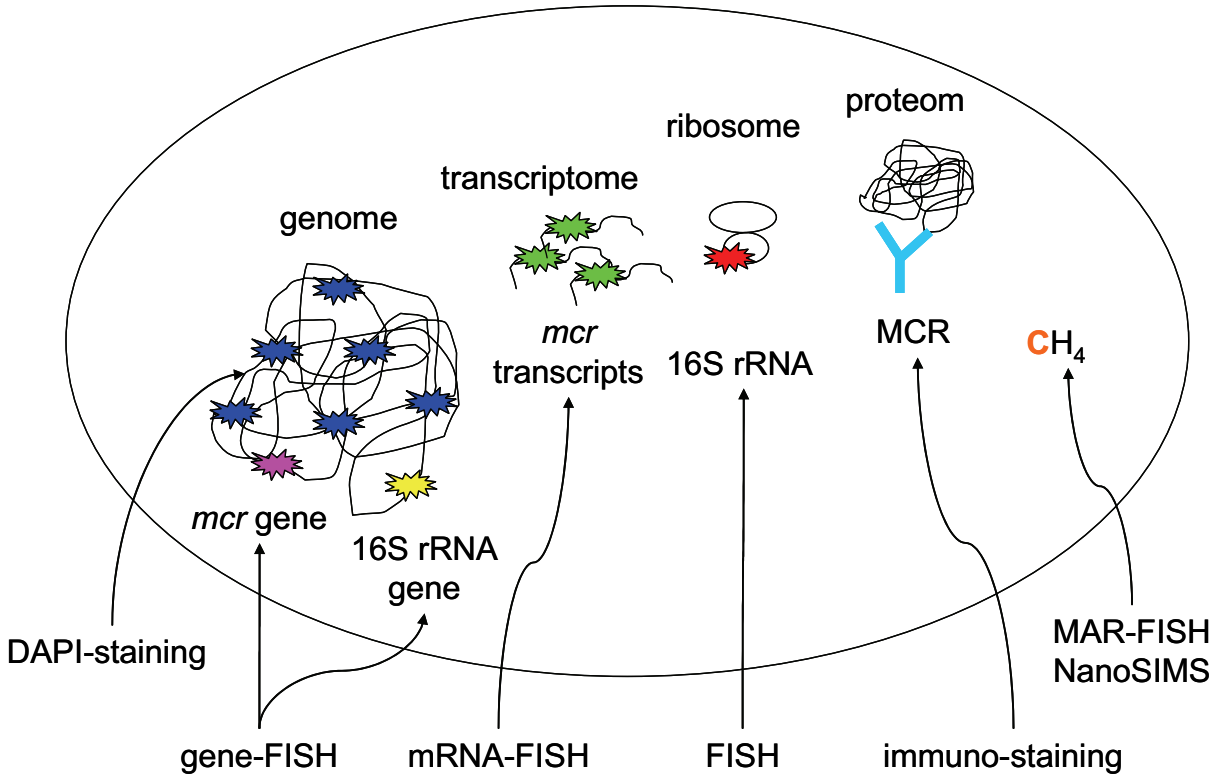


Figure 15: Visionary scheme of a multifaceted intracellular staining technique. Scheme depicts an ANME cell. Protocols for some combinations of some techniques (simultaneous hybridization of mRNA and rRNA, Pernthaler and Amann 2004; single genes and rRNA, C. Moraru, MPI Bremen, unpublished data; immuno-staining and rRNA FISH, J. Milucka, MPI Bremen, unpublished data) have been already established, but are not yet standardized. The combination of all techniques to study a single cell would yield new insights.

C. Publications
1. Publication list

1. **Methanotrophic communities from novel cold seeps at the continental Hikurangi margin (New Zealand`s North Island)**
   J. Arnds, K. Knittel, G. Wegener, H. Niemann, A. Boetius, and R. Amann
   in preparation
   Sampling, molecular analysis and data evaluation, writing of a first manuscript draft.
2. **Polyphasic comparative analysis of microbial reef communities along the northwestern Black Sea shelf** J. Arnds and P. Rossel, K. Knittel, T. Treude, T. Holler, M- Elvert, K.-U. Hinrichs, A. Boetius, and R. Amann
   in preparation
   Molecular analysis and data evaluation, writing of a first manuscript draft.
3. **Development of a 16S rRNA-targeted probe set for Verrucomicrobia and its application for fluorescence in situ hybridization in a humic lake**
   J. Arnds, K. Knittel, U. Buck, M. Winkel, and R. Amann
   Systematic and Applied Microbiology, in press
   Molecular analysis and data evaluation, writing the manuscript.

Not presented in this thesis:

4. **Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria**
   P. Rossel, J. Lipp, H. Fredricks, J. Arnds, A. Boetius, M. Elvert, and K.-U. Hinrichs
   published in Organic Geochemistry 39 (2008) 992–999
   CARD-FISH analysis and data evaluation.

Abstract: Previous biomarker studies of microbes involved in anaerobic oxidation of methane (AOM) have targeted non-polar lipids. We have extended the biomarker approach to include intact polar lipids (IPLs) and show here that the major community types involved in AOM at marine methane seeps can be clearly distinguished by these compounds. The lipid profile of methanotrophic communities with dominant ANME-1 archaea mainly comprises diglycosidic GDGT derivatives. IPL distributions of microbial communities dominated by ANME-2 or ANME-3 are consistent with their phylogenetic affiliation with the euryarchaeal order Methanosarcinales, i.e., the lipids are dominated by phosphate-based polar derivatives of archaeol and hydroxyarchaeol. IPLs of associated bacteria strongly differed among the three community types analyzed here; these differences testify to the diversity of bacteria in AOM environments. Generally, the bacterial members of methanotrophic communities are dominated by phosphatidylethanolamine and phosphatidyl-(N,N)-dimethylethanolamine species; polar dialkylglycerolethers are dominant in the ANME-1 community while in ANME-2 and ANME-3 communities mixed acyl/ether glycerol derivatives are most abundant. The relative concentration of bacterial lipids associated with ANME-1 dominated communities appears significantly lower than in ANME-2 and ANME-3 dominated communities. Our results demonstrate that IPL analysis provides valuable molecular fingerprints of biomass composition in natural microbial communities and enables taxonomic differentiation at the rank of families to orders.

5. **Anaerobic methanotrophic communities are non-sensitive to process shifts in high-pressure long-term batch and continuous incubation**

   C. Deusner, K. Knittel, J. Arnds et al.

   in preparation

   CARD-FISH analysis of Chloroflexi.

Anaerobic oxidation of methane (AOM) was studied in high-pressure gas phase-free continuous long-term incubation at 15 MPa with approximately 60 mM of dissolved methane using naturally enriched biomass from microbial mats from the Black Sea at a hydraulic retention time of 35 h.
AOM rates reached 6.0 $mmol \cdot g_{DW}^{-1} \cdot d^{-1}$ as measured by sulfide production and sulfate consumption. After the startup period the effluent sulfide concentration increased steadily in consequence of complete biomass retention. The highly active biomass was insensitive to rapid changes in incubation conditions resulting from process shifts between continuous and batch operation with varying substrate supply. The accumulation of dissolved sulfide of up to 18 mM did not influence AOM rates. The biomass could survive a starvation period without methane supply of 2.5 months without loosing its viability. The detected ANME cells maintained a high rRNA content as confirmed by FISH with mono-labeled probes. Also, mono-labeled FISH indicated decreased activity of Chloroflexi spp. after biomass starvation suggesting that the organism activity in AOM systems is enhanced when AOM activity is high. During starvation minor sulfide and methane production was detected. After starvation AOM activity was re-established without measurable lag phase after re-supply of methane. FISH analysis with specific probes indicated a higher activity of sulfate-reducing bacteria (SRB) under AOM conditions. Comparison of biomass sampled at day 185 and 262 indicated a strong increase in AOM active cells. The average consortia diameter changed from 12.16 to 17.07 µm. This study contributes to the understanding of AOM under dynamic environmental conditions and indicates that the still unknown mechanism of AOM is efficient in process stabilization.

6. **Identification and activity of widely distributed, gammaproteobacterial sulfur oxidizers in tidal sediments**

   S. Lenk, J. Arnds, K. Zerjatke, N. Musat, R. Amann, and M. Mußmann

   in preparation

   CARD-FISH analysis of Gammaproteobacteria.

Abstract: In coastal marine sediments the respiration of sulfate is a dominant process in carbon mineralization, which releases toxic hydrogen sulfide in substantial amounts. Therefore, the oxidation of sulfur compounds is a central process for the marine sulfur cycle. However, the key organisms driving sulfur oxidation in the sea are yet unknown. The aim of our study was to identify potential sulfur oxidizing bacteria (SOB) by molecular techniques in tidal flat sediments of the German Wadden Sea. The phylogenetic analyses of 16S rRNA and three functional gene sequences plus metagenomics suggest the prevalence of sulfur oxidizing Gamma- and Alphaproteobacteria. Subsequent fluorescence *in situ* hybridization (FISH) using novel probes revealed globally occurring, abundant gammaproteobacterial subpopulations that are potentially involved in sulfur oxidation. Consistently, comprehensive phylogenetic analyses of key enzymes of sulfur oxidation such as the reverse dissimilatory sulfite reductase (rDsrAB), the adenosine-5-phosphosulfate reductase (AprA) and sulfate thiohydrolase (SoxB) pointed at Gammaproteobacteria as potential SOB. Moreover, a deeply-branching rDsr sequence cluster with unclear affiliation was identified. In a metagenomic approach, this sequence cluster could be linked to Alphaproteobacteria via the colocalization of the rDsr and an alphaproteobacterial Sox gene cluster on a fosmid clone indicating an unknown contribution of this group to sulfur oxidation in marine sediments. In addition, we identified genomic fragments of Gammaproteobacteria that encode genes for hydrogen utilization as well as carbon assimilation. Our study gives first insights into the diversity, abundance and function of yet unknown, but important organisms of the marine sulfur cycle.

7. **Bacterial Diversity in a Calcareous Sandy Sediment of Mallorca and Community Response to Polyaromatic Hydrocarbon Contamination**

   A. Suárez-Suárez, A. Tovar-Sánchez, A. López-López, P. Yarza, A. Orfila, J. Terrados, J. Arnds, S. Marqués, H. Niemann, R. Amann, and R. Rosselló-Móra

   submitted to FEMS Microbiology Ecology under the number FEMSEC-09-11-0488

   help with CARD-FISH analysis.

Abstract: Calcareous sandy sediment with low organic content of one of the most pristine coasts of the island of Mallorca was studied by molecular tools combined with most probable numbers of sulfate reducers. Bacterial diversity was initially assessed by comparative sequence analysis of 214 16S rRNA gene clones. With respect to the dominance of Gammaproteobacteria and *Deltaproteobacteria* as well as overall high diversity, the bacterial community was similar to that encountered in other marine sediments. Subsequently, bacterial community changes occurring in two sediment mesocosms artificially contaminated with naphthalene or crude oil were *in situ* followed. Altogether, clone libraries, fluorescence *in situ* hybridization (FISH), catalyzed reporter deposition FISH (CARD-FISH), and culturing methods indicated that within three months the bacterial communities did not change strongly in composition. The Gammaproteobacteria, members of which are well known for their potential to degrade aromatics, did not increase, but rather decline in abundance. Overall, the contamination with naphthalene or crude oil caused a deleterious effect preventing an otherwise observed increase in total prokaryotic abundance over the summer. The contamination resulted in slightly enhanced sulfate reduction rates indicating that the sediments seem to harbor an autochthonous microbiota able to degrade the added contaminants.

Publications

Manuscript I

Title:

Methanotrophic communities from novel cold seeps at the continental Hikurangi margin, New Zealand

Authors:

J. Arnds, K. Knittel, G. Wegener, H. Niemann, A. Boetius, and R. Amann

Abstract

Continental margins host numerous cold seeps characterized by methane-rich porewater emission. Around New Zealand, novel cold seep habitats have been discovered only recently. Here, high methane concentrations have been measured in both bottom waters and hydrate-bearing sediments. Some sites are populated by typical seep fauna, while others are characterized by extremely high gas fluxes and dominated by a new species of ampharetid polychaetes. This study provides first molecular insights into microorganisms populating these two habitats in the Wairarapa area at the eastern Hikurangi margin off New Zealand`s North Island. High AOM activity was distributed over broad sediment horizons harboring diverse microorganisms involved in methane-turnover and sulfur-cycling, e.g. anaerobic methanotrophs of the ANME groups and sulfate-reducing *Desulfosarcina/Desulfococcus*.

Introduction

Although continental margins store enormous amounts of the greenhouse gas methane, the ocean contributes only little to global warming (<2%), because most methane is consumed via the microbially mediated anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) prior to emission (reviewed by Reeburgh 2007).

In the last decade, molecular tools provided many insights into the composition and distribution of methanothrophic microbial communities populating methane-rich marine habitats (reviewed by Knittel and Boetius 2009). Many of these habitats host associations of anaerobic methane-oxidizing archaea (ANME) related to *Methanosarcinales* and sulfate-reducing *Desulfosarcina/Desulfococcus* (DSS), which apparently mediate AOM with sulfate in a poorly understood syntrophic relation (Boetius et al. 2000). To date, three ANME groups, ANME-1, ANME-2 with the subgroups -2a, -2b, and -2c, and ANME-3, are identified (Knittel et al. 2005). Neither ANME nor associated SRB have been yet cultured and the biochemical mechanism of AOM still remains elusive. However, methane is oxidized with sulfate, yielding sulfide and bicarbonate as products (reviewed by Thauer and Shima 2008).

Ecosystems known as cold seeps are found where methane-rich pore waters rise through the sediments forced by pressure gradients and emerge from the seafloor without substantial temperature rise (Levin 2005). At such sites, AOM-derived sulfide, availability of hard carbonate substratum, and increased food supply provide the basis for complex light-independent seep fauna dominated by various bivalves, tube worms, sponges, gastropods, and shrimp (Levin 2005). Similar oases of life are found at hot vents (reviewed by Jørgensen and Boetius 2007). Since the discovery of cold seeps in the Gulf of Mexico (Paull et al. 1984), numerous active, but also fossil seeps have been identified on both active and passive continental margins around the globe (e.g. Kojima 2002, Campbell et al. 2002).

Recently, cold seep sites have been discovered at the accretionary Hikurangi margin in the East of New Zealand`s North Island, far away from all known cold seep sites (Figure 1). The Hikurangi margin is formed by intense compressional tectonics related to the subduction of the Pacific Plate under the Australian Plate. Before 2006, seep sites around New Zealand were indicated only accidentally by fisherman

recovering seep fauna and detecting flares in fish-finding sonar (Lewis and Marshall 1996). In the recent years, high seawater methane concentrations (up to 3200 nM) and several methane seep sites along the deep continental margin have been detected in this area (Faure et al. 2006, 2008, Greinert et al. 2008), some of which were populated by characteristic seep fauna (Nelson et al. 2008, Jones et al. 2008). Adjacent seep sites are characterized by 41-fold higher seepage fluxes (207±66 mmol m^{-2} d^{-1}) and dominated by a newly discovered species of ampharetid polychaetes (Sommer et al. 2008). *Ampharetidae* have already been found at other cold seep sites and whale falls, but never constituted the key fauna. However, the seep microbial communities have never been studied in this area.

Here, we report first molecular insights into benthic microbial communities from two adjacent active seep sites in the Wairarapa area located at the Hikurangi margin, offshore the southern tip of New Zealand`s North Island. One seep site was covered by a white microbial mat, whereas the other sites` surface was populated by the ampharetid polychaetes. Hot spots of AOM and SR were determined by biogeochemical rate measurements and selected for further molecular microbial analysis.

Materials and Methods

Site description and sample collection

Sediment samples were obtained offshore New Zealand`s North Island (Hikurangi margin, Wairarapa area; water depth 1057 m, Figure 1) in March 2007. Two seep sites were chosen for the analysis: (1) square-decimeter scaled habitats covered by white microbial mats, probably formed by sulfur-oxidizing bacteria (SOB site: MUC47, 315: 41:46.32S/175:25.69E) and (2) square-meter scaled habitats populated by heterotrophic ampharetid polychaetes (Polychaete site: MUC45a, 309, 41:46.35S/175:25.69E).

Sediments were sampled aboard the German research vessel Sonne during cruise SO191, leg 3, using a TV-guided multicorer system (IFM-Geomar Kiel, Germany). Onboard, cores were stored at 4 °C until further processing. Subcores were subsequently taken and sliced in one or two centimeter intervals.

Biogeochemistry

For determination of sulfate reduction and methane oxidation rates, subcores from the SOB and Polychaete site were analyzed in triplicates. Radiolabeled methane (5 kBq) and sulfate (50 kBq) were injected and cores incubated at 4 °C (*in situ* temperature) for 36 h in the dark. Reactions were stopped by transferring samples into NaOH and zinc acetate solution, respectively.

Further processing of AOM and SR rates was performed according to Treude et al. (2003) and references therein. Activities of the reactants (sulfate and methane) and products were measured and concentrations of the reactants detected via flame ionization detection gas chromatography for methane and via barium sulfate precipitation.

16S rRNA gene library construction

Sediment samples were frozen onboard at -20 °C. DNA was extracted from 10 g sediment (SOB site 0-10 cm, Polychaete site 0-10 cm and 10-20 cm) as described by Zhou et al. (1996). Archaeal and bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) with the primer sets Arch20F/Univ1392R (Massana et al. 1997, Lane 1991) and GM3F/GM4R (Muyzer et al. 1995), respectively. PCR conditions were according to Ravenschlag et al. (1999) with the following modifications:

initial denaturation was carried out for 3 min at 95 °C, only 15 cycles were performed, annealing temperatures were 58/44 °C (archaeal/bacterial primers), and a final elongation step was conducted for 59 min at 60 °C. Twenty replicates were pooled and purified using a QiaQuick PCR purification kit (Qiagen, Hilden, Germany). Amplicons were ligated into pGEM-T-Easy vectors (Promega, Madison, Wis., USA) and subsequently transformed into *Escherichia coli* Top10 cells (Invitrogen, Karlsruhe, Germany) according to manufacturers` recommendations.

Sequencing and phylogenetic analysis

Sequencing was performed by Taq cycle sequencing with a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). The presence of chimeric sequences in the libraries was determined with Bellerophon (Huber et al. 2004). Sequence data were phylogenetically analysed with the ARB software package (Ludwig et al. 2004) using the dataset provided by ARB_SILVA (Prüsse at al. 2007, release_98). The nucleotide sequence data reported in this paper will be deposited in the EMBL, GenBank, and DDBJ nucleotide sequence database under accession numbers.

Catalyzed reporter deposition fluorescence *in situ* hybridization

Samples were fixed in formaldehyde (4% final concentration) for 24 h at 4 °C. Fixative was washed off twice with 1x PBS (130 mM NaCl, 10 mM sodium phosphate; pH 7.2) and samples were finally stored in 1x PBS-ethanol (1:1) at -20 °C. Fixed cells were detached from sediment particles by sonicating the samples on ice at the following settings: twice 20 sec (Polychaete site) and four times 30 sec (SOB site) at an amplitude of 42 µm, <10 kW, with a 30 sec break between each sonication (Sonopuls HD70, MS73 probe; Bandelin, Berlin, Germany).

Catalyzed reporter deposition *in situ* hybridizations (CARD-FISH) with horseradish peroxidase (HRP)-conjugated probes (Biomers, Ulm, Germany) followed by tyramide signal amplification were carried out as described by Pernthaler et al. (2002) with minor modifications: endogenous peroxidases were inhibited with Methanol for 30 min at RT and cell walls permeabilized with HCl (0.1 M) for 20 sec at RT. Hybridizations were carried out for 2 h at 46 °C. Carboxyfluorescein-, $Alexa_{488}$-, and $Alexa_{594}$-labeled tyramides were added as substrates for the HRP. Hybridized cells were stained with 4´.6´-diamidino-2-phenylindole (DAPI). Cells were counted in 40 independent

microscopic fields using an Axiophot II epifluorescence microscope (Carl Zeiss, Jena, Germany). Cells in dense aggregates could not be counted and were estimated semi-quantitatively in 200 independent microscopic fields as described previously (Lösekann et al. 2007). Sequences of probes used and formamide concentrations required for specific hybridization are given in Table 1.

Most cells were only weakly DAPI stained and not distinguishable from the sedimentary background, but showed bright CARD-FISH signals (Figure 2). Consequently, the abundance of Bacteria and Archaea is given in total numbers (Figure 3c) and the relative abundance of subgroups related to CARD-FISH detection rates (sums of EUB338 I-IV and Arch915). At the SOB site, most cells in the upper 10 cm of the sediment were embedded in matrices and did not evenly distribute on filters. Distribution was improved only little by elongated sonication and matrices could neither be dissolved with acid nor enzymes. Therefore, numbers were estimated by focusing through the matrices.

Results

Biogeochemistry

Methane concentrations were constantly low at the SOB site (up to 0.4 mM) and only little higher, but more variable at the Polychaete site (up to 0.6 mM). At both sites, sulfate concentrations decreased from 28 mM in the seawater to <10 mM within the upper 10 cm of the sediments (Figure 3a).

Maximum rates of AOM (almost 200 nmol cm^3 d^{-1}) were comparable at the SOB and the Polychaete site. High AOM activities were measured over the upper 10 cm of sediment at the SOB site and decreased with further depth, whereas high activity at the Polychaete site was detected only at around 15 cm sediment depth (Figure 3b). Consequently, integrated AOM rates were higher at the SOB site than at the Polychaete site (11 versus 6 mol m^{-2} yr^{-1}). Also SR activity was significantly higher at the SOB site peaking with >500 nmol cm^3 d^{-1} in 1 cm depth. It decreased strongly below 10 cm. At the Polychaete site, SR activity peaked in 1 cm depth, but here, the decrease in activity with depth was more continuous. Also integrated SR rates were much higher at the SOB than at the Polychaete site (14 versus 2 mol m^{-2} yr^{-1}). However, at both sites, AOM and SR activity was detected over a relatively broad sediment interval of >10 cm (Figure 3b).

