Biogeochemical activity and associated biodiversity at reduced deep-sea hotspot ecosystems

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I dedicate this PhD thesis to my parents, Draga Maneli Pop Ristova and Aleksandar Pop Ristov for their endless support, love, friendship and believe in me.

“The world is my country, science my religion”

(Christiaan Huygens)

Summary

Understanding biodiversity and its patterns across space, time and environmental gradients is central in order to assess the functioning of natural systems and their resilience to external perturbations. To date, the deep-sea floor and its associated ecosystems remain one of least explored environments on Earth, hence, little is known about their biodiversity, and especially that of microbes. Here we investigated the biogeochemical activity and associated bacterial biodiversity of two hotspot ecosystems in the deep-sea, the cold seeps and wood falls. Cold seeps and wood falls are peculiar ecosystems at the deep-sea floor, at which unique sources of energy such as methane and wood-derived cellulose fuel high biomasses of faunal communities with special diversity, typically not encountered at the deep-sea floor. In this study we specifically focused on the investigation of microbial communities since on one hand their diversity patterns are the least understood, and also because their capabilities to utilize methane and cellulose are crucial for the supply of energy to these ecosystems. Cold seeps and wood falls are isolated and fractured ecosystems with only small areal coverage, which poses a challenge for the dispersal of their associated communities and maintenance of their populations. The interconnectivity of these isolated ecosystems and the biodiversity patterns of microbes across different spatial scales are largely unknown. The combination of molecular fingerprinting and geochemical approaches used in this study helped to understand how microbial communities of seep and wood falls are connected over different spatial scales and identify the main environmental factors shaping their diversity.

In Chapter I the local-scale patterns of bacterial community structure and their relation to the sediment heterogeneity was investigated at the REGAB cold seep pockmark. Strong variations in the sediment geochemistry and the core biogeochemical processes were detected between different reduced habitats, which were related to differences in the methane effluxes that ranged over two orders of magnitude. Variation in the structure of bacterial communities was linked to local sediment heterogeneity. Methane, the main energy source at cold seeps, was identified as the most important factor that shaped the seep bacterial community structure and distributions of chemosynthetic megafauna.

The link between the relative abundance of symbiotic bacteria in the gills of a cold seep chemosynthetic mussel and their energy sources was investigated in Chapter II. Mussels inhabiting gassy sediments laden with methane had relatively higher abundance of

methanotrophic symbionts that take up methane, in comparison to thiotrophic symbionts that rely on sulfide as their source of energy. In addition, the abundances of methanotrophs seemed to be linked to variations in methane concentrations in the bottom water, and were correlated with the content of methane-derived carbon in the mussel biomass.

The results presented in Chapter III reveal how seep bacterial communities vary on local and regional scales, within and between cold seep sites. Variation in the seep bacterial community was not correlated to geographic distance, and instead communities displayed patchy structure reflecting on the variability in the sulfide content, as their main energy source. Highest bacterial turnover, with > 50% replacement with new bacterial types was evident between reduced habitats, separated by few meters to hundreds of meters. This result suggest that small reduced habitats, not more than few meters in diameter, represent biodiversity hotspots and contribute substantially to the overall diversity of the deep-sea floor.

In the last chapter (IV) the temporal and spatial variations of communities associated to wood falls were investigated using experimental wood deployments. The results of this study suggest that biogeography plays an important role for the composition of both bacteria and fauna of wood-associated communities. Temporal succession of bacterial and faunal communities occurred within a period of 1 to 2 y. During the whole immersion period the bacterial communities associated to the wood fall remained distinct from the surrounding background sediments, indicating that wood falls represent an important source of diversity in the deep-sea floor.