Microbial diversity

Archaeal and bacterial 16S rRNA gene analysis were conducted with three sediment samples: the surface and subsurface Polychaete site (0-10 cm and 10-20 cm), and the SOB site (0-10 cm). In total, 19/16/14 bacterial phyla were identified analyzing 117/80/108 partial sequences (~500 bp) retrieved from these three samples. The largest fractions in all libraries grouped with Delta- and Gammaproteobacteria, minor fractions with Alpha- and Epsilonproteobacteria (Table 2).

Several Proteobacteria potentially involved in methane-oxidation and sulfur-cycling were represented. Sequences affiliated with ones of diverse sulfate-reducing Deltaproteobacteria, e.g. of the genera *Desulfobulbus, Desulfosarcina, Desulfococcus,* and others. Among the Gammaproteobacteria, sequences grouped with ones of e.g. *Methylobacter, Methylophaga, Thiomicrospira, Thioploca,* and *Thiothrix* spp.. Among the Epsilonproteobacteria, sequences with similarity to *Arcobacter* spp. sequences were

found. Notably, several sequences of symbionts from invertebrates such as *Olavius, Riftia, Alvinella, Oligobrachia, Sedimenticola,* and *Bathymodiolus* spp. were identified.

Further, Bacteroidetes and Planctomycetes related 16S rRNA genes occurred in high frequency. Other groups found were Lentisphaera, WS3, and Acidobacteria. Rarely, Spirochaetes, Fusobacteria, Verrucomicrobia, OP3, Caldithrix, Nitrospirae, Nitrospina, Firmicutes, OP8, Actinobacteria, Cyanobacteria, Thermomicrobia, and members of the OD1-OP11-WS6-TM7 cluster were represented.

Archaeal 16S rRNA genes were retrieved only from the subsurface of the Polychaete site and the SOB site (49 versus 63 sequences), but not from the surface of the Polychaete site, probably due to low archaeal abundance and the low number of PCR cycles used for amplification. The archaeal diversity was low compared to the bacterial one. Most sequences grouped with members of ANME-3 (26 versus 42 sequences), ANME-2a (22 versus 17 sequences), and ANME-2c (1 sequence versus 4 sequences). Thermoplasmatales genes were retrieved as well, but only from the SOB site (2 sequences).

Microbial i*n situ* abundance

Only in the upper sediment layers of the Polychaete site, total cell numbers determined by AODC-staining were higher than the ones determined by DAPI staining and CARD-FISH (Figure 2). In deeper sediment layers, numbers determined by CARD-FISH (sums of EUB338 I-IV and Arch915) were higher than ones enumerated by AODC- and DAPI-staining. At the SOB site, total cell numbers revealed by CARD-FISH were highest throughout the entire sediment core, except for the top layer (<1 cm). Consequently, the *in situ* abundance of Bacteria and Archaea is presented in total numbers (Figure 3c). The relative abundance of the subgroups is related to the sums of Bacteria and Archaea (Figure 3d).

SOB site. Total cell numbers at the SOB site were highest with 1 x 10^{10} cells ml^{-1} in the top sediment layer (0.5 cm) and decreased strongly within the upper three centimeter (Figure 2). In the top layer, more than 90% of all DAPI stained cells were CARD-FISH detectable, but only half in the layer below (1.5 cm). The microbial community was always dominated by Bacteria comprising up to 8 x 10^9 cells ml^{-1}. Archaea made up to 2 x 10^9 cells ml^{-1} (Figure 3c).

ANME and DSS were omnipresent and mostly associated. An upper layer (1.5 cm) hosted the largest ANME-2 population (1 x 10^9 cells ml^{-1}), but the highest relative abundance of ANME-2 (15% of all CARD-FISH detectable cells) differed only little throughout the core with peaks in 2 cm and 8 cm depth. The relative abundance of DSS was up to two-fold higher than the one of ANME-2 in the upper 10 cm, but the distribution profile was positively correlated with that of ANME-2 (Figure 3d). While single ANME-2 cells accounted for only 1-4% of all detected cells, the abundance of single DSS cells ranged from 1-15% with a peak in 2 cm depth, where their total and relative abundance was highest.

Highest aggregate numbers were found in 2 cm depth, where aggregates were on average 8 µm in diameter. Larger aggregates with an average diameter of 16 µm occurred in 6 cm depth. Quantification of ANME-2 was performed by group-specific probing. Subgroup-specific probes were used to further identify the subclades of ANME-2 and estimate their abundance at hot-spots. ANME-2a dominated the uppermost layers (>80% in 0.5 cm and >60% in 1.5 cm). In 2.5 cm depth, ANME-2a and ANME-2c were equally abundant.

ANME-2a were coccoid and variable in size (0.6-1 µm diameter). Aggregates were most often surrounded by shells of smaller coccoid DSS (0.5 µm diameter). These shell-type consortia were 3 to 10 µm in diameter (average 7 µm). Further, ANME-2a formed loose associations with rod-shaped or vibrioform DSS (1 µm by 0.7 µm). Coccoid ANME-2c formed spherical mixed-type consortia variable in size with coccoid cells (both ~1 µm in diameter). In contrast to most ANME-2a/DSS aggregates, only few ANME-2c partners were identified as DSS (Figure 4). Also CARD-FISH with the general bacterial probe mix EUB338 I-IV resulted in the detection of only few bacterial partners, but most ANME-2c partners were not be hybridized at all.

Neither ANME1 nor ANME3 were detected by CARD-FISH after permeabilising cells with HCl (0.1 M, 30 sec at RT) and proteinase K (15 µg ml^{-1}, 2 min at RT), but few cells (<0.1%) with rectangular shape, typical for ANME-1, were DAPI-stained. MS1414-positive cells made up 2-3% of all cells in the horizon between 2 to 9 cm, but were absent above and below.

Potential sulfate-reducing, sulfur-oxidizing and aerobic methanotrophic Bacteria were analyzed in greater detail in the surface layer (0.5 cm). Here, DSS accounted for 12% of all CARD-FISH detected cells and the majority of Deltaproteobacteria (14%). Gammaproteobacteria as detected by probe GAM42a constituted 7%, potential sulfur-oxidizing Gammaproteobacteria (probe Gam660) 3%, and aerobic methanotrophic Gammaproteobacteria (probe MetI-444) 1% to the community. Other aerobic methanotrophic Gammaproteobacteria (probe MPH732) were not detected at all. Giant filamentous cells stained by probe Gam42a were rare (<0.5%), but formed a significant biomass. Sulfur-oxidizing Epsilonproteobacteria (probe EPSI682) made up 4% of all CARD-FISH detectable cells.

Polychaete site. Total cell numbers at the Polychaete site were with up to 2 x 10^9 cells ml^{-1} (0.5 cm depth) significantly lower than the ones at the SOB site, but decreased only little with depth (Figure 2). Bacteria made up the major fractions of all microorganisms in all depths peaking with up to 2 x 10^9 cells ml^{-1} in the top layer (0.5 cm depth). Archaea were almost absent in the upper 10 cm (Figure 3c).

The largest archaeal communities were strongly dominated by ANME-2, which accounted for up to 25% of all CARD-FISH detected cells in around 15 cm depth (Figure 3d). Most ANME-2 occurred in small, loosely associated aggregates with DSS and only few (up to 1%) occurred as single cells. Most cells were further identified as ANME-2c by subgroup-specific probing. No ANME-2a were detected. Notably, CARD-FISH signals of single ANME cells were weak in the upper layers and intense only in the deeper layers. The highest abundance of DSS (14%) correlated with the highest ANME-2 abundance (Figure 3d). However, single DSS were found in all sediment layers and they were also abundant (8%) in the uppermost layers, where ANME-2 were absent. ANME1 and ANME3 were not detected. MS1414-positive cells accounted for 1% of all CARD-FISH detectable cells in the upper horizon (1.5 and 2.5 cm), but were absent above and below.

In the surface sediment layer (0.5 cm), bacteria potentially involved in methane-turnover and sulfur-cycling were analyzed in greater detail. All Deltaproteobacteria were assigned to DSS (6% of detectable cells). Gammaproteobacteria made up 11% of all CARD-FISH detectable cells and 3% were further identified by the use of probe

Gam660. Only few MPH732-positive (0.4% of detected cells) and no MetI-444-positive cells were detected. EPSI682-positive cells accounted for 3% of all detected cells.

Discussion

Diverse microbial seep communities

Microbial communities and activities were studied at two adjacent active cold seep sites (Polychaete site and SOB site) at the Hikurangi margin in the East of New Zealand`s North Island. The sediment of the Polychaete site was covered by ampharetid polychaetes and populated by nematodes in greater depth. The worms likely play a key role as ecosystem engineers (Jones et al. 1994).

It is known that bioturbation enlarges metazoan diversity (Meysman et al. 2006) and it might also explain the high bacterial diversity of the microbial community (19 bacterial phyla, Table 2) found in the upper horizon of the Polychaete site, but little is yet known about the factors controlling microbial diversity. The diversity observed was high for the low number of clones analyzed and was likely due to the high number of replicates as well as low number of PCR cycles used to amplify the DNA. However, all detected phyla have been found previously in marine sediments and the diversity was unlikely covered with the clone numbers analyzed. Pyrosequencing might reveal a significant higher diversity (Kim et al. 2008). In contrast, the subsurface of the Polychaete site and the SOB site hosted a less diverse community. For the subsurface Polychaete site, this is likely due to the lower number of clones analyzed. The lower diversity at the SOB site might reflect a more specialized community associated with high AOM activities at these two sites, which could only be generalized by sequencing a larger number of clones.

Microbial population size

At the SOB site, most microbial cells were embedded in matrices complicating their quantification. However, cell numbers were rather under- than overestimated. The matrices might be composed of complex extrapolymeric substrates and provide several advantages: (i) adhesion, (ii) protection from grazers, toxins, harsh changes of environmental parameters, and hydraulic pressure, (iii) nutrient conservation or source (Costerton et al. 1995, Birch and Bachofen 1990, Appan et al. 1995, Decho 2000, Flemming and Wingender 2001, Tian 2008). In this study, the matrices were highly resistant against mechanical and chemical treatment suggesting that they have a complex composition and efficiently protect embedded cells.

AODC, DAPI, and CARD-FISH detection rates differed strongly in both sediment cores. DAPI stained cells were hardly distinguishable from the high sedimentary background, probably, because DAPI stains also sedimentary matter and due to the low intensity of the dye. The fact that total numbers determined by AODC-staining were mostly lower than ones enumerated by CARD-FISH is astonishing, because CARD-FISH stained cells should generally be detectable by AODC staining and can be best explained by the high sensitivity of CARD-FISH.

The top sediment layers (0.5 cm) hosted the largest microbial communities at both the SOB and the Polychaete site (1 x 10^{10} versus 2 x 10^{10} cells ml^{-1}) suggesting favorable environmental conditions, such as high nutrient load and intense exchange of porewater with seawater at the surface replenishing oxygen and sulfate or removing sulfide. The significantly higher numbers at the SOB site are best explained by high AOM-derived sulfide, which favored development of mat forming SOB. AOM nearby surface sediments is known to trigger the development of microbial mat forming sulfide-oxidizers, e.g. *Beggiatoa* species (Treude et al. 2003, De Beer et al. 2006, Niemann et al. 2006).

Indeed, a white microbial mat was monitored *in situ*, but not visible after retrieving the core onboard, probably due to sediment disturbance. Moreover, the top sediment layer harbored many bacteria potentially involved in sulfur-cycling, mostly sulfate-reducing Deltaproteobacteria, but also potential sulfur-oxidizing Gamma- and Epsilonproteobacteria. Also at the Polychaete site many Proteobacteria were detected and the *in situ* abundance of known potential sulfur-oxidizers was comparable at both sites. Contrarily, the SOB and Polychaete site were dominated by Delta- and Gammaproteobacteria, respectively.

At the SOB site, only half of all cells were CARD-FISH detectable below the top sediment layer in 1.5 cm depth suggesting a community shift, which might reflect significant changes of environmental parameters. Probably, the resident community suffered from oxygen depletion and/or sulfide accumulation after sediment coverage (Weber et al., MPI Bremen, in preparation). Below 5 cm depth, the numbers of detected cells were comparable at both sites suggesting stable environmental conditions in the depth.

Resident ANME and DSS

The area studied here is far away from all known ANME sites and the occurrence of closely related ANME suggests their global distribution. The novel cold seeps harbored both ANME-2a and ANME-2c. At the SOB site, ANME-2a dominated the upper surface layers (~1 cm depth), whereas ANME-2a and ANME-2c were equally abundant in the layer below indicating that ANME-2a and 2c compete for AOM substrates. *In situ* abundance did not correlate with the gene frequency of ANME-2a and ANME-2c indicating a DNA extraction, PCR, and/or cloning bias in favor of ANME-2a. Moreover, ANME-3 related sequences were most frequent in archaeal libraries, but no ANME-3 cells were detected by CARD-FISH suggesting methodological artifacts or that their gene frequency was overestimated in clone libraries, or many cells were starving, dormant or dead (Table 2, Figure 2). MS1414-positive cells accounted for 3% maximal.

Interestingly, ANME-2a/DSS occurred as shell-type or mixed-type aggregates, whereas ANME-2c/DSS formed only mixed type aggregates. In all other AOM habitats, shell-type forming ANME were identified as ANME-2c (Knittel and Boetius 2009). This raises the question why the ANME occurring far away from all other known AOM habitats developed another morphotype, which is exactly the opposite. However, the shell-type association seems to provide some advantage.

While ANME-2a associated DSS were mostly detectable, ANME-2c associated DSS were rarely CARD-FISH detectable (Figure 3) and probably starving, dormant or dead in the vast layers suggesting unfavorable environmental conditions, at least for the ANME partners. On the other hand, both ANME-2c and their undetectable partners might have been inactive. However, in greater depth, the associated cells were even detectable by FISH with fluorescently labeled probes and hence apparently active.

In comparison to other cold seeps (reviewed by Knittel and Boetius 2009), AOM activities were comparable, but the total and relative abundance of AOM key players was low. However, some ANME were relatively large (up to 1 µm) and their cell-specific activity was probably higher. On the other hand, other, yet unknown microorganisms might be involved in AOM at these novel cold seeps. Bacteroidetes, Proteobacteria, Acidobacteria, or Verrucomicrobia are probably involved in AOM (Beal et al. 2009).

Distribution of AOM activity and anaerobic methanotrophs

AOM activity was distributed over a relatively broad sediment horizon of >10 cm (Figure 2), whereas it is typically restricted to narrow surface horizons of a few centimeters due to diffusion limits raising the question how sulfate is transported into the deeper sediment layers. At the SOB site, convective influx of seawater due to focused fluid upflow might be an explanation. At non-reduced sites in the seep area, porewater might be mixed with bottom water down to several meters due to rising methane bubbles (Haeckel et al. 2007, Haeckel et al. 2008). At the Polychaete site, the width of the AOM horizon could be explained by bioturbation replenishing the sediment with sulfate.

Maximal AOM rates were comparable at the SOB and the Polychaete site with up to 200 nmol cm^{-3} d^{-1}, which is in the common range for hydrate bearing seep sediments (Treude et al. 2003, Joye et al. 2004). The AOM peaks correlated with the highest ANME and DSS abundance. AOM hot spots were located in different sediment depths. At the SOB site, AOM activity peaked at the surface, whereas high activity was restricted to a deeper and less broad sediment horizon at the Polychaete site yielding higher integrated AOM rates at the SOB than at the Polychaete site. At the SOB site, the abundance of ANME and DSS increased three-fold in a depth of 1-2 cm compared to the layer above indicating favorable conditions for AOM, probably high methane flux and sulfate availability. The restriction of AOM and ANME to deeper layers at the Polychaete site is best explained by bioturbation replenishing the sediment not only with sulfate, but also with oxygen, which inhibits AOM. Oxygen data are not available for these sampling sites, but at the Polychaete sites, sulfate is generally depleted below sediment depths of 5-10 cm (Haeckel et al. 2008) suggesting that oxygen is present above.

The Polychaete sites were characterized by up to 41-fold higher seepage fluxes compared to adjacent seep sites (Sommer et al. 2008). No visible seepage flux, higher integrated AOM rates, and higher total abundance of anaerobic methanotrophic key players at the SOB site suggest that these habitats form filters against methane emission (e.g. Niemann et al. 2006). It is known form other sites that SOB form efficient filters. For example, bottom water methane concentrations decreased 100-fold to almost background values in *Beggiatoa* covered areas at the Haakon Mosby Mud Volcano

(Bogdanov et al. 1999, Damm and Budeus 2003, Milkov et al. 2004, Schlüter et al. 2004).

At the surface of both the SOB and Polychaete site, SR rates were higher than that of methane oxidation indicating AOM independent sulfate reduction. Most SRR peaks were located little above the AOM peaks, which is common, because methane diffuses faster than sulfate. In greater sediment depth, methane oxidation and sulfate reduction rates showed almost 1:1 ratios. At the Polychaete site, the AOM peak in 15 cm depth did not correlate with a high SR rate, which might be due to sulfate detection limits.

Habitat formation

Aerobic methanotrophic cells were rarely detected suggesting that their 16S rRNA genes, which were frequently found in the clone libraries constructed in this study, derive from starving, dormant or ancient populations. At the Polychaete site, AOM independent SR seemed to play, if at all, a minor role. Sommer et al. (2008) hypothesized the Polychaete site as initial stage of metazoan colonization creating a pthway for high microbial methane turnover. Indeed, worms might replenish the sediment with sulfate initiating growth of deeply buried AOM communities, which support SOB. If true, the question raises on what the worms feed. Many Ampharetidae are deposit feeders. They might feed on aerobic methanotrophs if such used to be indeed present. Strongly negative carbon isotopic signatures of the ampharetid tissues indicate a methane derived diet (Sommer et al. 2009). Sommer et al. (2009) suggested that organic carbon generated via aerobic methane oxidation represents their major carbon source. Yet, it can not be excluded that the worms populate the nutrient-rich SOB site.

In conclusion, we showed that cold seeps in the South Pacific Ocean harbor the same anaerobic methanotrophic key players, ANME and DSS, as all known AOM habitats. In contrast to other AOM sites hosting different ANME groups, only ANME-2 were detected by CARD-FISH. Moreover, ANME-2a formed the same shell-type aggregates as ANME-2c at other AOM sites. ANME numbers were comparably low suggesting a high cell-specific activity or the presence of yet unknown anaerobic methanotrophs.

Acknowledgements

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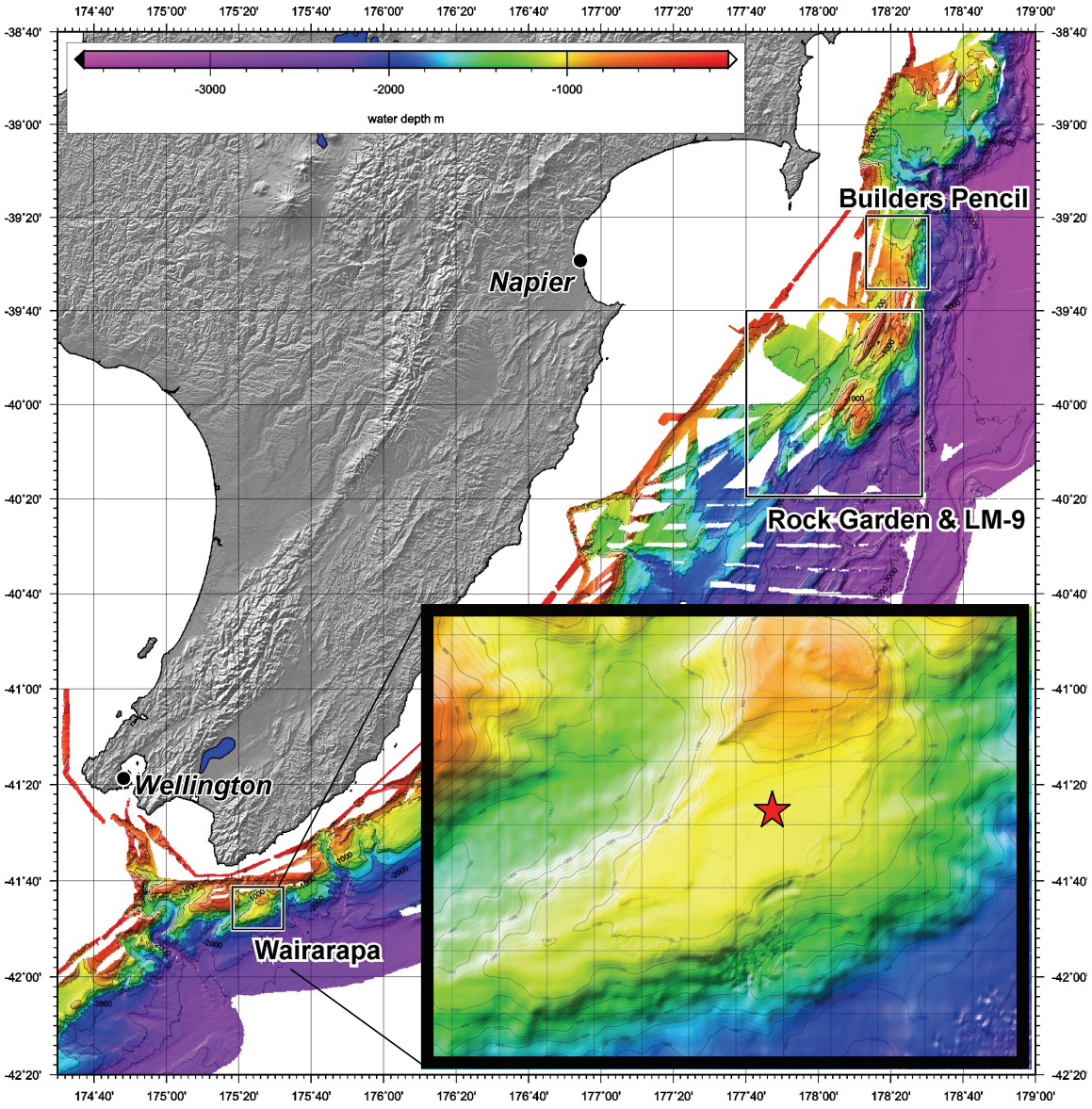