Zusammenfassung

Um die Funktionsweise natürlicher Systeme und deren Widerstandsfähigkeit gegenüber externen Störungen bewerten zu können, ist es wichtig, Biodiversität innerhalb räumlicher und zeitlicher Muster, wie auch entlang bestimmter Umweltgradienten zu verstehen. Bis heute sind die Tiefsee und damit verbundene Ökosysteme die am wenigsten erforschten Gebiete der Erde und wenig ist bisher über deren Biodiversität, insbesondere der mikrobiellen Diversität, bekannt. Tiefsee-Ökosysteme wie kalte Quellen und Holzeinträge, sind durch ihre hohen Konzentrationen an Methan und Zellulose gekennzeichnet. Diese Energieressourcen sind verantwortlich für einen überdurchschnittlich hohen Biomasse-Aufbau innerhalb der Tiergesellschaften, welche wiederum durch eine einzigartige Diversität gekennzeichnet sind. In der vorliegenden Arbeit wurde ein besonderer Fokus auf die biogeochemischen Aktivitäten und die damit verbundene bakterielle Biodiversität an kalten Quellen und Holzeinträgen gelegt. Diese Studie befasste sich mit der Untersuchung mikrobieller Gemeinschaften, da deren Diversität und Struktur bisher nur in geringem Maße untersucht und verstanden ist, und weil die Fähigkeiten der Bakterien Methan und Zellulose umzuwandeln entscheidend für den Energiekreislauf dieser Ökosysteme ist. Kalte Quellen und Holzeinträge stellen isolierte, fragmentierte und heterogene Ökosysteme von geringer Fläche dar. Sie stellen somit besondere Herausforderungen für die Verteilung, Zusammensetzung und Erhaltung der bakteriellen Lebensgemeinschaften und der damit verbundenen Überlebensstrategien dar. Die Vernetzung dieser isolierten Ökosysteme und mikrobielle Diversitätsmuster über unterschiedliche räumliche Distanzen sind bisher weitgehend unbekannt. Die vorliegenden Ergebnisse, erzielt durch eine Kombination aus molekularen Fingerprinting- und geochemischen Methoden, zeigen auf, wie mikrobielle Gemeinschaften an kalten Quellen und Holzeinträgen über unterschiedliche räumliche Skalen hinweg verbunden sind. Zudem tragen die vorgestellten Ergebnisse dazu bei, die wichtigsten ökologischen Faktoren, die auf die Zusammensetzung der mikrobiellen Gemeinschaften Einfluss nehmen, zu identifizieren.

In Kapitel I wurde der Zusammenhang zwischen der lokal begrenzten bakteriellen Gemeinschaftsstruktur und der Sediment-Heterogenität der kalten Quelle REGAB untersucht. Starke Schwankungen wurden sowohl in der Sediment-Geochemie, wie auch bei wichtigen biogeochemischen Prozessen zwischen den unterschiedlich reduzierten Lebensräumen festgestellt. Diese Schwankungen waren zurückzuführen auf Unterschiede in den

Methanflüssen, die sich über zwei Größenordnungen erstreckten. Die Variationen innerhalb der bakteriellen Gemeinschaftsstrukturen standen in Zusammenhang mit der lokalen Sediment-Heterogenität. Methan, die wichtigste Energieressource kalter Quellen, wurde als der wichtigste Faktor identifiziert, der sowohl die bakterielle Gemeinschaftsstruktur, als auch die Verteilung der chemosynthetisch aktiven Megafauna beeinflusste.

Der Zusammenhang zwischen der relativen Abundanz symbiotischer Bakterien in den Kiemen einer chemosynthetisch aktiven Muschel und deren Energiequellen wurde in Kapitel II untersucht. Muscheln, die methanangereicherte Sedimente bevölkerten, wiesen eine relativ höhere Anzahl an methanotrophen Symbionten auf, die das Methan umsetzen, im Vergleich zu thiotrophen Symbionten, die Sulfide als Energiequelle nutzen. Darüber hinaus schien die Häufigkeit der methanotrophen Bakterien mit Unterschieden in den Methankonzentrationen im Bodenwasser verknüpft zu sein und korrelierte außerdem mit dem aus Methan stammenden Kohlenstoff in der Muschelbiomasse.