Figure 1: Sampling area Wairarapa at the eastern Hikurangi margin off New Zealand`s North Island. Bathymetric map by J. Greinert (IFM Geomar, Kiel, Germany)

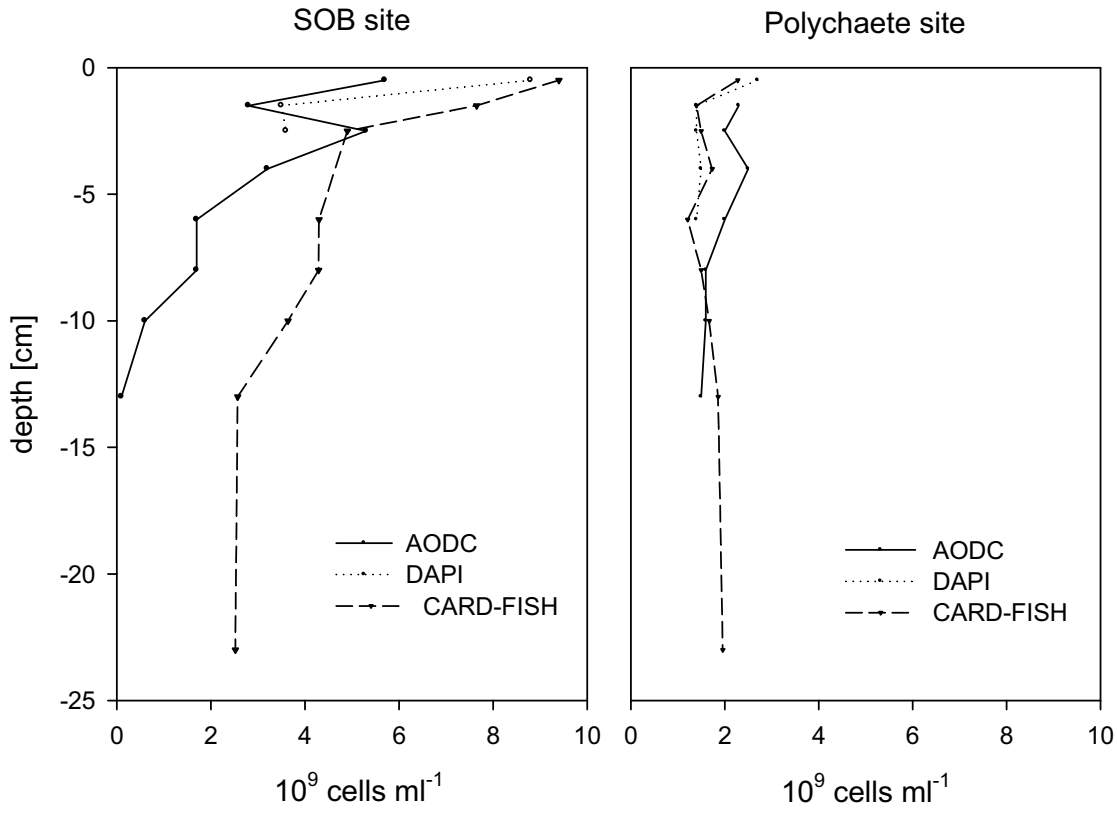
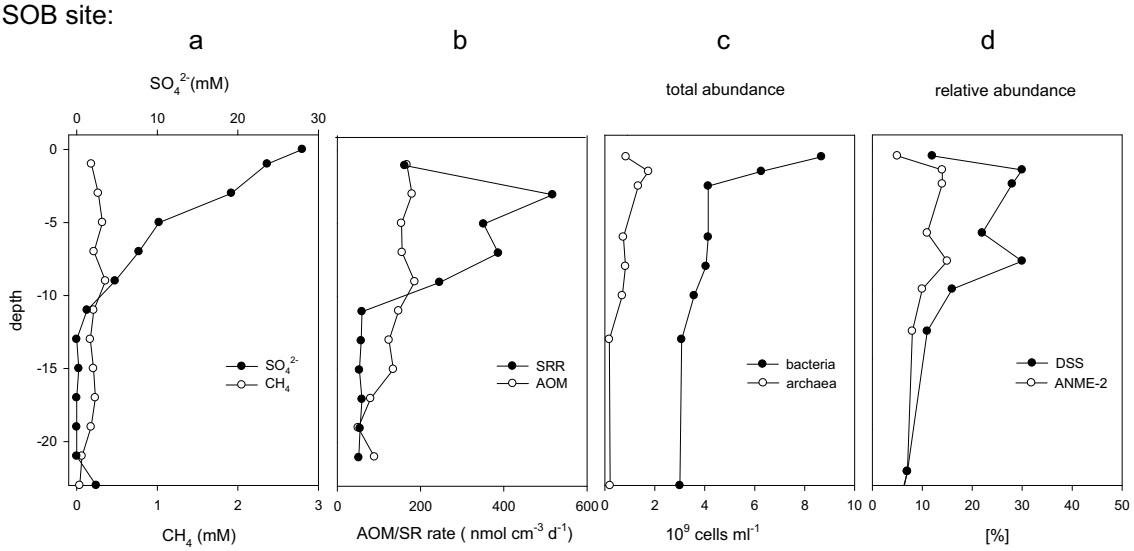


Figure 2: Comparison of total cell numbers in Wairarapa cold seep sediments (SOB and Polychaete site) determined by AODC-staining, DAPI-staining, and CARD-FISH.

SOB site:



Polychaete site:

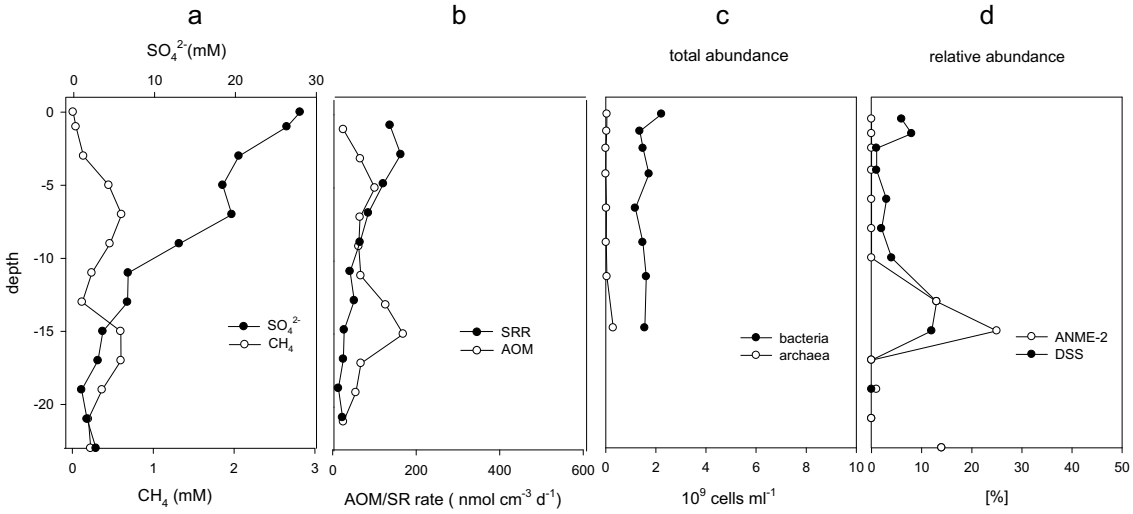


Figure 3: (a) Methane and sulfate concentrations, (b) methane oxidation and sulfate reduction rates, (c) total bacterial and archaeal *in situ* abundance, and (d) relative *in situ* abundance of ANME-2 and DSS cells in Wairarapa cold seep sediments (SOB site and Polychaete site).

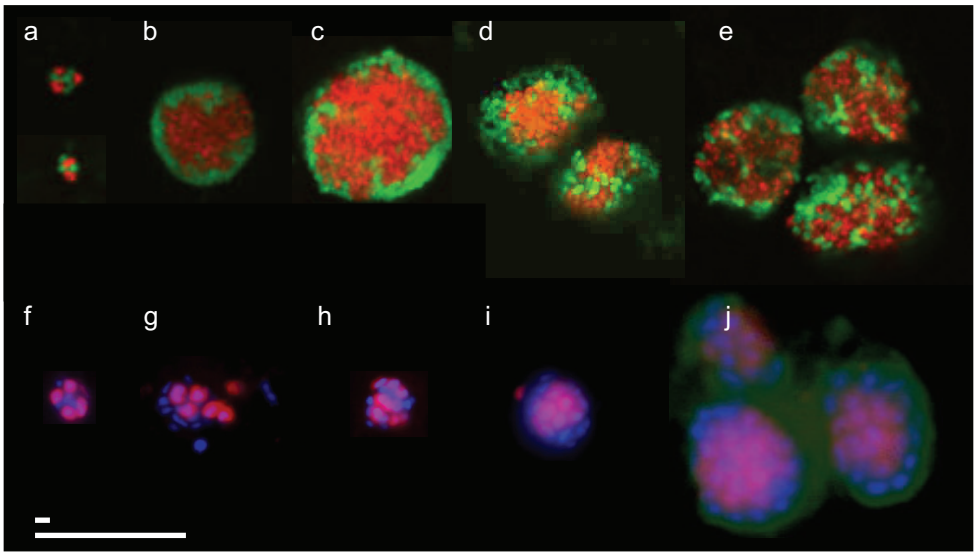


Figure 4: Micrographs of ANME-2 (red) and associated DSS (green) visualised by CARD-FISH and Laserscanning (a-e) and Epifluorescence microscopy (f-j). a-e show shell-type ANME-2a/DSS consortia. f-h show mixed-type ANME-2c associated with cells that were not hybridized at all, but DAPI stained (blue). i and j show ANME-2a consortia with associated cells undetectable by CARD-FISH. Scale bars 1 and 10 µm.

Table 1: Oligonucleotide probes used in this study

Probes	Target organisms	Probe sequences (5` to 3`)	FA	References
Arch915	most Archaea	GTG CTC CCC CGC CAA TTC CT	35	Stahl and Amann 1991
ANME1-350	ANME-1 archaea	AGT TTT CGC GCC TGA TGC	40	Boetius et al. 2000
EelMS932	ANME-2 archaea	AGC TCC ACC CGT TGT AGT	50	Boetius et al. 2000
ANME2a*	ANME2a archaea	TCT TCC GGT CCC AAG CCT	50	Knittel et al. 2005
ANME2c-760	ANM2c archaea	CGC CCC CAG CTT TCG TCC	60	Knittel et al. 2005
ANME3-1249	ANME3 archaea	TCG GAG TAG GGA CCC ATT	30	Niemann et al. 2006
ANME-3-1249 H3	Helper probe for ANME-3-1249	GTC CCA ATC ATT GTAGCC GGC		Lösekann et al. 2007
ANME-3-1249H5	Helper probe for ANME-3-1249	TTA TGA GAT TAC CATCTC CTT		Lösekann et al. 2007
MS1414	Methanosarcinaceae, some ANME-3	CTC ACC CAT ACC TCA CTC GGG	50	Raskin et al. 1994
hMS1395	Helper probe for MS1414	GGT TTG ACG GGC GGT GTG		Crocetti et al. 2005
Eub338 I	most Bacteria	GCT GCC TCC CGT AGG AGT	35	Amann et al. 1990
Eub338 II	most Planctomycetetales	GCA GCC ACC CGT AGG TGT	35	Daims et al. 1999
Eub338 III	most Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	35	Daims et al. 1999
EUB338 IV	Lentisphaerae	GCA GCC TCC CGC AGG AGT	35	this study
DSS658	*Desulfosarcina/Desulfococcus* and related SRB	TCC ACT TCC CTC TCC CAT	50	Manz et al. 1998
Gam42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	35	Manz et al. 1992
Bet42a competitor	Betaproteobacteria	GCC TTC CCA CTT CGT TT		Manz et al. 1992
Gam660	Potential sulphur-oxidizing Gammaproteobacteria	TCC ACT TCC CTC TAC	35	Ravenschlag et al. 2001
MetI-444	HmmvMetI *(*Gammaproteobacteria*)*	CCT GCC TGT TTT CCT CCC	60	Niemann et al. 2006
MPH732	HMMV-MPH (Gammaproteobacteria)	GTA ATG GCC CAG TGA GTC	40	Niemann et al. 2006
EPSI682	Epsilonproteobacteria	CGG ATT TTA CCC CTA CAC M	20	Moussard et al. 2006

FA: formamide concentration in hybridisation buffer [%, vol/vol]
*HRP-labeled probe hybridized at 35% FA

Table 2: Clone frequencies of bacterial 16S rRNA gene sequences retrieved from Wairarapa cold seep sediments

	SOB site	Polychaete site	
Phylum	0-10 cm depth	0-10 cm depth	10-20 cm depth
Alphaproteobacteria	1	3	1
Gammaproteobacteria	16	27	13
Deltaproteobacteria	45	20	40
Epsilonproteobacteria	11	10	5
Bacteroidetes	11	8	13
Planctomycetes	5	9	5
Verrucomicrobia	0	1	3
Lentisphaera	1	1	3
Acidobacteria	1	3	1
WS3	1	1	1
OD1-OP11-WS6-TM7	4	4	0
Thermomicrobia	0	1	5
Nitrospirae	0	3	1
OP3	0	3	1
Actinobacteria	0	1	3
Spirochaetes	2	0	1
Fusobacteria	0	2	1
Caldithrix	1	2	0
Cyanobacteria	0	1	0
Nitrospina	1	0	0
Firmicutes	1	0	0
OP8	0	1	0
Others	0	2	3
total clones	**108**	**117**	**80**
total phyla	14	19	16

Manuscript II

Title:

Polyphasic comparative analysis of microbial reef communities along the northwestern Black Sea shelf

Authors:

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Abstract

Massive microbial reefs, fueled by the anaerobic oxidation of methane (AOM) with sulfate, grow along the deep anoxic shelf of the northwestern Black Sea. The key methanotrophic microbes have been identified as ANME-1, ANME-2, and DSS, but little is known about their *in situ* abundance. In this study, we used two (semi)quantitative methods (i.e. intact polar membrane lipid and catalyzed reporter deposition fluorescence *in situ* hybridization analysis) to analyze the community composition in various reef samples. The data mostly correlated well and are discussed in relation to the biogeochemical conditions. Methane seeping sediment was included in the analysis. While ANME-2 and DSS dominated the top zones of the reefs, as well as the surface sediment layers, which were characterized by high AOM activity, ANME-1 were abundant in a reefs` ex- and interior zone, and in the subsurface sediment layers. Differently colored reef mat layers were either dominated by ANME-2 and DSS or ANME-1 depending on the reef zone. Individual reefs differed little suggesting stable environmental conditions along the shelf. Moreover, diverse other Bacteria (11 divisions and 6 candidate divisions) were identified by 16S rRNA gene analysis, e.g. members of the JS1 cluster, Thermomicrobia, Planctomycetes, and Bacteroidetes.

Introduction

Although continental shelf and slope sediments emit enormous amounts of methane, the ocean adds only minor amounts to the atmosphere, where methane is the most abundant hydrocarbon and acts as important greenhouse gas. Most of the methane is microbially oxidized prior to emission: vastly by the anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) and to minor portions aerobically, where oxygen becomes available, e.g. at the benthic boundary layer or in seawater (Reeburgh 2007). AOM is mediated by different microbial associations of anaerobic methanotrophic archaea (ANME-1, -2, -3) with relation to Methanosarcinales and sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* (DSS) branch (Michaelis et al. 2002) or *Desulfobulbus* spp. (ANME-3; Niemann et al., 2006; Lösekann et al. 2007). No ANME archaea could be obtained in pure culture yet and their metabolism is mainly still unknown.

AOM sets in anoxic habitats, which are in the ocean mostly restricted to surbsurface sediment horizons. In the nearly landlocked Black Sea, waters are poorly mixed and permanently anoxic below the chemocline located in around 150-180 m water depth (Reeburgh et al. 1991). This unique environment allows anaerobic methanotrophs to extend their habitat from the sediments into permanently anoxic water as evidenced by meter high microbial reefs growing along the shelf west of the Crimea peninsula. Here, hundreds of active cold seeps occur between 35 and 800 m water depth (Ivanov et al. 1991). Moreover, anaerobic methanotrophs have also been detected in the deep Black Sea water (Wakeham et al. 2003, Durisch-Kaiser et al. 2005, Schubert et al. 2006, Schubert and Durisch-Kaiser et al. 2006).

Microbial reefs are built by anaerobic methanotrophs and associated microorganisms (Knittel et al. 2005, Reitner et al. 2005[a,b]) covering up to 10 cm thick microbial mats, which are internally stabilized by carbonate precipitates deriving from AOM (Thiel et al. 2001, Michaelis et al. 2002). Microbial mats have are layered structure with black mats at the water-mat interphase. These mats cover pink mats, which enclose gray or greenish mats towards the reefs` interiors (Michaelis et al. 2002, Reitner et al. 2005[b]). Microscopic observations showed that rectangular shaped ANME-1

flourish in pink mat layers (Blumenberg et al. 2004, Michaelis et al. 2002, Reitner et al. 2005[b], Knittel et al. 2005, Krüger et al. 2008), whereas coccoid ANME-2 dominate black mat layers (Blumenberg et al. 2004, Krüger et al. 2008, Reitner et al. 2005[b]). Conversely, Treude and colleagues (2007) found a dominance of ANME-1 and ANME-2 in the ex- and interior of a similar mat, respectively. However, both groups also co-occur in the ex- and interior of reefs (Knittel et al. 2005, Reitner et al. 2005[b], Treude et al. 2007, Blumenberg et al. 2004, Krüger et al. 2008). Mat-forming ANME are commonly associated with DSS (Michaelis et al. 2002, Knittel et al. 2005, Reitner et al. 2005[b], Treude et al. 2005[a] and 2007), although large populations of ANME-1 have also been found without any association to DSS (Treude et al. 2007). CARD-FISH and Intact polar lipid analysis showed that ANME-2 dominate nodules on top of the reefs, whereas ANME-1 thrive in the reefs` ex- and interior (Rossel et al. 2008). The spatial arrangement of different ANME populations dominating different mat layers and reef zones suggests distinct physiological requirements, which needs to be further investigated.

Only few studies have so far hypothesized on growth modes of microbial reefs. Treude et al. (2005[a]) postulated that reefs begin to take shape where a subsurface methanotrophic mat rises above the sediment surface. Nodules growing on top of the reefs were identified as youngest structures, suggesting that reefs develop relatively vertically (Lein et al. 2002). Recent results of radiocarbon dating indicate that reef carbonates, which mostly derive from AOM (Thiel et al. 2001, Reitner et al. 2005[a]), are between 3.500 to 13.000 years old (Lein et al. 2007).

In this study we investigated the diversity and spatial distribution of the microbial community in different zones of a Black Sea microbial reef (i.e. top, ex- and interior), for which activity measurements were conducted. Comparative sequence analysis of bacterial and archaeal 16S rRNA genes was used to get phylogenetic information. Intact polar lipid (IPL) analysis, and quantification of whole fixed cells by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) were used in parallel as two independent molecular methods to obtain (semi)quantitative information on community composition in different layers of various reef samples. Data were correlated with methane and sulfate profiles, as well as with AOM and SR rates and discussed with

respect to reef development and ecophysiological properties of different ANME groups. In a second part of this study, the microbial community structure of individual reefs from adjacent and distant geographic locations were compared to study reef diversity along the shelf. All yet studied reefs originate from the Dnepr Area off the Ukraine. Here, reefs from further west of the Dnepr Area (Northwest Shelf and Danube Canyon) are firstly described.

Materials and Methods

Site description and sample collection

Microbial mat and sediment samples were collected from several areas in the northwestern Black Sea in October 2004 (Table 1). In these areas, giant microbial reefs are formed by thick microbial mats, internally stabilized by carbonates (Michaelis et al. 2002). The western Crimea area of the Black Sea has been studied in detail and is characterized by active seepage of free gas containing 95 to 99% methane and minor amounts of N_2, CO_2, and H_2 (e.g. Pimenov et al. 1997, Michaelis et al. 2002). Samples were obtained during a cruise onbord the German research vehicle Poseidon, cruise POS317, leg 3, using the manned submersible JAGO.

Usually, black reef surfaces are covering exterior pink or orange mat layers which are up to 10 cm thick and often incrusted with carbonates. These exterior mat layers enclose interior gray, green or black mat horizons, which are characterized by porous carbonates forming irregular cavities. Reefs from the Danube Canyon were gas-filled, but did not emit gases during sampling. Mats had black, gray or brown surfaces and were often additionally covered by filamentous structures, which might represent accumulation of organic particles. Moreover, some reefs were covered with sediment, re-suspended by landslides on the Danube slope. Dark and soft nodules have been observed commonly on top, but also in deeper reef zones.

Differently colored mat layers were separated prior to analysis. Two neighboring mat pieces were subsampled from each layer and used as replicates. Nodules were always sampled from the reefs` top, with exception of reef P795, from which a nodule was sampled from the reef side at half-height.

Sediment samples were retrieved with a pushcore from an area characterized by a black spot on the surface, surrounded by soft gray sediment. The sediment visibly released gas during sampling, thus it was referred to as methane seeping sediment.

Biogeochemical analysis

AOM rates were determined for two subcores after incubation with ^{14}C radiotracers by $^{14}CH_4$-combustion and $^{14}CO_2$-acidification according to Treude et al. (2005[a]) and references therein. For SRR analysis, cores were incubated with $^{35}SO_4^{2-}$ radiotracers as also described previously (Treude et al. 2005[a]). Sulfate concentrations were determined in the supernatant of centrifuged sediment fixed with zinc acetate using nonsuppressed ion chromatography (Treude et al. 2005[a]).