Die Ergebnisse, die in Kapitel III vorgestellt werden, verdeutlichen, wie Bakteriengemeinschaften kalter Quellen auf lokaler und regionaler Ebene variieren, sowohl innerhalb, als auch zwischen den unterschiedlichen kalten Quellen. Variationen innerhalb der Bakteriengemeinschaft korrelierten nicht mit geographischer Distanz. Die fleckenhafte Verteilung der Bakteriengemeinschaft wiederum spiegelte die Variabilität des Sulfidgehaltes, die Hauptenergiequelle der Bakterien, wieder. Der höchste bakterielle Durchsatz, mit >50% Austausch durch neue Bakterienarten, zeigte sich im Vergleich der unterschiedlichen reduzierten Habitate, die wenige Metern bis zu Hunderten von Metern voneinander entfernt waren. Diese Ergebnisse deuten darauf hin, dass kleinskalige, reduzierte Habitate, nicht mehr als wenige Meter im Durchmesser, Biodiversitäts-Hotspots darstellen und erheblich zur gesamten biologischen Vielfalt der Tiefsee beitragen.

Im letzten Kapitel (IV) werden die zeitlichen und räumlichen Veränderungen Holz-assoziierter Gemeinschaften mithilfe von experimentellen Holzeinträgen untersucht. Die Ergebnisse dieser Studie legen nahe, dass Biogeographie eine wichtige Rolle im Hinblick auf die Zusammensetzung der Bakterien- und Tiergemeinschaften spielt. Innerhalb von 1-2 Jahren fand eine Sukzession der Bakterien- und Tiergemeinschaft statt. Während der gesamten Zeit am Meeresgrund waren die mit dem Holz assoziierten bakteriellen Gemeinschaften von denen im umliegenden Sediment vorhandenen Bakterien deutlich zu

unterscheiden. Holzeinträge sind demnach wichtige Faktoren, die zur allgemeinen Tiefsee-Diversität beitragen.

Table of contents

1 Introduction

1.1 Hot spot ecosystems at the deep-sea floor

Cold seeps and hydrothermal vents of the deep-sea floor represent some of the most productive ecosystems in the whole oceans (Jørgensen and Boetius, 2007). In contrast to the largely oligotrophic deep-sea floor, where communities directly depend on the sparse input of organic matter produced in the euphotic zone, seep and vent communities depend only indirectly on photosynthesis (e.g. for access to oxygen as an electron acceptor) and instead rely on local production of organic matter. At seeps and vents, some bacteria and archaea utilize chemical energy from the oxidation of reduced compounds (e.g. methane, sulfide and hydrogen), to fix carbon dioxide into organic biomass through the process known as chemosynthesis. Hence, this microbial transformation of reduced compounds, and especially methane in the case of cold seeps, provides the basis for the establishment of prolific chemosynthetic ecosystems dominated by large biomasses of chemosynthetic bivalves, tubeworms and bacterial mats. The chemosynthesis-based life was confirmed three decades ago with the discovery of lush communities of animals and microbes at hydrothermal vents (Lonsdale, 1977; Corliss et al., 1979), followed by the exploration of cold seeps in 1984 (Paull et al., 1984; Kennicutt II et al., 1985; Suess et al., 1985; Kulm et al., 1986). Few years after the discovery of cold seeps, new type of reduced habitats which support chemosynthetic organisms was discovered (Smith et al., 1989). These habitats, commonly known as large organic falls, include sunken wood and whale carcasses at the deep-sea floor. Organic falls supply unusually large quantities of organic matter to the deep-sea floor, which causes local oxygen depletion and production of sulfide that nourishes chemosynthetic communities. In comparison to typical deep-sea sediments all three types of ecosystems i.e. cold seeps, hydrothermal vents and cold seeps, have distinct biogeochemistry and support specialized and highly diverse communities. Therefore, they are regarded as hotspot ecosystems at the deep-sea floor.

This PhD study investigated sunken woods and cold seep habitats, with the aim to provide better understanding of the biogeochemical activity and biodiversity of reduced hotspot ecosystems in the deep-sea.

1.2 Cold seep habitats as geological hotspots

Cold seeps are geologically induced structures, at which seabed fluids, including liquid and gas, are transported to the sediment-water interface, and can eventually come in contact with the water body. Furthermore, cold seep sites are characterized by precipitation of carbonates, formation and dissociation of gas hydrates (see text below for explanation), and hence represent geologically very dynamic areas, which can be considered as **geological hotspots** in the deep-sea. Fluids emanating at cold seeps are mainly composed of methane, but also higher hydrocarbons, oil, brines, freshwater and may contain nutrients and hydrogen sulfide. Fluid flow velocities can be highly variable between individual seepage sites, and can range from few tens of centimeters to a few meters per year (Tryon et al., 1999; Torres et al., 2002; Boetius and Suess, 2004; Niemann et al., 2006). As the name indicates, these sites are usually not associated to thermal anomalies, or not to the extent observed at hydrothermal vents (Feseker et al., 2008; Suess, 2010). Cold seeps are widespread along passive and active continental margins, as well as at transform plate boundaries which are covered by thick sedimentary sequences exist (Judd, 2003; Suess, 2010) (Figure 1).