16S rRNA gene library construction

Samples were frozen on board at -20 °C. Microbial mats obtained from three distinct zones of reef P822 (i.e. top, ex- and interior) were selected for clone library construction. DNA was extracted according to the method described by Lueders et al. (2004), using 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA). Archaeal and bacterial 16S rRNA genes were amplified by PCR with the primer sets Arch20F/Univ1392R (Massana et al. 1997, Lane et al. 1985, Lane 1991) and GM3F/GM4R (Muyzer et al. 1995), respectively. PCR conditions were as described previously (Ravenschlag et al. 1999) with following modifications: initial denaturation step for 3 min at 95 °C, 15 cycles, annealing at 58 °C (Arch20F/Univ1392R)/44 °C (GM3/GM4), and a final elongation step for 59 min at 60 °C. Twenty replicates per sample were pooled and purified using a QiaQuick PCR purification kit (Qiagen, Hilden, Germany). Amplicons were ligated into pGEM-T-Easy vector (Promega, Madison, Wisconsin, USA) and subsequently transformed into *Escherichia coli* Top10 cells (Invitrogen, Karlsruhe, Germany) according to manufacturers` recommendations.

Sequencing and phylogenetic analysis

Sequencing was performed by Taq cycle sequencing with a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Presence of chimeric sequences in the libraries was determined with Bellerophon (Huber et al. 2004). Sequence data were phylogenetically analysed with the ARB software package (Ludwig et al. 2004) using the dataset provided by ARB_SILVA (Prüsse at al. 2007, release_92). Phylogenetic trees were calculated by RaxML analyses using filters considering only those positions that are conserved in at least 50% of all Archaea/Bacteria. For tree calculation, only full-length sequences were considered. Partial sequences (~600 bp) were inserted into the

reconstructed tree by parsimony criteria without allowing changes in the overall tree topology. The nucleotide sequence data reported in this paper will be deposited in the EMBL, GenBank, and DDBJ nucleotide sequence database.

Catalyzed reporter deposition fluorescence *in situ* hybridization analysis

Sediments and microbial mats were fixed on board and stored as described previously (Rossel et al. 2008). Prior to quantification, fixed mat samples were physically homogenized by first pressing them with syringes through needles of decreasing diameters (1.2, 0.9, 0.65, and 0.4 mm) followed by loose and tight grinding (25.4-76.2 µm) with Dounce Tissue Grinders (Wheaton, Millville, New Jersey, USA). Finally, the cell suspensions were sonicated on ice at the following settings: three times 20 sec at an amplitude of 42 µm, < 10 kW, with a 20 sec break between each sonication (Sonopuls HD70, MS73 probe; Bandelin, Berlin, Germany). Fixed sediment samples were sonicated only once at the same settings.

In situ hybridizations with horseradish peroxidase (HRP)-conjugated probes (Biomers, Ulm, Germany) followed by tyramide signal amplification (CARD) were carried out as described by Pernthaler and colleagues (2002) with following modifications: endogenous peroxidases were inhibited with methanol (30 min), archaeal and *Planctomycetes* cell walls were permeabilized with proteinase K (15 µg ml^{-1}, for 2 min), and other Bacteria with lysozyme (10 mg ml^{-1}, for 10 min at 37 °C). All hybridizations were carried out for 2 h at 46 °C. Carboxyfluorescein-, Alexa488-, or Alexa594-labeled tyramides were added as substrates for the HRP. Total cell counts were determined by 4´,6´-diamidino-2-phenylindole (DAPI)-staining as described previously (Snaidr et al. 1997). Hybridized and DAPI-stained samples were examined with an Axiophot II epifluorescence microscope (Carl Zeiss, Jena, Germany). For each probe and sample 700-1000 DAPI-stained cells in around 70-100 independent microscopic fields with approximately 100 cells each were counted. Cells were counted only in the upper row of each field to avoid bleaching. Quantification of ANME-2 and DSS within the aggregates was possible as for free-living cells by direct cell counts. Sequences of probes used and formamide concentrations required for specific hybridization are given in Table 2.

Intact polar lipid analysis. Intact polar membrane lipid (IPL) analysis was performed as described previously (Rossel et al. 2008 and references therein). Total lipid extracts were obtained with an automated microwave assisted extraction system (MARS-X, CEM, USA). Structural identification of IPLs were based on mass spectral interpretation. Due to limited availability of commercial standards, we did not use response factors for IPLs quantification. Thus, IPLs are reported in relative amounts (percentage of total identified IPLs) with a signal to noise ratio higher than six.

Results

Methane seeping sediment and reef P822 - an in-depth study

Three distinct zones of an individual reef (P822) were selected to study the potential development of such a reef assuming zone “top” to harbor the youngest mats, zone “exterior” to harbor mats of medium age and zone “interior” as oldest mats. Methane seeping sediment samples were also included in the analysis and represent the initial reef stage.

Biogeochemical conditions

In the top 5 cm of sediment, methane concentrations ranged between 0.08 mM and 0.15 mM (Figure 1a). Sulfate concentrations varied between 10 mM and 13 mM. Highest AOM and high SR activity was detected between 0 and 3 cm sediment depth with 0.4 to 0.7 µmol ml^{-1} d^{-1} and 1.4 to 2.1 µmol ml^{-1} d^{-1}, respectively, indicating a 1:5 (surface) to 1:2 coupling (2.5 cm depth) between the two processes (Figure 1b). Below 3 cm depth, AOM activities were negligible, while SR activity was high throughout the core. A second AOM-independent SRR peak was determined in 8 cm depth with highest values of 2.6 µmol ml^{-1} d^{-1}. Incubations of the sediment revealed high AOM activity (5 µmol g_{dw}^{-1} d^{-1}) in the top 5 cm showing that rates are higher *in vitro* than *ex situ*.

In the reef, methane concentration in the pore water taken from the interior zone was 4 mM. The d13C value of methane was -62‰. The DIC concentration was 5 mM and the d13C DIC -17‰. Neither acetate nor dissolved iron (Fe^{2+}/Fe^{3+}) was detected. Sulfate and sulfide concentrations were 6 and 3 mM, respectively. AOM rates were measured *in vitro* for the top, ex- and interior zone of the reef. The rates were three-fold higher (940 µmol g_{dw}^{-1} d^{-1}) in the reefs` top than in the exterior (330 µmol g_{dw}^{-1} d^{-1}) and interior (300 µmol g_{dw}^{-1} d^{-1}) zone. These rates are extremely high, probably, because cells were homogenized prior to incubation.

Reef microbial diversity

To get first insights into reef microbial diversity and identify further potential key players involved in reef formation, archaeal and bacterial 16S rRNA gene libraries were constructed from the three zones of reef P822. Of the archaeal libraries, a total of 207

clones (top: 71, exterior: 82, interior: 54) and of the bacterial libraries, 174 clones (top: 48, exterior: 70, interior: 56) were analysed. Archaeal diversity was remarkably low: Euryarchaeota of the ANME-1 clade dominated with 65 sequences the clone library from the reefs` top, with 69 the library from the exterior, and with 48 sequences that of the interior. ANME-2a (top: 4, exterior: 0, interior: 0), ANME-2c (top: 0, exterior: 3, interior: 6), and Crenarchaeota of the marine benthic group B (top: 0, exterior: 1, interior: 0) were rare in all three libraries. In contrast, comparative sequence analysis suggested that reef bacterial diversity was high: 11 bacterial divisions and 6 candidate divisions were identified. The largest fraction of sequences grouped with ones of Deltaproteobacteria. Sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* branch, known to be the syntrophic partner bacteria of the ANME groups, were most abundant and present in all parts of the reef (top: 1, exterior: 26, interior: 16). Desulfobulbaceaea (top: 4, exterior: 2, interior: 0), *Desulfobacterium* (top: 0, exterior: 5, interior: 0), and Desulfovibrionales (top: 4, exterior: 0, interior: 1) were detected in lower frequency and not in all reef samples (Figure 2). The second largest fraction of clones grouped with sequences of the JS1 (23 sequences), which currently comprises only sequences from uncultivated organisms and has been repeatedly found in deep marine sediments (Webster et al. 2004). The bacterial phyla Planctomycetes and Thermomicrobia were detected with 19 sequences each, Planctomycetes related 16S rRNA genes were affiliated with three different lineages: *Pirellula* (4 sequences), *Isosphaera-Nostocoida* (13 sequences), and *Kuenenia* (2 sequences). They were mostly obtained from the reefs` top and rarely from its exterior. Thermomicrobia related sequences were retrieved from all reef zones. In lower frequencies were identified: sequences of Bacteroidetes (15 sequences, all reef zones), Spirochaetes (5 sequences, reefs` top), WS3 (5 sequences, reefs` exterior), Chloroflexi (4 sequences, all reef zones), Gemmatimonadetes (3 sequences, reefs` top and exterior), Acidobacteria (3 sequences, reefs` exterior), OP8 (3 sequences, reefs` ex- and interior) and OP11 (3 sequences, reefs` interior), OD1 (3 sequences, reefs` ex- and interior), Caldithrix (2 sequences, reefs` interior), Actinobacteria (2 sequences, reefs` top), Thermotoga, BRC1, and Nitrospirae (1 sequence each).

Microbial *in situ* abundance

In the top 3 cm of sediment, the microbial community was characterized by high cell numbers with 1 to 2.7 x 10^{10} (Figure 1 c). Cell counts dropped by one order of magnitude within the first 5 cm to 0.1 x 10^{10} cells ml^{-1}. Due to technical reasons, cell numbers were probably underestimated in these layers. High load of presumably inorganic particles and enclosure of cells in thick matrices hindered cell detection. Detection rates for Archaea and Bacteria were high ranging from 76% to 94% of total cells along the vertical profile (Figure 1c). While the upper layers were strongly dominated by Bacteria (up to 64% of total cells), archaeal cell numbers increased with depth and contributed more than 50% to total cells in the deepest layer analyzed.

Using probes for the known anaerobic methanotrophic archaea, ANME-1 to ANME-3, the major fraction of detected Archaea was assigned to ANME-1 and ANME-2. While the upper layers, which showed highest AOM rates, were dominated by ANME-2 archaea (18% of total cells, 90% of archaea), the deeper layers were characterized by a dominance of ANME-1 archaea (11-17% of total cells, 21-53% of Archaea) and the absence of ANME-2 (Figure 1d). ANME-2 cells mostly occurred as coccoid single cells and only few formed small aggregates. ANME-1 cells showed the well-known typical rectangular morphology. Sulfate-reducers of the *Desulfosarcina/Desulfococcus* branch (DSS) were highly abundant and accounted for around half of all Bacteria (up to 29% of total cells) throughout all layers. They did not only occur tightly associated with ANME-2 archaea, but mainly as single cells and most were vibrioform.

Of each reef zone, mat pieces were analyzed in replicates to tackle a possible heterogeneity of the mat. Abundance of specific groups varied only little between the parallel samples, however, in few mats, high heterogeneity was indicated by strong variation in bacterial counts (Table 3; e.g. 48% vs. 72% of total cells in reef P780). Mats from the reefs` top were characterized by highest total cell numbers of up to 3.0 x $10^{11}$ cells $g^{-1}$ and CARD-FISH detection rate of nearly 70% of total cells (Table 3). In contrast, cell numbers within the reefs` ex- and interior were more than 20-fold lower with averaged 1.1 and 0.8 x 10^{10} cells g^{-1}, respectively. The reefs` top and exterior were dominated by Bacteria (ca. 40% of total cells vs. ca. 20% Archaea) while in the interior, Bacteria and Archaea were equally abundant (35 to 34% of total cells).

ANME-1 and ANME-2 archaea were detected in all reef zones, however, their numbers varied remarkably. ANME-2 archaea dominated only the top of the reef with 24% and 15% in the subsamples, while they were rarely detected in the ex- and interior zone. Most ANME-2 from the reefs` top were assigned to the ANME-2a subgroup. ANME-2c were only sporadically detected at the reefs` top and in the reefs` exterior, and were of low abundance in the reefs` interior (~1%). ANME-2 archaea were, as in the sediments, coccoid and, if at all, only loosely aggregated with DSS (Figure 3), which might be due to the intense homogenization procedure applied prior to quantification. In contrast, in the reefs` ex- and interior, ANME-1 archaea dominated with averaged 30 and 27% of total cells (80-100% of total Archaea), respectively. Some ANME-1 cells showed CARD-FISH signals without corrresponding DAPI-signals. Probably, they did not contain DNA.

The highest total and relative abundance of DSS (2.6 x 10^7 and 3.4 x 10^7 cells ml^{-1}, 21 and 15% of total cells) was found at the reefs´ top. These numbers correspond to half of all Bacteria detectable with CARD-FISH (Table 3). Most DSS occurred as single vibrioform cells (4 µm by 1 µm) without tight association to ANME. Vibrioform DSS have so far been detected only in microbial reefs of the Black Sea (Knittel et al. 2005, Reitner et al. 2005[b]). Further CARD-FISH analyses were performed to quantify Crenarchaeota, Beta-, Gamma-, Delta-, and Epsilonproteobacteria, as well as Planctomycetes (Table 3).

Intact polar lipid signatures

The microbial community structure was further studied by the composition of their intact lipids (IPLs). An apparent change in IPL diversity and abundance between top, ex-, and interior zone of reef P822 was observed (Table 4). Archaeal IPLs of the reefs` top were largely based on high amounts of archaeol (AR) and hydroxyarchaeol (OH-AR), which have been assigned to ANME-2 (Rossel et al. 2008). Diglycosyl glyceroldialkylglyceroltetraether (2-Gly-GDGT) was as abundant as AR and OH-AR with 35% of total archaeal IPLs. Lipid composition of mats from the reefs` ex- and interior was highly dominated by 2-Gly-GDGT with 98% and 79% of the total identified archaeal IPLs, respectively (Table 4). Latter derive from ANME-1 (Rossel et al. 2008). Additionally, small amounts of 2-Gly-archaeol (2-Gly-AR), 2-Gly-OH-AR,

phosphatidylserine- (PS-) OH-AR, phosphatidylglycerol- (PG-) AR, and phosphatidylethanolamine- (PE) OH-AR were detected.

Bacterial IPLs varied with respect to structural diversity and abundance. PE of diacyl glycerol or mixed acyl/ether glycerol (DAG/AEG) types were present in variable amounts in all zones of reef P822. The ANME-2-dominated top of the reef mainly contained AEG-PE lipids or DAG-PE lipids (51% of total bacterial lipids) and only minor amounts of dietherglycerol lipid types (DEG, 8%, Rossel et al., 2008). Phosphatidylcholine (PC) and other PEs were also abundant (30%). The ANME-1 dominated interior of the reef showed a similar IPL profile as the top mainly differing by the absence of PEG-PE and PCs. IPL composition of the reefs` exterior was dominated by DEG-PE lipids (19%), but also comprised remarkably amounts of diverse DAG/AEG-PEs.

Inter-reef diversity

Microbial mats from four additional reefs were selected for CARD-FISH analysis to study similarities and dissimilarities in their microbial community structure. The mats derived from three different areas, i.e. the Dnepr Area (P822 and P795), the Northwest Shelf (P787), and Danube Canyon (P784 and P780). From all sites, nodules from top of the reefs were sampled, except for reef P795, where the material originated from the reef side at half-height (exterior). If possible, fine-scale analyses were conducted by separation of differently colored mat layers (i.e. black, pink, and gray; Table 1, 3).

Microbial i*n situ* abundance

Cell numbers varied strongly with up to three orders of magnitude between different reefs and mat layers ranging between 18 x 10^{10} cells g^{-1} in the pink mat layer of reef P795 to as low as 0.02 x 10^{10} cells g^{-1} in the black mat layer of reef P784. The known key players in AOM, ANME-1, ANME-2, and DSS bacteria, were present in all samples. However, all nodules were dominated by ANME-2 archaea and DSS bacteria, whereas ANME-1 cell abundance was always <1% (Table 3). In contrast, a dominance of ANME-1 was found in mats from P795 and the ex- and interior zone of P822, as described above. Here, ANME-1 accounted for up to one-third of total cells (almost 100% of all Archaea), while ANME-2 archaea were below the CARD-FISH detection limit. In layers

dominated by ANME-1, DSS cells were always rare, in layers dominated by ANME-2, DSS cells largely contributed to the total microbial community.

Fine-scale analysis of differently colored mat layers from P780 and P795 showed that an individual mat was either ANME-1 or ANME-2 dominated. The relative ANME abundance seemed to increase from the mats surface towards their interiors (6% to 12% and 5% to 36% of total cells). Conversely, the relative abundance of DSS slightly decreased in both mats (2% to <1% and 19% to 14% of total cells).

Intact polar lipid signatures

ANME-2 dominated microbial mats from reef P784 and P780 were characterized by high percentages of AR and hydroxyarchaeol OH-AR with either glycosidic or phosphate-based headgroups (Table 4). 2-Gly-AR made up 33%, PG-AR 31%, and tentative phosphor-AR 20% of total archaeal lipids in P784 mats (46%, 26%, and 8% in reef P780 mats). Polar dialkylglycerolethers (2-Gly-GDGT), typically being associated with ANME-1 communities, were only found in minor abundance of 9% (P784) and 6% (P780) of total archaeal lipids. In contrast, 2-Gly-GDGT contributed almost 100% of total archaeal lipids in the ANME-1 dominated reef P795 mats.

DEG-PE contributes with 22% and 16%, while DAG/AEG-PE with 78% and 84% to the total bacterial IPLs in reef P784 and reef P780, respectively. However, the previously perceived small contribution of bacterial IPLs in ANME-1 dominated samples compared to ANME-2 systems (Rossel et al. 2008) was also observed in this study (<5% and >20% of the total identified IPLs were associated to Bacteria in ANME-1 and ANME-2 systems, respectively, Table 4).

Discussion

Reefs host diverse microbial communities

In contrast to the low archaeal diversity, the diversity in the bacterial 16S rRNA gene libraries was high with 11 bacterial divisions and 6 candidate divisions. Since our libraries were too small to discuss absence we only focus the discussion on abundant sequences. The largest fraction was assigned to diverse DSS bacteria, which were also abundant *in situ* and are frequently found in AOM habitats (Boetius et el. 2000, Knittel et al. 2005, Mills et al. 2005, Lösekann et al. 2007, Wegener et al. 2008, Omoregie et al. 2008, Orcutt 2008).

Planctomycetes related genes were frequent in the libraries and cells were also consistently detected in the methane seeping sediment and all reef samples. Several sequences grouped with ones of Bacteroidetes, which were also found *in situ*. They might have settled from the waters (Vetriani et al. 2003, Lin et al. 2006) or immigrated from the surrounding sediment. Bacteroidetes are frequently found at AOM sites (Lösekann et al. 2007, Wegener et al. 2008) and in other marine sediments, and probably degrade organic matter (Bissett et al. 2008). Most of the other groups have been often identified also in other AOM habitats including Actinobacteria, Chloroflexi, and Spirochaetes, OP11 (Mills et al. 2005, Wegener et al. 2008, Lösekann et al. 2007), Acidobacteria, and Nitrospirae (Lösekann et al. 2007, Wegener et al. 2008). Genes of the OP8, OD1, and BRC1 cluster, Caldithrix, and Thermotoga have yet been detected only rarely, if at all, at AOM sites. The high diversity of bacterial 16S rRNA genes retrieved in our libraries might be due to the PCR protocol applied that was characterized by large numbers of replicates and only 15 PCR cycles used to amplify the DNA. Both aimed at retrieving high diversity.

Spatial distribution and niche formation of ANME-1 and ANME-2

In the top layers (0-3 cm) of methane seeping surface sediment, cell numbers were two orders of magnitudes higher than those found in the sediments` subsurface (10^8 cells ml^{-1}), one magnitude higher than ones in other Black Sea shelf/slope sediments (10^9 cells ml^{-1}, Thamdrup et al. 2000, Leloup et al. 2007) and only one order of magnitude lower (10^{10} cells ml^{-1}) compared to reefs (10^{11} cells ml^{-1}). This suggests that methane seeping sediment might represent an initial reef stage. AOM and SR rates were highest within the top layers, where ANME-2 dominated the community, while ANME-1 were only rarely detected. In turn, ANME-1 dominated in the subsurface (3-5 cm), but based on the low AOM rates (up to 0.1 µmol ml^{-1} d^{-1}), they were probably inactive. Sulfate concentrations were sufficient to allow AOM throughout the entire core and methane concentrations comparable at the surface and subsurface. However, the surface sediment likely contains higher concentrations of methane, but lower concentrations of sulfate than the surface layers *in situ.* ANME abundance, and AOM rates were low (up to 0.7 µmol ml^{-1} d^{-1}) compared to those described for microbial reefs (average 18±12 µmol g_{dw}^{-1} d^{-1}, Michaelis et al. 2002) and ones determined in a subsurface methanotrophic mat (1.5 µmol cm^{-3} d^{-1}), a suggested preliminary stage of reef formation (Treude et al. 2005[a]), which further supports that this sediment represents an initial reef stage. DSS were abundant in all sediment layers and might response the high AOM independent SRR detected in the sediment.

Sensing and motility of ANME have never been described. However, ANME-2 in the surface of methane seeping sediment could have passively drifted up from the subsurface with gas bubbles (Schubert and Durish-Kaiser et al. 2006), settled from the water (Michaelis et al. 2002, Wakeham et al. 2003, Durisch-Kaiser et al. 2005, Schubert et al. 2006) or over from reefs nearby driven by strong methane outbursts. ANME-1 in the subsurface of methane seeping sediment were probably part of a deeper located methanotrophic mat similar to the one described by Treude et al. (2005[a]). The growth of ANME into an aggregated mat could support gas trapping and initiate reef development.

CARD-FISH and IPL analysis, which mostly correlated well, revealed that all reefs along the shelf hosted ANME-1, ANME-2, and DSS supporting their importance as reef-forming microorganisms and suggesting relatively stable environmental conditions

in this area. However, not all reefs formed large structures: the small reef from the Northwest Shelf probably once used to be well supplied with nutrients, but was under-supplied long before sampling or another factor was suboptimal to support microbial fitness, indicated by the fuzzy, presumably damaged structure of most cells.

Distribution of the key methanotrophs varied in distinct reef zones: either ANME-1 or ANME-2 were dominant. High ANME-2 abundance correlated with high numbers of DSS, whereas no significant numbers of DSS or other Deltaproteobacteria were observed in ANME-1 dominated samples. ANME-2 and DSS are commonly tightly associated, while ANME-1 often occur as single cells (e.g. Boetius et al. 2000, Knittel et al. 2005). In this study, many ANME-1 and ANME-2 were not at all associated with DSS, likely due to the intense homogenization of the mats prior to quantification. However, also in the sediment, which was homogenized by sonication only, many cells occurred solely. The high abundance of DEG-PEs in ANME-1 dominated systems indicates that these lipids do not derive from DSS bacteria as previously suggested (Rossel et al. 2008), because DSS were rare in ANME-1 dominated zones. Moreover, these lipids were absent in the reefs` interior. If DEG-PEs indeed belong to ANME-1 partner, there is no partner in the interior. DAG/AEG in combination with PE is consistent with the IPL composition of *Desulfosarcina variabilis* (Rütters et al. 2001) although the chain length distribtuion and degree of unsaturation differ and might be the lipids associated with DSS.

The top nodule of reef P822 and upper layer of methane seeping sediment revealed certain parallels: although these niches presumably contain less methane than the reefs` interior and subsurface sediment, both habitats were characterized by highest AOM rates and largest ANME populations. Further, both habitats were dominated by ANME-2a and DSS, while ANME-1 numbers were very low. Also top nodules from all other reefs were dominated by ANME-2. The higher amount of ANME-1 associated intact polar lipids in this zone might be explained by the presence of intact, but inactive and thus not CARD-FISH detectable cells or higher amounts of IPL in ANME-1 than ANME-2 cells. Higher lipid fractions are confirmed by the Archaea/Bacteria ratios. However, nodules represent the youngest structures of the reefs (Lein et al. 2002) and are probably the most rapidly growing reef zones.

Our data support the hypothesis of Reitner et al. (2005[a]), who claim that the reefs` interior is the oldest zone. It hosted the smallest population, suggesting limitation of microbial growth. Further, AOM rates measured in the reefs` interior were low compared to the reefs` top, although methane concentrations were high (4 mM in the reefs` porewater sampled in the interior). However, cell-specific AOM rates of the resident ANME-1 were rather higher than those of ANME-2. AOM activity might be higher *in vitro* than *ex situ*. Nevertheless, the ANME-1 in the reefs` interior must have been active *in situ*. Additionally, ANME-1 cells without DAPI signals (likely inactive ANME-1) were most abundant in the interior (~8%), but the number was low compared to another study of a reefs` interior, in which even 80 vol% empty sheaths were reported (Reitner et al. 2005[a]). High abundance of inactive ANME-1 towards a mats` interior was also described in a small-scale study and probably related to low sulfate supply (Treude et al. 2007). The interior pore water contained sufficient sulfate to enable AOM activity, but sulfate concentrations were lower (6 mM) than ones in the surrounding seawater (around 16 mM) and its availability might have locally exert selective pressure within the irregular interior of the reefs. Few ANME-2c cells were detected in this zone, whereas they were almost absent in the reefs` top and exterior zone. An increasing ANME-2c abundance towards mats interiors was also observed by Treude et al. (2007) and Knittel et al. (2005). The microorganisms inhabiting the reefs` interior are likely limited by space. In comparison to the reefs` interior, the AOM rate and ANME-1 abundance was slightly higher in the exterior, although it is the zone most likely under-supplied with methane. This might be due to higher sulfate availability in the reefs` exterior.

The dominance of active and large populations of ANME-2a/DSS in the surface sediment as well as in the reefs` top and the dominance of highly active ANME-1 in the subsurface sediment as well as in the reefs` ex- and interior suggests that a reef grows upward and moreover outward from its point of origin in the sediment upon precipitated carbonates, driven by ANME-2a in the upper zone, whereas ANME-1 response the outward growth. Probably, ANME-2a are wiped out when methane concentrations strongly increase due to temporarily intense upward methane fluxes. It was observed occassionally that gas-filled nodules sometimes take off the reefs and float away driven by intense gas outbursts, which might be explained by local gas trapping or temporarily

increased fluxes. The low abundance of ANME-2 in the exterior zone can probably be explained by too low methane concentrations. However, without comparable data on different reefs and *in situ* rate measurements, this hypothesis remains speculative.

Small-scale analysis of differently colored mat layers of reefs from the Dnepr Area and Danube Canyon revealed an increasing ANME abundance from the mats` ex- towards their interiors. Also Treude et al. (2007) found an increase of ANME abundance and highest AOM activity in the interior of mats located close to the water-mat interface and explained this by grazing. Even permanently anoxic areas of the Black Sea host grazers (Sergeeva and Gulin 2007). In contrast, relative abundance of DSS bacteria strongly decreased towards the mats` interiors. Decreasing sulfate availability towards the mats interiors might explain the decrease of DSS bacteria in our mats. The partly high fractions of cells neither identified as Archaea nor as Bacteria indicate that several cells in the mats are dead or inactive, which suggests that cells are preserved within mat matrices. However, if mats grow outwards, inwards, or simultaneously out- and inwards needs to be further investigated.

Probably, ANME-2a can neither cope with very high or very low methane fluxes and depend on high fluxes of sulfate. ANME-1 might prefer high methane fluxes, but seem to cope with low fluxes of both methane and sulfate. ANME-2c might survive, but not thrive when methane fluxes are high and sulfate fluxes are low. In a continuous flow reactor enriched ANME-1 and ANME-2 showed highest growth rates in high and low porewater flow rates, respectively (Girguis et al. 2005). Aside from nutrient availability, the removal of products deriving from AOM and other pathways might also exert selective pressure: e.g. high sulfide concentrations might be toxic as already considered by Krüger and his colleagues (2008).

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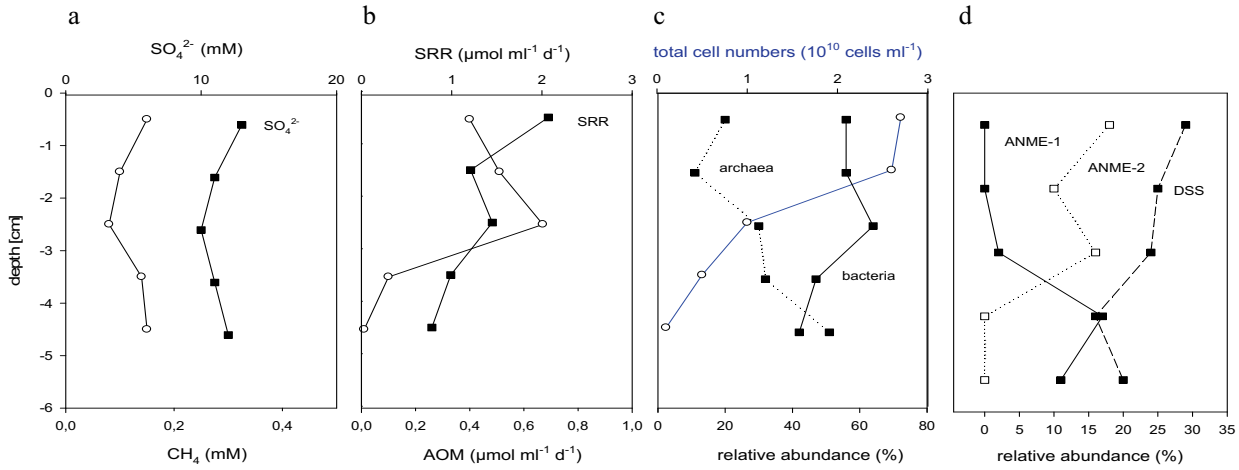

Figure 1: *Ex situ* methane/sulfate concentrations and AOM/SR rates, and *in situ* microbial abundance in methane seeping sediment from the northwestern Black Sea (Dnepr Area).

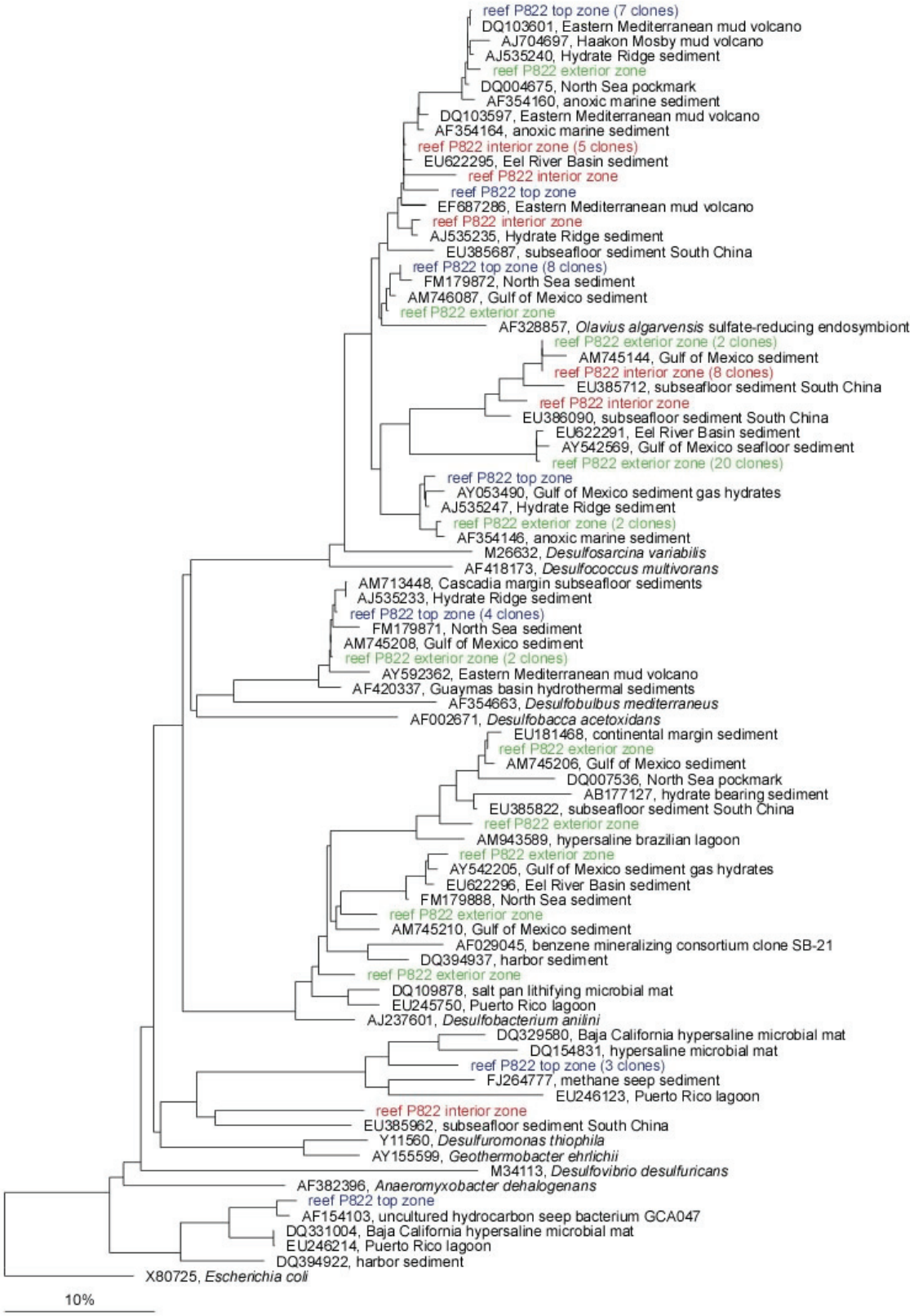

Figure 2: Phylogenetic tree showing affiliations of Black Sea microbial reef SRB 16S rRNA gene sequences (blue: reef top, green: reef exterior, red: reef interior) to selected reference sequences of (potential) sulfate-reducing Deltaproteobacteria.

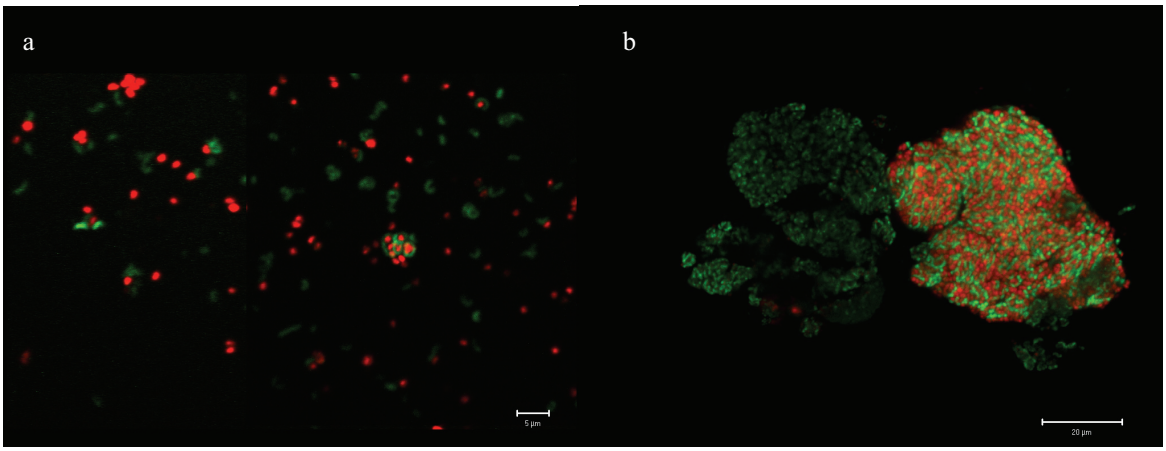

Figure 3: Laserscanning micrographs of (a) single ANME-2 (red) cells and DSS (green) and (b) aggregates of both.

Table 1: Sampling sites and description of samples obtained from the northwestern Black Sea shelf/slope

sampling site	sample name	sample type	north	east	water depth	sample description
Dnepr Area	sediment	sediment	44:46.569	31:58.967	192 m	black surface, gassy, 0-10 cm
	reef P822	microbial mat	44:46.542	31:58.978	191 m	top, ex- and interior zone
	reef P795	microbial mat	44:46.775	31:59.164	189 m	tricolored nodule, exterior
Northwest Shelf	reef P787	microbial mat	44:37.756	31:08.635	248 m	slimy pink nodule, top zone
Danube Canyon	reef P784	microbial mat	43:50.590	30:27.657	370 m	soft brown nodule, upper zone
	reef P780	microbial mat	43:57.474	30:17.262	295 m	tricolored nodule, top zone

Table 2: Oligonucleotide probes used in this study

Probes	Target organisms	Probe sequences (5` to 3`)	FA*	Reference
Arch915	most Archaea	GTG CTC CCC CGC CAA TTC CT	35	Stahl and Amann 1991
GI-554	marine crenarchaeotal group I	TTA GGC CCA ATA ATC MTC CT	20	Massana et al.1997
ANME1-350	ANME-1 archaea	AGT TTT CGC GCC TGA TGC	40	Boetius et al. 2000
EelMS932	ANME-2 archaea	AGC TCC ACC CGT TGT AGT	50	Boetius et al. 2000
ANME-2a-647	ANME2a archaea	TCT TCC GGT CCC AAG CCT	50**	Knittel et al. 2005
ANME-2c-760	ANM2c archaea	CGC CCC CAG CTT TCG TCC	60	Knittel et al. 2005
Eub338-I	most Bacteria	GCT GCC TCC CGT AGG AGT	35	Amann et al. 1990
EUB338-II	most Planctomycetales and some other bacteria	GCA GCC ACC CGT AGG TGT	35	Daims et al. 1999
EUB338-III				
Competitor		GCT GCC ACC CGT AGG TGT		
EUB338-III	most Verrucomicrobiales, many Chloroflexi and some other bacteria	GCT GCC ACC CGT AGG TGT	35	Daims et al. 1999
EUB338-II				
Competitor		GCA GCC ACC CGT AGG TGT		
AMX-820	*Candidatus* Kuenenia stuttgartiensis	AAA ACC CCT CTA CTT AGT GCCC	40	Schmid et al. 2000
DSS658	*Desulfosarcina/Desulfococcus* and related SRB	TCC ACT TCC CTC TCC CAT	50	Manz et al. 1998
Alf968	Alphaproteobacteria, except for Rickettsiales	GGT AAG GTT CTG CGC GTT	35	Neef 1997
Bet42a	Betaproteobacteria	GCC TTC CCA CTT CGT TT	35	Manz et al. 1992
Gam42a competitor		GCC TTC CCA CAT CGT TT		
Gam42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	35	Manz et al. 1992
Bet42a competitor		GCC TTC CCA CTT CGT TT		
EPSI682	Hydrothermal vent Epsilonproteobacteria	CGG ATT TTA CCC CTA CAC M	20	Moussard et al. 2006
EPSY549	Most Epsilonproteobacteria	CAG TGA TTC CGA GTA ACG	35	Lin et al. 2006
CF319a	most Flavobacteria, some Bacteroidetes, some Sphingobacteria	TGG TCC GTG TCT CAG TAC	35	Manz et al. 1996

*FA= formamide concentration in hybridisation buffer [%, vol/vol], ** 35% FA used for CARD-FISH

Table 3: *In situ* abundance of diverse groups of archaea and bacteria in sediment and microbial reefs from the northwestern Black Sea as revealed by DAPI staining and CARD-FISH

Sample	Subsample	Total cells	Arch915	EUB338 I-III	ANME-1	ANME-2	GI554	DSS658	Gam42a	EPSI682	CF319a	EUB338-II
		[10^{10} g^{-1}]					[% of total cells]					
P817	0-1	2.7	20	56	0	18	n.a.	29	n.a.	n.a.	n.a.	n.a.
	1-2	2.6	11	56	0	10	n.a.	25	n.a.	n.a.	n.a.	n.a.
	2-3	1.0	30	64	2	16	n.a.	24	n.a.	n.a.	n.a.	n.a.
	3-4	0.5	32	47	17	0	n.a.	16	n.a.	n.a.	n.a.	n.a.
	4-5	0.1	51	42	11	0	n.a.	20	n.a.	n.a.	n.a.	n.a.
P822	top	13/30	23/14	44/43	<1/<1	24/15	<1/<1	21/15	<1/<1	<1/<1	3/7	3/7
	exterior	0.9/1.4*	24/30	34/32	28/32	<1/<1	<1/<1	<1/<1	<1/<1	<1/3	<1/<1	<1/<1
	interior	0.8/0.9*	33/35	36/35	28/27	<1/<1	<1/<1	<1/<1	<1/<1	<1/<1	<1/<1	<1/<1
P795	out	6.9/8.0	11/9	23/17	13/5	<1/<1	n.a.	2/<1	n.a.	11/3	n.a.	1/4
	mid	6.2/4.4	46/42	18/20	33/31	<1/<1	n.a.	<1/<1	n.a.	6/5	n.a.	<1/2
	centre	18/n.a.	30/37	19/13	30/36	<1/<1	n.a.	<1/1	n.a.	4/8	n.a.	2/n.a.
P787	pink	2.1/n.a.	14/n.a.	63/n.a.	<1/n.a.	16/n.a.	n.a.	36/n.a.	n.a.	<1/n.a.	n.a.	n.a.
P784	brown	4.7/n.a.	10/n.a.	45/n.a.	1/n.a.	6/n.a.	n.a.	26/n.a.	n.a.	<1/n.a.	n.a.	n.a.
P780	out	0.4/2.8	21/12	48/72	n.a./1	n.a./6	n.a.	n.a./19	n.a.	<1/<1	n.a.	<1/<1
	mid	2.2/0.5	10/16	64/53	n.a./<1	n.a./12	n.a.	n.a./15	n.a.	<1/<1	n.a.	1/1
	centre	0.7/0.02	19/24	58/62	n.a./<1	n.a./12	n.a.	n.a./14	n.a.	<1/<1	n.a.	4/1

*of these numbers, $5.1x10^8$/$8.3x10^8$ and $6.9x10^8$/$6.0x10^8$ ANME-1 signals g^{-1} in mats from the exterior and interior zone, respectively, were found without corresponding DAPI signal. n.a. not analyzed

Table 4: IPLs distribution of both Bacteria and Archaea from different reefs from the northwestern Black Sea shelf. Concentrations of IPLs are based on the peak area in mass chromatograms relative to the internal standard C16-PAF. Bacterial IPLs named according to the type of bond between alkyl moieties and glycerol backbone (DEG = diether, DAG = diacyl, AEG = mixed), followed by head groups, the sum of carbon atoms in both alkyl chains and number of double bonds.

Reef	Subsample	Archaeal IPL diversity (µg/gdw mat)	Bacterial IPL diversity (µg/gdw mat)[a]	Source
P822	top	PG-AR (404), 2-Gly-GDGT (385), tentative phospho-AR (221), 2-Gly-AR (48), PS-OH-AR (20), 2-Gly-OH-AR (48), PE-OH-AR (12)	DAG/AEG-PE C31:2 (65), DEG-PE C32:1 (24), DAG/AEG-PE C31:1 (22), DAG/AEG-PE C32:2 (18), DEG-PE C31:1 (16), other PEs and PCs (62)	Rossel et al. (2008)
	exterior	2-Gly-GDGT (12372), 2-Gly-AR (86), PS-OH-AR (33), PG-AR (33), 2-Gly-OH-AR (13)	DEG-PE C33:1 (35), DAG/AEG-PE C29:1 (26), DAG/AEG-PE C31:1 (16), DAG/AEG-PE C30:1 (14), DAG/AEG-PE C28:0 (14), other PEs and PCs (81)	This study
	interior	2-Gly-GDGT (723), tentative phospho-AR (65), PG-AR (64), 2-Gly-AR (35), 2-Gly-OH-AR (8), PS-OH-AR (7), PE-OH-AR (2)	DAG/AEG-PE C31:2 (10), DAG/AEG-PE C32:2 (8), DAG/AEG-PE C31:1 (5), DAG/AEG-PE C30:1(3), other PEs (16)	This study
P795	pink	2-Gly-GDGT (13308), 2-Gly-AR (56)	DEG-PE C30:0 (39), DAG/AEG-PE C33:2 (24), DEG-PE C31:1 (23), DAG/AEG-PE C35:2 (14), DAG/AEG-PE C32:2 (13), other PEs (12.3)	Rossel et al. (2008)
P784	brown	2-Gly-AR (141), PG-AR (190), tentative phospho-AR (123), 2-Gly-GDGT (58), 2-Gly-OH-AR (29), PE-OH-AR (9), PS-OH-AR (8), PS-2OH-AR (4)	DAG/AEG -PE C31:2 (40), DAG/AEG-PE C32:2 (22), DAG/AEG-PE C31:1 (19), DAG/AEG-PE C29:1 (10), DEG-PE C32:1 (9), other PEs (57)	This study
P780	brown	2-Gly-AR (64), PG-AR (56), tentative phospho-AR (18), 2-Gly-OH-AR (16), 2-Gly-GDGT (13), PS-OH-AR (10), PE-OH-AR (3)	DAG/AEG-PE C31:2 (21), DAG/AEG-PE C29:1 (16), DAG/AEG-PE C31:1 (8), other PEs (36)	This study

[a] Distinction between DAG and AEG was not possible under HPLC-MS conditions applied, alkyl chains are provided for DAG derivatives

Manuscript III

Title:

Development of a 16S rRNA-targeted probe set for Verrucomicrobia and its application to *in situ* quantification in a humic lake

Authors:

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Abstract

Members of the highly diverse bacterial phylum *Verrucomicrobia* are globally distributed in various terrestrial and aquatic habitats. They are key players in soils, but little is known about their role in aquatic systems. Here, we report on the design and evaluation of a 16S rRNA-targeted probe set for the identification of *Verrucomicrobia* and of clades within this phylum. Subsequently, the probe set was applied for a study on the seasonal abundance of *Verrucomicrobia* in waters of the humic lake Große Fuchskuhle (Germany) by catalyzed reporter deposition fluorescence *in situ* hybridization. The lake hosted diverse *Verrucomicrobia* clades in all seasons. Either *Spartobacteria* (up to 19%) or *Opitutus* spp. (up to 7%) dominated the communities, whereas *Prosthecobacter* spp. were omnipresent in low numbers (<1%). Verrucomicrobial abundance and community composition varied between the seasons, and between more and less humic basins, but little between oxic and seasonally anoxic waters.

Key words

Verrucomicrobia, 16S rRNA, oligonucleotide probes, fluorescence *in situ* hybridization, humic lake, Große Fuchskuhle

Introduction

Members of the bacterial phylum *Verrucomicrobia* were first observed more than 70 years ago (Henrici and Johnson 1935). To date, six monophyletic subdivisions are distinguished, based on 16S rRNA gene analysis (Hugenholtz et al. 1998, Schlesner et al. 2006, Wagner and Horn 2006). A seventh subdivision has recently been proposed (Dunfield et al. 2007, Islam et al. 2008, Pol et al. 2007). *Verrucomicrobia* inhabit various terrestrial and aquatic habitats including oligotrophic, eutrophic, extreme, polluted, and man-made ones (Schlesner et al. 2006, Wagner and Horn 2006). Representatives of five subdivisions have been isolated so far from soil, freshwater, and marine habitats, as well as from fecal samples. Isolates are aerobic, facultative anaerobic, or obligate anaerobic heterotrophs using various carbon compounds such as plant polymers, e.g. cellobiose, xylan, pectin, and starch (Chin et al. 1999, Janssen et al. 1997), sugars (Schlesner 1987), or methane (Dunfield et al. 2007, Islam et al. 2008, Pol et al. 2007).

Verrucomicrobia are morphologically diverse. Some form fimbriated prostheca (Schlesner 1987). Highly specialized members are ectosymbionts that form epixenosomes, harpoon-like organelles, to protect their host ciliate (Petroni et al. 2000). Species of at least three verrucomicrobial subdivisions have intracellular compartments (Lee et al. 2009), a feature that was among *Bacteria* so far known only from *Planctomycetes* (Fuerst 2005) and *Poribacteria* (Fieseler et al. 2004). Pilhofer et al. (2008) suggested an FtsZ-based cell division mechanism in all members of *Verrucomicrobia*. Genomes of *Akkermansia, Opitutus,* and *Methylacidiphilum* species have been completed and three further ones released in draft form (Galperin 2008, Hou et al. 2008). Some *Verrucomicrobia* have been found associated with algae (Bruckner et al. 2008), protozoa (Petroni et al. 2000), sponges (Scheuermayer et al. 2006), worms (López-García et al. 2002, Vandekerckhove et al. 2000, 2002), arthropods (Corby-Harris et al. 2007), fish, and mammalians including humans (Derrien et al. 2004, Ley et al. 2008). Several studies have revealed syntrophic relations between *Verrucomicrobia* and eukaryotes (López-García et al. 2002, Petroni et al. 2000, Vandekerckhove et al. 2000). Interestingly, *Verrucomicrobium spinosum* carries genes coding a protein secretion apparatus, which might translocate bacterial proteins into eukaryotic cells (Pallen et al. 2005).

Verrucomicrobia constitute significant parts (up to 10%) of microbial soil communities as revealed by e.g. competitive quantitative polymerase chain reaction of rRNA genes, most-probable-number strategies, or hybridization against extracted rRNA (Buckley and Schmidt 2001, Chin et al. 1999, Lee et al. 1996, O`Farrell and Janssen 1999). Distribution of

verrucomicrobial rRNA in soil suggested that *Verrucomicrobia* are affected by environmental parameters that change with time, soil history, and soil depth, and a statistically significant amount of the variation in verrucomicrobial rRNA abundance can be explained by changes in soil moisture content (Buckley and Schmidt 2001). Whole cell fluorescence *in situ* hybridization (FISH) has only rarely been applied to identify *Verrucomicrobia* (Daims et al. 1999, Dedysh et al. 2006, Petroni et al. 2000, Pol et al. 2007, Vandekerckhove et al. 2002). Little is reported on the abundance and ecology of aquatic *Verrucomicrobia*. Verrucomicrobial prevalence might be positively correlated with nutrient-richness (Haukka et al. 2006) and phosphorus availability (Lindström et al. 2004) in lakes. In soils, they might be adapted to low substrate concentrations (Noll et al. 2005).

In this study, we used published verrucomicrobial 16S rRNA gene sequences and developed a set of oligonucleotide probes. Some of the probes were applied to water samples from the humic lake Große Fuchskuhle (Germany). We selected this habitat because several studies indicated the importance of *Verrucomicrobia* in humic lakes (Haukka et al. 2005, Lindström et al. 2004, Newton et al. 2006). Verrucomicrobial genes frequently occurred in two independently constructed gene libraries from this lake and a rather large fraction of the lake microbial community had remained unidentified in FISH studies, which did not cover *Verrucomicrobia* (Burkert et al. 2003, Glöckner et al. 2000).

Materials and Methods

Site description and sample collection

Lake Große Fuchskuhle is located in the large Mecklenburg-Brandenburg lake district near Berlin (53°10`N, 13°02`E). In 1990, the lake was artificially divided into four basins (northwest, northeast, southwest, and southeast; Fig. 1) which differ with respect to hydrogeology, biogeochemistry, microbial activity, and structure of the microbial food web as in detail described elsewhere (Allgaier and Grossart 2006, Bittl et al. 1996, Bittl 1999, Buck et al. 2008, Burkert et al. 2003, 2004, Grossart et al. 2008, Hehmann et al. 2001, Kasprzak et al. 1988, Kasprzak 1993, Koschel 1995, Sachse et al. 2001, Simek et al. 1998).

Here, we chose the two most contrasting basins, the acidotrophic humic southwestern (SW) basin with a high influx of allochthonous dissolved organic carbon (DOC) and the more mesotrophic northeastern (NE) basin to study abundance and seasonality of *Verrucomicrobia*. Lake water from the SW and NE basin was collected from 0.5 m (oxic) and 4.5 m (seasonally anoxic) depth approximately trimonthly (Mar-01 = spring, Jun-29 = summer, Sep-12 = autumn, and Dec-11 = winter) in 2000.

Fig. 1

Phylogenetic analysis

A phylogenetic tree of the phylum *Verrucomicrobia* was reconstructed using ~3000 almost full length sequences of 16S rRNA genes available in the ARB SILVA database (SILVA release_98, 26th of March 2009 [Prüsse et al. 2007]). The tree was calculated using maximum likelihood analyses (PhyML) as implemented in the ARB software package (Ludwig et al. 2004). Selected sequences of *Lentisphaera* and *Chlamydia* were used as outgroups, and a filter considering only those positions that are conserved in at least 50% of all *Verrucomicrobia* was applied in the calculations.

Design of oligonucleotide probes

More than 1000 almost full length 16S rRNA gene sequences of *Verrucomicrobia* (SILVA release_91, 15th of August 2007 [Prüsse et al. 2007]) were aligned, and oligonucleotide probes for individual verrucomicrobial clades designed using the probe design tool of the ARB software (Ludwig et al. 2004). The criteria were high coverage of the target group, and as few

as possible hits to organisms outside the target group. In addition, the probe target position should be located within a region of the 16S rRNA molecule having a high *in situ* accessibility (Behrens et al. 2003, Fuchs et al. 1998). Probe specificity was checked again using the actual dataset (SILVA release_100, 3rd of August 2009 [Prüsse et al. 2007]). Probe sequences and hybridization conditions used for FISH are given in Table 1. Aligned fullmatch and mismatch reference 16S rRNA sequences showing probe binding sites are shown in Figure 3.

Optimization of hybridization conditions

For our newly designed subgroup-specific verrucomicrobial probes (Table 1), optimal conditions in FISH were established by evaluating the fluorescence intensities of the target cells after hybridization with Cy3-labeled probes at increasing concentrations of formamide in the hybridization buffer (Pernthaler et al. 2001). For the two already published group-specific verrucomicrobial probes (EUB338-III and VER47, Table 1), i*n situ* hybridizations with horseradish peroxidase (HRP)-conjugated probes followed by catalyzed reporter deposition (CARD) were conducted to determine the optimal formamide concentrations. Hybridization conditions were considered to be optimal at the last formamide concentration before significant amounts of probes dissociated off the target organism. Since Hoshino et al. (Hoshino et al. 2008) had shown that HRP-labeled probes may dissociate at lower formamide concentrations compared to Cy3-labeled probes, we validated hybridization of our new probes at optimized formamide concentrations in CARD-FISH.

CARD-FISH quantification of *Verrucomicrobia* in lake water

Water samples were fixed with formaldehyde, filtered (0.2 µm pore size filter), and stored as described previously (Burkert et al. 2003). CARD-FISH was carried out as described by Pernthaler et al. (Pernthaler et al. 2002) with following modifications: endogenous peroxidases were inhibited prior to hybridization by overlaying filters with pure methanol for 30 min. Subsequently, verrucomicrobial cell walls were permeabilized with 15 µg ml^{-1} proteinase K for 2 min at room temperature. Total cell counts were determined by 4´,6´-diamidino-2-phenylindole (DAPI)-staining as described previously (Snaidr et al. 1997). Hybridized and DAPI-stained samples were examined with an epifluorescence microscope (Axiophot II microscope; Carl Zeiss, Jena, Germany). For each probe and sample, 1000 DAPI-stained cells in 100 independent microscopic fields were counted. Only the upper 10 squares of a field were

analyzed to avoid bleaching. For all time points, duplicates were analyzed with probe EUB338-III (Daims et al. 1999).

Results and Discussion

Basis of probe design - phylogeny of *Verrucomicrobia*. Six verrucomicrobial subdivisions (V1 to V6) are distinguished by Hugenholtz et al. (1998), Schlesner et al. (2006), and Wagner and Horn (2006). The subdivisions V1, V2, V3, and V4 currently consist of eight, two, one, and eight genera, respectively (Fig. 2). For subdivision V5 and V6, no representatives have been yet cultured. Subdivision V1, *Verrucomicrobiaceae*, encompasses the genera: *Prosthecobacter* (Hedlund et al. 1996, Staley et al. 1976), *Verrucomicrobium* (Schlesner 1987), *Akkermansia* (Derrien et al. 2004), *Rubritalea* (Scheuermayer et al. 2006), *Luteolibacter*, *Persicirhabdus*, *Roseibacillus* (Yoon et al. 2008[a]), and *Haloferula* (Yoon et al. 2008[b]). Numerous 16S rRNA gene sequences of uncultured *Verrucomicrobia* from terrestrial, freshwater and marine systems cluster with these genera. Within subdivision V2, *Xiphinematobacteriaceae*, *Chthoniobacter flavus* (Sangwan et al. 2004) and the not yet validly described *Spartobacterium* sp. (Im and Lee 2006, unpublished) have been isolated. Further, sequences of uncultured *Verrucomicrobia* mainly from soil and freshwater systems affiliate with these groups. Subdivision V3 includes the isolate Ellin5102 (Joseph et al. 2003) and many sequences of uncultured *Verrucomicrobia* mostly from soil and freshwater habitats. Subdivision V4 encompasses the eight cultured genera *Fucophilus* (Sakai et al. 2003), *Lentimonas* (Choo and Cho 2006, unpublished), *Coraliomargarita* (Yoon et al. 2007[a]), *Cerasicoccus* (Yoon et al. 2007[b]), *Puniceicoccus* (Choo et al. 2007), *Opitutus* (Chin et al. 1999, 2001, Janssen et al. 1997), *Alterococcus* (Shieh and Jean 1998), and *Pelagicoccus* (Yoon et al. 2007[c]). These organisms group with sequences of uncultured *Verrucomicrobia* from various terrestrial and aquatic habitats. *Methylacidiphilum* spp. are cultivated members of a recently proposed new subdivision, hereafter referred to as “V7” (Dunfield et al. 2007, Islam et al. 2008, Pol et al. 2007).

Design and evaluation of oligonucleotide probes for *Verrucomicrobia*

New oligonucleotide probes were developed and evaluated together with already published probes for *Verrucomicrobia*. Several probes were targeted to individual phylogenetic clades of *Verrucomicrobia* on the family and genus level (Fig. 2 and 3, Table 1).

Probes targeting the phylum *Verrucomicrobia*. Probes EUB338-II and EUB338-III (Daims et al. 1999) have originally been developed to supplement the general bacterial probe EUB338 (Amann et al. 1990). A mixture of these three probes in equimolar amounts is used to target most bacteria. EUB338-III is fully complementary to most *Verrucomicrobia* (coverage 96%), but

like many probes for higher taxa (Amann and Fuchs 2008), it also targets members of several other phyla. It perfectly matches many *Chloroflexi*, members of the OP11, NKB19, and OP10 cluster, and several other bacteria, and has a single mismatch to most *Planctomycetes*. Unspecific bindings of EUB338-III to *Planctomycetes* can be avoided by hybridizing under stringent conditions and adding EUB338-II as unlabeled competitor probe (Daims et al. 1999). However, hybridization of EUB338-III can only be used as a first hint for *Verrucomicrobia*.

Another verrucomicrobial probe mixture, VER47, was initially used for hybridization against extracted rRNA (Buckley and Schmidt 2001). In this study, the probes were applied for the first time in FISH, and proved to be suitable for this technique. VER47 seems to cover less (coverage 78%) *Verrucomicrobia,* but coverage might be underestimated, because several of the almost full length sequences do not include any information or inaccurate information at the target position (region: 47-63 according to the *Escherichia coli* numbering system [Brosius et al. 1981]). With respect to outgroup hits, VER47 is currently much more specific for *Verrucomicrobia* than EUB338-III; the two probe variants have 61 and 38 outgroup hits. Furthermore, VER47 mostly has other outgroup hits (mainly *Planctomycetes, Acidobacteria,* and *Proteobacteria*) than EUB338-III. Hybridization conditions for this probe were optimized using *Verrucomicrobium spinosum* (strain DSM 4136, [Schlesner 1987]) as target organism. A formamide concentration of 15% was optimal for hybridization at 46 °C. Co-localization of VER47 and EUB338-III signals in dual hybridizations should be used to corroborate identification of cells as *Verrucomicrobia*.

Specific probes for *Spartobacteria*. The clade *Spartobacteria* is part of *Verrucomicrobia* subdivision V2. It comprises 471 sequences mostly from arid (e.g. EU861830, [Nemergut et al. 2008]) and wet soils (e.g. DQ450784, Costello et al. database release), but also from rivers (e.g. EF494343, Macalady et al., database release) and lakes (e.g. AJ575731, [Burkert et al. 2003]). Isolated representatives are *Chthoniobacter flavus* (Sangwan et al. 2004) and not yet validly described members of *Spartobacteria* (e.g. AB245342, Im and Lee, database release). We developed probe SPA714 (Fig. 2, Fig. 3, Table 1) which is currently covering 78% of the sequences in this clade. The probe has few outgroup hits to *Firmicutes* and some other *Bacteria*. A melting curve for this probe was conducted using *Chthoniobacter flavus* (strain Ellin428), kindly provided by M. Sait and P. Janssen. Optimal formamide concentration was 35% at 46 °C. A tight cluster (97-99% sequence similarity) within this clade comprised only sequences from Lake Große Fuchskuhle (Burkert et al. 2003, Glöckner et al. 2000) and

another humic lake (Newton et al. 2006). This tight cluster is targeted by the new probe SPA649. Hybridization conditions for this probe were optimal using a formamide concentration of 35% at 46 °C.

Specific probes for *Opitutus* spp.. Another target group was the *Opitutus* clade within subdivision V4 including almost 100 sequences from anoxic or arid soils (e.g. AF234133, Holmes et al., database release), rocks (e.g. AB179492, Yoshida et al., database release), freshwater (e.g. groundwater: AY661974, Fields, database release; rivers: AY212598, [Simpson et al. 2004]; lakes: AJ289985, [Glöckner et al. 2000]; sludge: AB286411, [Sadaie et al. 2007]), antarctic sea water (e.g. FJ169213, Riesenfeld et al., database release), and even a termite`s intestine (e.g. AB062814, [Shinzato et al. 2005]). Isolated representatives of this clade are *Opitutus terrae* (Chin et al. 1999, 2001) and *Opitutus* sp. (ultramicrobacteria, [Janssen et al. 1997]). The newly developed probe OPT1169 covers 86% of the entire clade and has only few outgroup hits to other bacteria. However, it has single mismatches to outgroup *Verrucomicrobia* from marine habitats. Hybridization conditions for this probe were optimized using *Opitutus* sp. (strain DSM 14424, [Janssen et al. 1997]) as target organism. A formamide concentration of 35% was optimal for hybridization at 46 °C.

Specific probes for *Verrucomicrobium-Prosthecobacter* spp.. The clade *Verrucomicrobium-Prosthecobacter* within the *Verrucomicrobia* subdivision V1 comprises 68 sequences from various environments such as lakes, soils (e.g. EF516716, Cruz et al., database release), rock biofilms (e.g. FM253612, Zielenkiewicz, database release), sludge (e.g. EU104166, Brown et al., database release), or rivers (e.g. FJ230907, Li et al., database release). Isolated representatives are *Verrucomicrobium spinosum* (Schlesner 1987) and *Prosthecobacter* spp. (Hedlund et al. 1996). The new probe VP403 (Fig. 2 and 3, Table 1) covers 90% of this clade and has only two outgroup hits to other *Bacteria*. This probe was optimized using *Verrucomicrobium spinosum* (strain DSM 4136, [Schlesner 1987]) as target organism. The optimal formamide concentration was determined as 35% at 46 °C.

General consideration for probe application. The design of new probes is always aiming at a compromise of good target group coverage and high specificity. This was also the case for the design of probes targeting individual clades of *Verrucomicrobia.* Since the bacterial diversity is still largely unknown we recommend applying the clade-specific probes initially in dual and triple hybridizations together with the probes EUB338-III and/or VER47. With the multiple probe approach, the risk for false positive identifications can be markedly reduced.

The possibility of cross-hybridization with non-target *Verrucomicrobia* still exists, e.g., for probe OPT1169 which targets a rather broad clade. OPT1169 has single mismatches to few marine outgroup *Verrucomicrobia*: *Pelagicoccus* spp.*, Fucophilus fucoidanolyticus,* and some uncultured *Verrucomicrobia* related to *Puniceicoccus vermicola*. Here, the use of competitor oligonucleotides (Table 1) might be helpful to avoid unspecific bindings (Fuchs et al. 2000).

In situ monitoring of *Verrucomicrobia* in Lake Große Fuchskuhle. As a first application to real world, the probe set was used to gain initial insights into the seasonal dynamics of *Verrucomicrobia* in two different basins and at two water depths of the humic Lake Große Fuchskuhle, known to host bacteria of the phylum. Hybridizations with the probes EUB338-III and VER47 showed that *Verrucomicrobia* were indeed present over the whole year in both basins and water depths. Four seasonal samples – one each in spring, summer, autumn, and winter - were selected for studies of the temporal variability. For all time points, duplicates were analyzed with probe EUB338-III. Most duplicates were highly comparable: standard deviations were below ±1% for most samples, and ±2% and ±3% for the samples NE 4.5 m autumn and winter (Table 2). An exceptional high deviation was observed for the sample NE 0.5 m winter (±10%) using probe EUB338-III, probably due to uneven distribution of cells on the filter. However, duplicate analyses of other sections of this filter with the probes SPA714 and SPA649 revealed comparable numbers.

Verrucomicrobia contributed between <0.1 and 19% to the total microbial community (Table 2). Population size and composition varied strongly between seasons, basins, and water depth. Populations making up at least 5% of all cells were detected during all seasons in at least one sample. The highest relative abundance (19% of total cells) thrived in winter, whereas the largest population (1.9×10^6 cells ml^{-1}) occurred in summer (NE 4.5 m). In autumn, populations were mostly small. The NE basin hosted significantly more *Verrucomicrobia* than the SW basin. While in the NE basin relative abundance peaked in winter, it was highest in the SW basin in summer. Populations in the SW basin were small in the other seasons. DOC concentrations were mostly only little lower in the NE basin hosting significantly larger populations of *Verrucomicrobia* than the SW basin.

From our preliminary data points, we hypothesize that the quality of DOC rather than its concentration influences verrucomicrobial growth in Lake Große Fuchskuhle. Recalcitrant humic substances form the main DOC compounds with higher concentrations in the SW basin, others are polysaccharides, proteins, peptides, amino acids, low molecular weight carboxylic

acids (Buck et al. 2008), and methane (Casper et al. 2003). As mentioned above, *Verrucomicrobia* isolates are physiologically diverse utilizing various carbon compounds such as poly- and oligosaccharides, proteins, or methane. High verrucomicrobial abundance in Lake Große Fuchskuhle did neither positively correlate with high phosphate nor nitrogen availability (Table 2).

We showed that *Verrucomicrobia* occur in both basins which differ significantly in pH. While the pH in the SW basin of Lake Große Fuchskuhle is acidic around 5, it is close to neutral (pH 6-7) in the NE basin, with exact values varying with depth (Burkert et al. 2004, Grossart et al. 2008, Simek et al. 1998). Most verrucomicrobial isolates obtained so far favor pH values between 6 and 8, but some species cope even with extreme values such as pH 1 (Pol et al. 2007) or 9 (Yoon et al. 2008[a]). Our data suggest that *Opitutus* spp. might be less tolerant to low pH than *Spartobacteria*.

The relative abundance of *Verrucomicrobia* was similar in oxic and seasonally anoxic waters (Table 2), but absolute population sizes were mostly much larger in anoxic waters. In anoxic waters of the NE basin, a large population formed also during autumn, when populations were small in all other samples. This suggests that anoxic conditions favor growth of *Verrucomicrobia* in Lake Große Fuchskuhle. In general, *Verrucomicrobia* inhabit various oxic as well as anoxic habitats (Schlesner et al. 2006). Different oxygen needs have been described for cultured *Verrucomicrobia*: some are aerobic (e.g. *Spartobacteria* and *Prosthecobacter* spp. [Sangwan et al. 2004, Schlesner et al. 2006]) or oxygen tolerant (*Opitutus* sp. (ultramicrobacteria) [Janssen et al. 1997]), whereas others are facultative anaerobic (*Verrucomicrobium spinosum* [Schlesner 1987]) or obligate anaerobic (e.g. *Opitutus terrae* [Chin et al. 1999, 2001]). The different clades of *Verrucomicrobia* in Lake Große Fuchskuhle have most probably different preferences for oxygen, which can only be revealed by further studies.

Abundance of *Verrucomicrobia* subgroups. *Spartobacteria* were quantified by using the probes SPA714 and SPA649 in dual CARD-FISH with two differently labeled tyramides. Only cells with overlapping signals were counted. *Spartobacteria* strongly dominated most verrucomicrobial populations found in both basins constituting up to 19% of all cells (62×10^4 cells ml^{-1}, NE 4.5 m in winter, Table 2) and also formed the sole large population detected during autumn. Cells were coccoid, 0.5 to 1 µm in diameter (Fig. 4), and often found in pairs. Some were attached to filamentous algae.

The dominance of the clade *Spartobacteria* is in line with earlier 16S rRNA sequence analyses. Most verrucomicrobial sequences detected in Lake Große Fuchskuhle (Burkert et al. 2003, Glöckner et al. 2000) are affiliated (91% sequence identity) with the 16S rRNA of a member of the *Spartobacteria* clade, *Chthoniobacter flavus*, an aerobic heterotroph described by Sangwan et al. (2004). Their patchy distribution suggests that environmental control of this group is complex. Interestingly, the relative abundance of *Spartobacteria* seems to correlate negatively with that of *Actinobacteria*, and positively with *Betaproteobacteria* when comparing our counts to those reported earlier on the same samples from Lake Große Fuchskuhle (Burkert et al. 2003). *Spartobacteria* were the smallest of all *Verrucomicrobia* detected in Lake Große Fuchskuhle. Their size fits that reported for the closest related cultured representative, *C. flavus*, well (oval-shaped, 0.9 by 1.4 µm in size [Sangwan et al. 2004]). Cells of this size are experiencing high grazing pressure (Pernthaler and Amann 2005). This might explain why cells of the clade *Spartobacteria* were low in abundance in summer surface water in which heterotrophic nanoflagellates and ciliates in Lake Große Fuchskuhle are abundant (Simek et al. 1998).

Opitutus spp., as quantified with probe OPT1169, made up major fractions of the verrucomicrobial populations and dominated several populations in the NE basin including the largest one found (1.3×10^6 cells ml^{-1}, 7% of total cells, NE 4.5 m in summer). In the NE basin, they were abundant during spring at both depths, during summer they were abundant only in anoxic waters (Table 2). All *Opitutus* spp. occurred as coccoid single cells or cell pairs and were 1 to 2 µm in diameter (Fig. 4). Some of the cells formed monospecific aggregates.

The FISH identification of *Opitutus* spp. also confirms earlier data from 16S rRNA sequence analyses (Burkert et al. 2003, Glöckner et al. 2000), in which sequences were retrieved that were 95% identical to the 16S rRNA sequence of *Opitutus* sp. (ultramicrobacteria) [Janssen et al. 1997]. *Opitutus* spp. ferment mono-, di-, and polysaccharides. Some degrade plant polymers, e.g. pectin suggesting their involvement in the break down of plant polymers (Chin et al. 1999, 2001, Janssen et al. 1997). Interestingly, *Opitutus* spp. detected in Lake Große Fuchskuhle water were with about 2 µm larger than those described in the literature (e.g. ultramicrobacteria 0.35 µm by 0.5 µm [Janssen et al. 1997) and *Opitutus terrae* 0.4-0.6 µm [Chin et al. 2001), *Puniceicoccus vermicola* 0.6-1 µm [Choo et al. 2007]).

CARD-FISH with probe VP403 indicated that members of *Verrucomicrobium/Prosthecobacter* constituted only minor fractions (<1%) of the microbial populations in Lake Große Fuchskuhle (Table 2), accounting for a maximum of only 57 x 10^3 cells ml^{-1} (NE 4.5 m in summer). All VP403-positive cells were fusiform, 1 to 6 µm long, and 0.5 to 1 µm wide (Fig. 4). The fusiform shape of cells detected by probe VP403 supports the close relation of these *Verrucomicrobia* to *Prosthecobacter* spp. (Hedlund et al. 1996). Many occurred as conspicuous pairs of dividing cells suggesting active growth (Fig. 4b). Chains of three or more cells were rarely detected. Some cells occurred in loose association with other cells, often with *Opitutus* spp., and some appeared attached to organic particles and algae. A rather low abundance of these well known *Verrucomicrobia* is in line with the study of Glöckner et al. (2000) who retrieved only a single 16S rRNA sequence with a similarity of 98% to the sequence of *Prosthecobacter fluviatilis* isolated by Takeda et al. (2008).

Prosthecobacter spp. have been isolated from various oligo- and eutrophic freshwaters (DeBont et al. 1970, Staley et al. 1976). They grow on various mono- and disaccharides as sole carbon sources. In Lake Große Fuchskuhle containing large amounts of polysaccharides (Buck et al. 2008), they might be involved in the degradation of sugars. The cells identified as *Prosthecobacter* spp. were large and thus formed a significant biomass despite their relative low abundance. Their large size and fusiform shape might protect them from protozoal grazing (Pernthaler and Amann 2005). The prosthecae were visible in our FISH images showing that they are cytoplasmatic. They might provide several advantages summarized by Hedlund et al. (1996): enhanced respiration and nutrient uptake (Schmidt 1971), decreased sedimentation (Pointdexter 1978, Stove and Stainier 1962), and attachment to solid substrates (Merker and Smit 1988).

We did not apply additional verrucomicrobial probes to Lake Große Fuchskuhle because “book keeping” indicated that the sum of counts with probes SPA714/SPA649, OPT1169, and VP403 added up to the counts obtained with EUB338-III. This suggests that in Lake Große Fuchskuhle, *Verrucomicrobia* are mostly from the three clades detected by these new probes. Since *Chloroflexi* and other *Bacteria* targeted by EUB338-III seem to be rare or absent in this lake, probe EUB338-III could here be used to quite reliably estimate abundance of *Verrucomicrobia*.

Outlook. The verrucomicrobial probe set developed in this study is far from being complete since we have focused on aquatic members of this interesting phylum. It nevertheless allowed

us to assign most of the *Verrucomicrobia* found in Lake Große Fuchskuhle to specific clades. Some of these clades accounted for sizable populations. We hope that this probe set will facilitate future experiments addressing the ecophysiology of distinct verrucomicrobial clades.

Acknowledgments

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Table 1: Oligonucleotide probes for FISH detection of *Verrucomicrobia*

Probe	Probe sequence (5`-3`)	Target group	target group (no. of sequences)	probe hits in target group	Coverage [%]	Out-group hits	FA [%]	Ref.
EUB338-III	GCTGCCACCCGTAGGTGT	most *Verrucomicrobia*, many *Chloroflexi,* several other bacteria	3030	2899	96	3996	35	[Daims et al. 1999]
cEUB338-II	GCAGCCACCCGTAGGTGT	most Planctomycetes, some other bacteria	-	-	-	-	-	[Daims et al. 1999]
VER47*	GACTTGCATGTCTTAWC	most *Verrucomicrobia*, some other bacteria	3030	2113/244	70/8	61/38	15	[Buckley and Schmidt 2001]
VP403	CGAAGACCTTATCCTCCACG	*Verrucomicrobium,* most *Prosthecobacter*, and uncultured relatives	68	61	90	2	35	this study
SPA714	CCTTCGCCACTGGTCTTC	most *Spartobacteria* and uncultured relatives	471	370	78	39	35	this study
SPA649	CTCTACAGTCCTCTAGCG	uncultured humic and acidic lake *Spartobacteria*	7	7	100	0	35#	this study
OPT1169	CCCACCTTCCCACCCTCA	*Opitutus* spp. and uncultured relatives	100	86	86	6	35	this study
c1 OPT1169 c2 OPT1169	CCCACCTTCCCACCCTAA CCCACCTTCCCACTCTCA	competitor oligonucleotides to block binding of probe OPT1169 to uncultured marine *Verrucomicrobia*	-	-	-	-	-	

*coverage was calculated based on incomplete sequence information (see text); c: unlabeled competitor probe; FA: formamide concentration in FISH hybridization buffer; #: melting curves conducted on water samples of Lake Große Fuchskuhle

Table 2: *In situ* abundance of *Verrucomicrobia* and known environmental parameters in Lake Große Fuchskuhle during year 2000

Sample	SW basin 0.5 m water depth				SW basin 4.5 m water depth				NE basin 0.5 m water depth				NE basin 4.5 m water depth			
	Mar-01	Jun-29	Sep-12	Dec-11	Mar-01	Jun-29	Sep-12	Dec-11	Mar-01	Jun-29	Sep-12	Dec-11	Mar-01	Jun-29	Sep-12	Dec-11
TCC [10^6 cells ml^{-1}]	1.0#	1.2	3.0	1.3	1.4#	3.0	8.6	1.0#	1.5#	2.6	4.1	2.0	1.8#	19.0	9.2	3.2#
Verrucomicrobia (probe EUB338-III*; % of TCC)	0.9±0.1	4.0±0.8	0.7±0.2	0.5±0.1	0.9±0.1	5.6±0.1	0.0	0.7±0.3	6.4±2.2	0.3±0.0	0.1±0.1	16.0±9.8	8.8±0.2	9.7±nd	8.9±2.5	18.6±3.4
Spartobacteria (probe SPA714/SPA649; % of TCC)	0.4	5.4	0.4	0.5	0.9	4.1	0.0	0.2	2.6	0.6	0.0	18.2	1.6	1.7	6.7	19.5
Opitutus spp. (probe OPT1169; % of TCC)	0.2	0.0	0.4	0.1	0.4	0.8	0.0	0.1	6.9	0.3	0.2	0.5	7.4	6.9	0.6	0.4
Verrucomicrobium/ *Prosthecobacter* spp. (probe VP403; % of TCC)	0.6	0.3	0.0	0.0	0.3	0.3	0.0	0.1	0.2	0.0	0.0	0.1	0.6	0.3	0.1	0.2
DOC [mg l^{-1}]	nd	16.2	12.2	12.0	nd	nd	21.0	14.7	10.4	16.5	9.1	11.2	nd	nd	13.0	13.5
pH	5.1	4.6	4.7	5.0	5.1	5.6	5.5	4.9	6.2	7.6	6.1	6.2	6.2	5.9	5.9	6.1
total N [mg l^{-1}]	1.1	0.9	0.7	0.9	1.1	1.6	1.3	0.7	1.2	0.8	1.1	1.2	1.5	1.3	5.2	1.3
total P [µg l^{-1}]	13.8	42.5	85.5	22.3	15.3	52.0	59.2	19.6	25.7	32.2	39.6	22.1	30.5	94.4	201.8	21.3
T [°C]	4.3	16.4	16.2	3.9	4.3	7.7	10.6	3.9	4.8	17.2	16.8	4.6	4.6	7.5	10.0	4.5
O_2 [%]	88	96.0	56.0	63.0	88.0	0.0	0.0	65.0	87.5	78.0	94.0	70.0	78.0	0.0	0.0	69.0

#: determined upon hybridization; *: used with cEUB338-II; nd: not determined; NE: northeast; SW: southwest; TCC: total cell counts
O_2: average SW 0.0 m and SW 1.0 m, SW 4.0 m; T: winter SW 4.0 m, winter average of NE 0.0 m and NE 1.0 m (Buck et al. 2008)

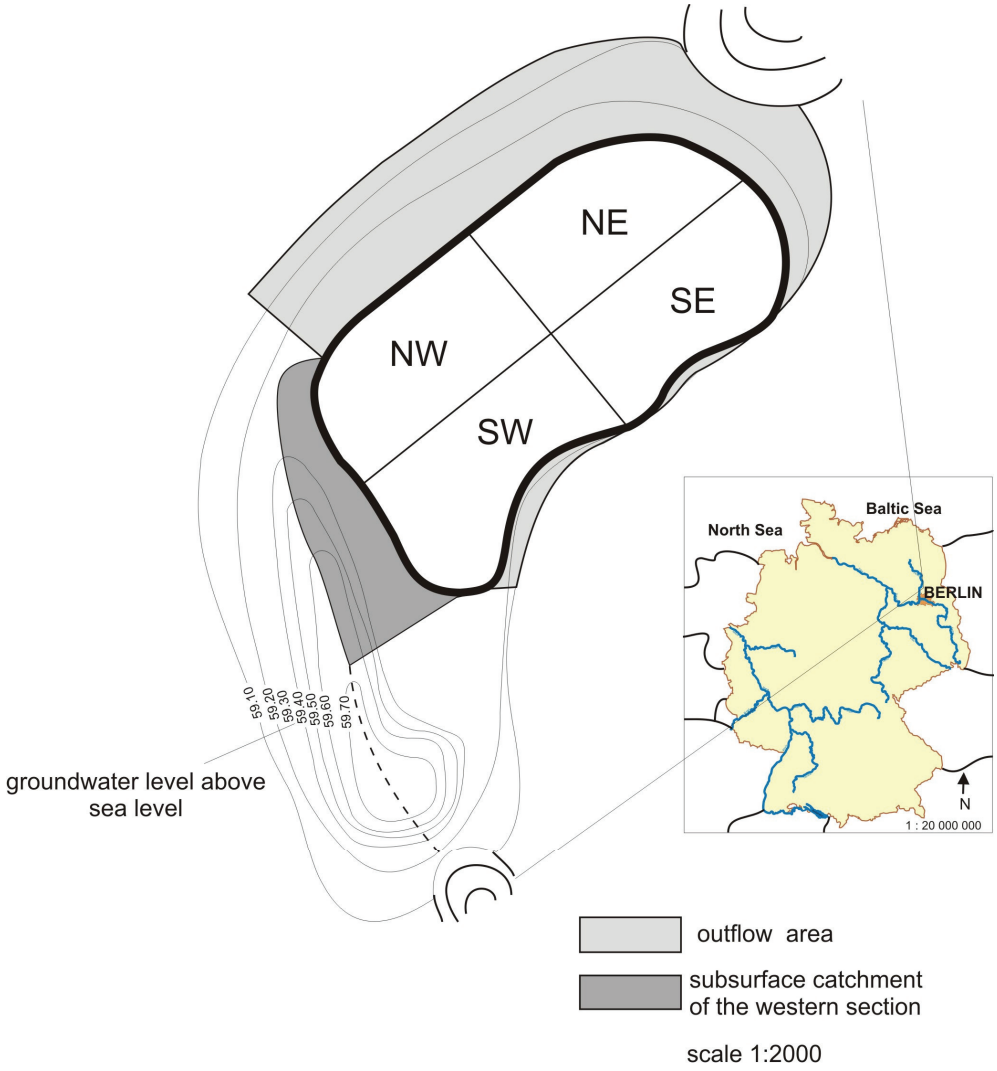

Figure 1: Lake Große Fuchskuhle is located in the Mecklenburg-Brandenburg lake district near Berlin. It is artificially divided into four basins. Water samples were taken from 0.5 m (oxic) and 4.5 m (seasonally anoxic) depth of the southwestern (SW) and northeastern (NE) basin approximately trimonthly in year 2000.

Arnds et al.,

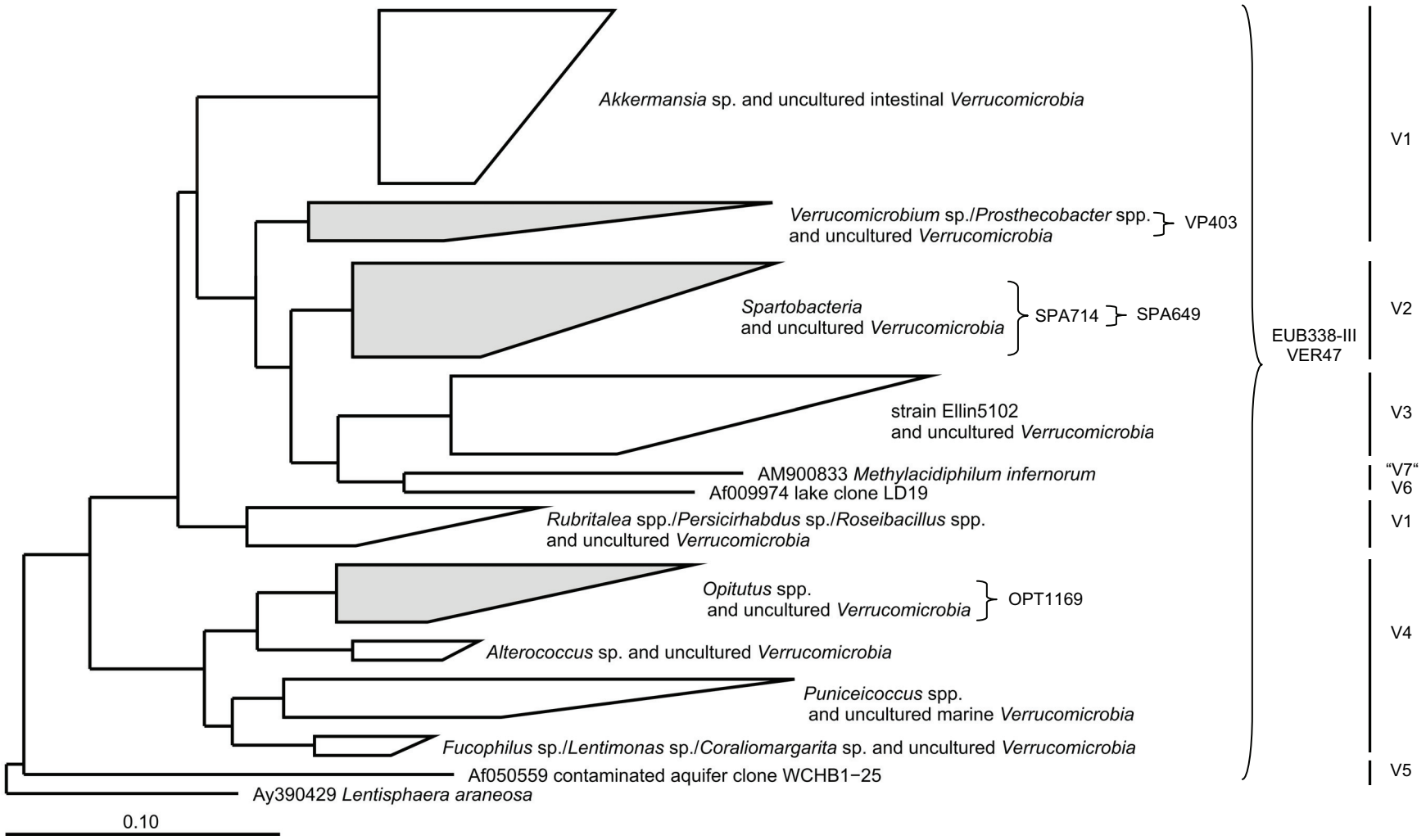

Figure 2: Phylogenetic tree of 16S rRNA gene sequences of *Verrucomicrobia* members showing the six informal subdivisions (V1 to V6, [Hugenholtz et al. 1998, Schlesner et al. 2006, Wagner and Horn 2006]) and the proposed subdivision "V7" [Pol et al. 2007]. Probe target groups are indicated by waved brackets. Arnds et al.,

Probe VER47	3`- CWA TTC TGT ACG TTC AG -5`
target	5`- GWU AAG ACA UGC AAG UC -3`
Prosthecobacter fluviatilis (AB305640) [a]	*** *** *** *** *** **
Chthoniobacter flavus (AY388649) [b]	*** *** *** *** *** **
Opitutus terraea (AJ229235) [c]	*** *** *** *** *** **
Alterococcus agarolyticus (AF075271) [d]	*C* *** *** *** *** **
Rhodopirellula baltica (BX294149) [e]	*** U** G** *** *** **
Roseobacter denitrificans (L01784) [f]	CC* **C *** *** *** **
Probe VP403	3`- GCA CCT CCT ATT CCA GAA GC -5`
target	5`- CGU GGA GGA UAA GGU CUU CG -3`
Prosthecobacter fluviatilis (AB305640) [a]	*** *** *** *** *** *** **
Uncultured soil clone (AJ863205) [g]	*** *** **U *** *** *** **
Rubritalea marina (DQ302104) [h]	*** *** *** ***G** **C *C* A*
Rhodopirellula baltica (BX294149) [e]	*** *CG *** ***G** **C *C* **
Roseobacter denitrificans (L01784) [f]	*** *AG U** ***G** **C *C* A*
Escherichia coli (X80725) [i]	*** *U* U** AG** **C *** **
Probe SPA714	3`- CTT CTG GTC ACC GCT TCC -5`
target	5`- GAA GAC CAG UGG CGA AGG -3`
Chthoniobacter flavus (AY388649) [b]	*** *** *** *** *** ***
Desulfovibrio hyrothermalis (AF458778) [j]	*** C** *** *** *** ***
Desulfacinum hydrothermale (AF170417) [k]	*** U** *** *** *** ***
Alterococcus agarolyticus (AF075271) [d]	*** C** *** *** *** ***
Rhodopirellula baltica (BX294149) [e]	*** C** *G* *** *** ***
Roseobacter denitrificans (L01784) [f]	*** C** *** *** *** ***
Escherichia coli (X80725) [i]	*** U** *G* *** *** ***
Probe SPA649	3`- GCG ATC TCC TGA CAT CTC -5`
target	5`- CGC UAG AGG ACU GUA GAG -3`
Uncultured lake clone FukuN18 (AJ575732) [l]	*** *** *** *** *** ***
Alterococcus agarolyticus (AF075271) [d]	G** *** **U **C *G* ***
Rhodopirellula baltica (BX294149) [e]	G** *U* *** *AG AC* *G*
Roseobacter denitrificans (L01784) [f]	GU* *U* **U U*G AG* ***
Escherichia coli (X80725) [i]	A** *U* **U CUC *** ***
Probe OPT1169	3`- ACT CCC ACC CTT CCA CCC -5`
target	5`- UGA GGG UGG GAA GGU GGG -3`
Opitutus terraea (AJ229235) [c]	*** *** *** *** *** ***
Fucophilus fucoidanalyticus (AB073978) [m]	*** *A* *** *** *** ***
Uncultured marine clone (U81738) [n]	*U* *** *** *** *** ***
Uncultured marine clone (CU919115) [o]	*U* **A*** *** *** *** ***
Alterococcus agarolyticus (AF075271) [d]	CA* *** *** *** *** ***
Rhodopirellula baltica (BX294149) [e]	AA* CC* GA* *** *** ***
Roseobacter denitrificans (L01784) [f]	AAG C** GA* *** *** *U*
Escherichia coli (X80725) [i]	AA* CU* GA* *** *** ***

[a] Takeda et al. 2008; [b] Sangwan et al. 2004; [c] Chin et al. 2001; [d] Shieh and Jean 1998; [e] Glöckner et al. 2003; [f] Fuerst et al., database release; [g] Graff and Conrad 2005; [h] Scheuermayer et al. 2006; [i]: Cilia et al. 1996; [j] Alazard et al. 2003; [k] Sievert and Kuever 2000; [l] Burkert et al. 2003; [m] Sakai et al. 2003; [n] Godon et al. 1997; [o] Riviere et al. 2009.

Figure 3: Aligned fullmatch and mismatch reference 16S rRNA sequences showing probe binding sites. Bold letters symbolize insertions.

Arnds et al.,

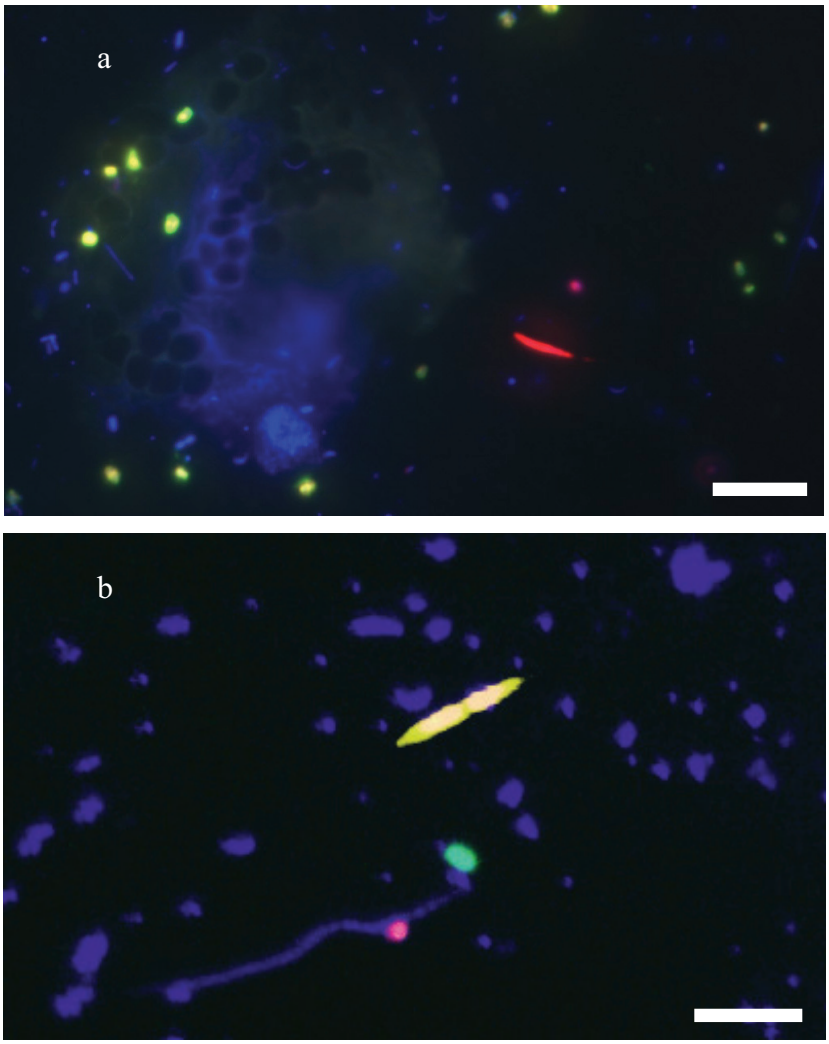

Figure 4: Photomicrographs of Lake Große Fuchskuhle *Verrucomicrobia*: (a) *In situ* identification of *Opitutus* spp. (yellow) with probes OPT1169 (tyramide Alexa488, green) and probe EUB338-III (tyramide Alexa594, red) by dual CARD-FISH. (b) *In situ* identification of *Opitutus* spp. (probe OPT1169, tyramide Alexa488, green), dividing *Prosthecobacter* spp. (probe VP403, tyramide Alexa546, yellow), and *Spartobacteria* (probe Ver714, tyramide Alexa633, red) by triple CARD-FISH. All other cells are DNA-stained (DAPI, blue). Scale bars 5 µm.

Arnds et al.,

Appendix

Materials and Methods

1. Appendix B1.5 Chloroflexi

- Primers used:

  EUB338 III, ACA CCT ACG GGT GGC AGC (Daims et al. 1999)

  V1492R, GGY TAC CTT GTT ACG ACT TCR TCC CA (based on primer 1492R, Lane 1991, modified in length and few bases)
- PCR conditions: initial denaturation for 5 min at 95 °C, 25 cycles of denaturation for 45 sec at 95 °C, annealing for 45 sec at 63 °C and extension for 2 min at 72 °C, followed by a final elongation step for 59 min at 60 °C
- Amplified 16S rRNA genes were ligated into „pGem®-T Easy“ vectors (Promega, Mannheim, Germany) and transformed into competent *Escherichia coli* (Top10)“ cells (Invitrogen, Karlsruhe, Germany) following the protocol „Novagen Competent Cells“. Plasmids were purified using the „QIAgen plasmid purification” kit (Qiagen, Hilden, Germany)
- Probes used:

  mCfx665, CCC GAA ATT CCA CCT CCC (35% formamide in hybridization buffer)

  Dhc1252 ATC ACT CCA TTG CCA CCT (10% formamide in hybridization buffer used, specific formamide concentration not determined)

2. Appendix B3.2.1 Real time PCR for quantification of ANME in marine sediments

- **Primer and probes.** Primer and TaqMan probes for ANME subgroups (Table A2) were designed based on 16S rRNA gene sequences (database release January 2006) with the probe_design tool of the ARB software (Ludwig et al. 2004). Primer/probe design protocols of Eurogentech, Qiagen, and Applied Biosystems were followed with minor modification. Oligonucleotides were checked for their potential to form secondary structures using a bioinformatic analysis tool (http://eu.idtdna.com). Primer and probes were obtained HPLC purified from Biomers (Ulm, Germany). The TaqMan probes had a fluorescent reporter dye (6-carboxyfluorescein) covalently attached to the 5`-end and a fluorescent quencher (black whole quencher-1) attached to the 3`-end.
- **Reference DNA.** Plasmids (Table A3, A4) were extracted using a plasmid purification kit (Qiagen, Hilden, Germany). The cloned 16S rRNA genes were sequenced with vector primers (M13F/M13R) to verify the sequence information. As standards, either the plasmids or 16S rRNA gene fragments amplified with Arch20F and Arc958R were used. PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany).
- **TaqMan approach.** The reaction mix (total volume 25 µl) was composed of: 12.5 µl Master Mix (qPCR MasterMix Plus, Eurogentech, Seraing, Belgium), 2.5 µl of bovine Serum Albumin (3 mg ml^{-1}), and 2 µl template DNA (various concentrations tested). Different primer, probe and template concentrations were tested. Triplicates for standard samples were performed on one plate. Real-time PCR was carried out in an IQ^{TM}5 Real Time PCR System (Biorad, Germany). Results were analysed using the IQ^{TM}5 Real Time PCR Detection System (Biorad, Germany).
- **SybrGreen approach.** The Power SYBR Green PCR Mastermix (Applied Biosystems, Warrington, UK) was used. The reaction mix was identical with that in the TaqMan approach, but the probe was replaced by sterile, deionized water.

Table 2: ANME specific probes and primers developed for real time PCR

target group	primer/probe	primer/probe sequence 5´-3´	tTm [°C]	eTm [°C]	bases	GC [%]
ANME-1	ANME1- 267F1	ATCA ACA AGC CTG CAA TC	59.05	64.3	18	44
	ANME1- 268F2	TCA ACA AGC CTG CGA T	59.26	n.d.	16	50
	ANME1- 268F3	TCA ACA AGC CTC CGA TC	59.58	n.d.	17	53
	ANME1- 269F4	CAA CAA GCC TAC GAT CG	58.36	n.d.	17	53
	ANME1- 351P	GCA TCA GGC GCG AAA ACT	63.16	n.d.	18	56
	C1ANME1-351P	GCA TCA AGC GCG AAA ACT	n.d.	n.d.	18	n.d.
	C2ANME1-351P	GCA TTA GGC GCG AAA ACT	n.d.	n.d.	18	n.d.
	C3ANME1-351P	GCA TCA GGC GCG AAG ACT	n.d.	n.d.	18	n.d.
	C4ANME1-351P	GCG TCA GGC GCG AAA ACT	n.d.	n.d.	18	n.d.
	C5ANME1-351P	GCA TCA GGC GCG AGA ACT	n.d.	n.d.	18	n.d.
	C6ANME1-351P	GCA TCA GGC GCG GAA ACT	n.d.	n.d.	18	n.d.
	ANME1-408R1	GAA CAG CCT CCA TAG GAG	59.53	63.0	18	56
	ANME1-408R2	CTGAA CAG CCT CCT TAG G	59.77	n.d.	18	56
	ANME1-408R3	A CAG CCC CCT TAG GG	59.86	n.d.	16	56
ANME-2a	ANME2a-544F1	CCG AGT GGT AAC CGT TT	59.83	n.d.	17	53
	ANME2a-544F2	CCG AGT GGT AGC CGT	60.82	n.d.	15	67
	ANME2a-648P1	TCT TCC GGT CCC AAG CCT	64.50	n.d.	18	61
	ANME2a-648P2	CTC CCG GTC CCA AGC CT	65.03	n.d.	17	71
	ANME2a-735R	GGA CCC GTT CTG GTA AG	59.58	63.9	17	59
ANME-2c	ANME2c-435F	GCT GTC CTT GTG CC	57.73	n.d.	14	64
	ANME2c-623P1	TCC CTT GGC AGT CTG ATT G	61.12	n.d.	19	53
	ANME2c-623P2	CC CTT GGC AGC CTG ATT	60.97	n.d.	17	59
	ANME2c-735aR	GAC CCG TTC TGG TAA G	57.31	61.1	16	56
ANME-3	ANME3-141F	GGA TTG GCA TAA CAC CG	58.66	63.0	17	53
	ANME3-217P	CAG ATC CAT CCT TAG GCA GC	61.80	n.d.	20	55
	cANME3-217P1	CAT ATC CAT CCT TAG GCA GC	n.d.	n.d.	20	n.d.
	cANME3-217P2	CAG ATC CAT CCT TAG GCA AC	n.d.	n.d.	20	n.d.
	ANME3-399R	ATA TGC TGG CAC TCA GT	58.43	64.3	17	47

c: competitor, eTM: melting temperature evaluated by gradient PCR, F: forward primer, n.d.: not determined, P: TaqMan probe, R: reverse primer, tTm: theroretical melting temperature determined with Kun`s Oligonucleotide Tm calculator (www.arep.med.harvard.edu). Primers were designed to have similar melting temperatures.

Table 3: Fullmatch and mismatch reference clones used for real time PCR

Number	Accession number	Clone
Ref01	n.a.	GoM140Arch30
Ref02	AM746094	Gom140Arch41
Ref03	n.a.	SortANME R1Arch14
Ref04	n.a.	HMMVBeg35
Ref05	n.a.	GoM161Arch81
Ref06	AJ704638	HMMVPog34
Ref07	n.a.	HMMVPog71
Ref08	FM179838	Tomm05-1274/3-Arch90
Ref09	n.a.	Sort ANME R1 Arch121
Ref10	AJ704631	HMMVBeg36
Ref11	AJ704651	HMMVBeg29
Ref12	AJ704653	HMMVBeg32
Ref13	AJ704650	HMMVBeg34
Ref14	n.a.	HMMVPog26
Ref15	n.a.	HMMV-GM83*MW#1142
Ref17	n.a.	HMMV-GM*MW#1298
Ref18	n.a.	HMMV-GM24Arch216
Ref19	n.a.	Karin15c
Ref20	n.a.	GoM_St.140_Arch1_Arch958R
Ref21	n.a.	HMMV-GM8*MW#1230
Ref22	n.a.	Tommeliten HE-180 clone Arch_1
Ref23	n.a.	GoM_58
Ref24	n.a.	GoM_St161_Arch29
Ref25	AJ578145	BS-SR-G10 (LOGA 0155-4)
Ref26	n.a.	GoM_St140_Arch82
Ref27	n.a.	GoM_St161_Arch86
Ref28	n.a.	GoM_St161_Arch47
Ref29	n.a.	GoM_St.140_Arch7_Arch958R
Ref30	n.a.	Tommeliten HE-180 clone Arch_25
Ref31	n.a.	Chefren Mud Volcano clone CfnMu283
Ref32	n.a.	HMMVPog-DS94
Ref33	M59127	*Methanococcoides methylutens* (strain DSM2657)

Table 4a: Fullmatch and mismatch references for ANME-1 specific primers and probe

	ANME1-351P	ANME1-267F1	ANME1-268F2	ANME1-268F3	ANME1-269F4	ANME1-408R1	ANME1-408R2	ANME1-408R3
Ref06	0	1	0	1	1	0	1	3
Ref22	0	1	0	1	1	0	1	3
Ref23	0	0	1	2	2	1	2	4
Ref24	1	0	1	2	2	0	1	3
Ref25	0	3	2	2	0	4	4	1
Ref26	0	2	1	0	1	1	0	2
Ref27	0	3	2	2	0	3	3	0
Ref28	0	0	1	2	2	0	1	3
Ref30	1	n.d.	0	n.d.	n.d.	0	n.d.	n.d.

F: forward primer, n.d.: not determined, P: probe, R: reverse primer, Ref: reference

Table 4b: Fullmatch and mismatch references for ANME-2a specific primers and probes

	ANME2a-648P1	ANME2a-648P2	ANME2a-544F1	ANME2a-544F2	ANME2a-735R
Ref09	0	1	1	0	0
Ref31	1	n.d.	n.d.	0	0
Ref18	1	0	0	1	0
Ref32	n.d.	1	0	n.d.	0
Ref19	1	0	0	1	1
Ref31	1	n.d.	n.d.	0	0
Ref32	n.d.	1	0	n.d.	0

F: forward primer, n.d.: not determined, P: probe, R: reverse primer, Ref: reference

Table 4c: Fullmatch and mismatch references for ANME-2c specific primers and probes

	ANME2c-623P1	ANME2c-623P2	ANME2c-435F	ANME2c-735aR
Ref01	0	1	0	0
Ref05	1	0	0	0
Ref20	0	0	0	1
Ref21	0	0	1	0

F: forward primer, P: probe, R: reverse primer, Ref: reference

Table 4d: Fullmatch and mismatch references for ANME-3 specific primers and probe

	ANME3-217P	ANME3-141F	ANME3-399R
Ref12	0	0	0
Ref04	3	1	0
Ref17	0	0	1
Ref15	1	0	0
Ref33	1	3	1

F: forward primer, P: probe, R: reverse primer, Ref: reference

3. Appendix B3.3 Construction of clone-FISH references by cloning probe targets

- **Plasmid.** An amplified 16S rRNA gene of *Congregibacter litoralis* (strain KT71) from a previous study (Fuchs et al. 2007) were ligated into a „pGem®-T Easy“ vector (Promega, Mannheim, Germany) and transformed into *Escherichia coli* „NovaBlue (DE3)“ cells (Novagen/VWR International, Darmstadt, Germany). Plasmids were purified using the „QIAgen QIAprep® Miniprep Kit“ (QIAGEN, Hilden, Germany). A plasmid with the correct insert orientation, namely KT71-D-25.
- **Enzymatic restriction of plasmid.** Plasmid DNA was linearized with restriction endonuclease *Sph*I (unique restriction site: GCATG`C; activity of 10.000 U $\mu l^{-1}$; Promega, Mannheim, Germany). Linearized plasmid DNA was purified with Micropure®-EZ-columns and Microcon® YM-100 columns (Millipore, Schwalbach, Germany) according to manufacturers` recommendation.
- **Hybridization of complementary oligonucleotides.** Equimolar amounts of the oligonucleotide „403“ (5′-CGT GGA GGA TAA GGT CTT CG CATG-3′) and its reverse complement („403 reverse“) were hybridized in a buffered solution (900 mM NaCl, 20 mM Tris-HCl, pH 7.6) for 14 h at RT. Underlined are the linker bases. The hybrids were separated from the non hybridized oligonucleotides by gelelectrophoresis using „Certified Low Range Ultra Agarose“ of Bio-Rad (Munich, Germany). Hybrids were purified using Microcon® YM-30 columns (Millipore, Schwalbach, Germany). Hybrids were ligated to the linearized plasmids and constructed plasmids, referred to as KT71-403, were transformed as described above.
- Constructed clones were analyzed by clone-FISH as described previously (Schramm et al. 2002) with minor modifications. Probe Non338 (Wallner et al. 1993) was used as negative control. Probes Gam42a (Manz et al. 1992) and NOR5-130 (Eilers et al. 2001) were used as positive controls. Melting curves were conducted for probe VP403 using the constructed clone and formaldehyde-fixed cells from a pure culture of *Verrucomicrobium spinosum* (DSM4136).

4. Appendix B3.4 Establishment of CARD-FISH of ANME in liquid and subsequent flow sorting

CARD-FISH of ANME-3 in solution was carried out according to the methods described by Schönhuber et al. (1997) and Pernthaler et al. (2002) with minor modifications.

- **Sample pretreatment.** Enriched ANME-3 cells were fixed with ethanol (50% v/v, 1 h at RT; K. Knittel, unpublished data), detached from residual sediment particles by sonication on ice (3x30 sec at an amplitude of 42 µm, <10 kW, with a 30 sec break between each sonication (MS73 probe, Sonopuls HD70; Bandelin, Berlin, Germany), and stored at -20 °C until further processing.
- **Permeabilization.** To permeabilize cell walls and bleach endogenous peroxidases in one step, a suitable volume of an enrichment culture was centrifuged (13.000 rpm for 2 min at 4 °C) and the pellet resuspended in 300 µl HCl (0.1 M for 30 sec at RT) followed by a short centrifugation (13.000 rpm for 30 sec at 4 °C). Residual HCl was washed off with 300 µl 1xPBS. Washed cells were centrifuged (13.000 for 2 min at 4 °C) and finally resuspended in 1xPBS (original sample volume).
- **Hybridization.** Hybridization buffer (810 µl, 20% formamide) was mixed with 18 µl of probe ANME3-1249 and each 9 µl of the helper probe ANME3-1249 H3 and ANME3-1249 H5 (each 50 ng μl^{-1}, Lösekann et al. 2007). The cell suspension was added to prewarmed hybridization mix. Hybridization was carried out for 3 h at 46 °C on a rotor.
- **Washing.** Hybridized cells were centrifuged (13.000 rpm for 1 min at 37 °C) and washed in 1 ml prewarmed washing buffer for 5 min at 48 °C. Washed cells were centrifuged (13.000 rpm for 2 min at 37 °C). The pellet was resuspended in 1 ml 1xPBS (for 15 min at RT) and centrifuged (13.000 rpm for 2 min at RT).
- **Amplification.** Washed cells were incubated in 1 ml amplification buffer containing 1 µl Carboxyfluorescein-tyramide and 0.0015% H_2O_2. Amplification was carried out for 20 min at 46 °C. Cells were centrifuged (13.000 rpm for 5 min at RT), resuspended in 300 µl cold 1xPBS, and stored on ice for subsequent flow cytometric sorting.

Flow sorting. Cells were sorted using a MoFlow flow cytometer (Cytomation Inc., Fort Collins, CO) according to the procedure described by Sekar et al. (2004) with minor modifications. The instrument was sterilized by subsequent runs of 70% ethanol and autoclaved distilled water. It was kept sterile during sorting by use of an in-line filter.

- **Purity check.** Purity of the sorted cell fraction was checked by epifluorescence microscopy and 16S rRNA gene analysis. For microscopic analysis, two sorted cell fractions (>10.000 cells) were spotted on a small filter section (PC-filter with 0.2 µm pore size; Millipore, Schwalbach, Germany). Cells were enumerated with an Axiophot II epifluorescence microscope (Carl Zeiss, Jena, Germany).

  Two sorted cell fractions (>10.000 cells) were pooled and diluted in 10 µl sterile deionized water. Archaeal and bacterial 16S rRNA gene libraries were constructed and selected clones sequenced according to the methods described by Lösekann et al. (2007) with minor modifications.

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Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

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