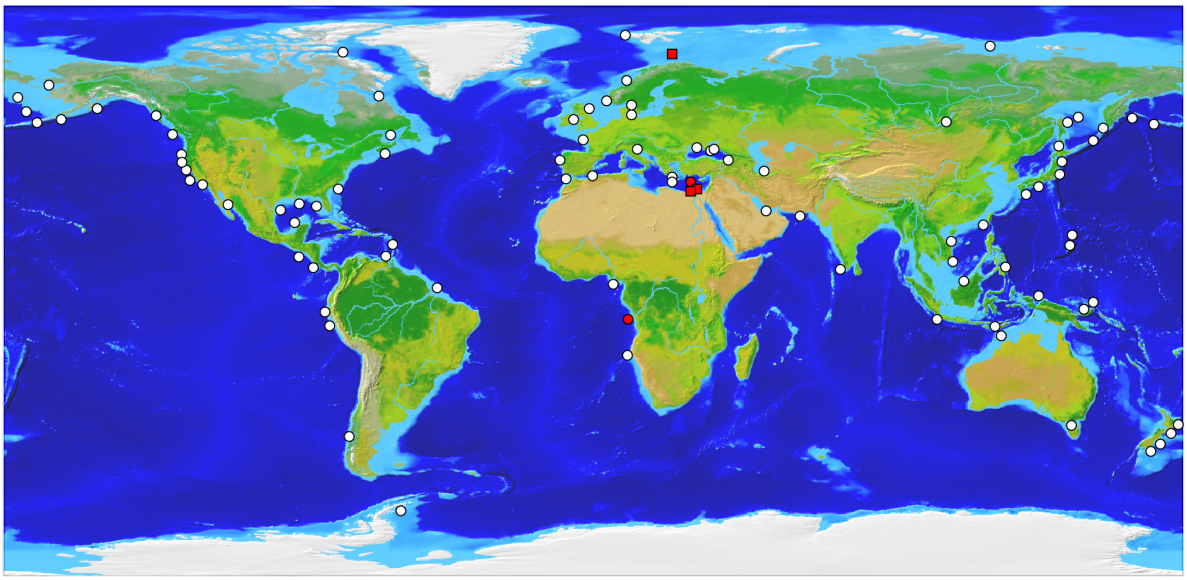

Figure 1 Global distribution of hydrocarbon seeps (white and red symbols). Cold seep sites investigated in this study are depicted with red symbols: the Amon Mud Volcano, the Amsterdam Mud Volcano and the Pockmark area in the Eastern Mediterranean sea, as well as the REGAB pockmark off the west coast of Africa. Square symbols depict sites of wood experiment deployments explored in this study (Map Courtesy of M. Römer).

Numerous cold seeps have been detected in every ocean and sea, in a broad range of oceanographic settings from the coast (e.g. Eckernförde Bay at 20 m water depth, Anderson et al., 1998; Treude et al., 2005) to the deep ocean (e.g. Japan Trench at water depth > 7000 m; (Fujikura et al., 1999; Kobayashi, 2002), and their number is steadily increasing with the ongoing exploration of new deep-sea areas and further improvement of deep-sea technologies (Figure 1). Fluid discharges can be associated to a variety of types of seep seafloor expression phenomena, of which the most prominent include mud volcanoes, pockmarks, gas seeps and brine/oil pools. The development of seafloor expression mainly depends on the geological setting, driving force for the seep formation, intensity of fluid flow and the nature of fluid discharge. Consequently, depending on the width of conduits through which mobilized fluids ascend, the seafloor expressions can range in size from only few centimeters (sites of individual gas bubbles escapes) to more than a couple of kilometers in diameter (e.g. mud volcanoes) (Kopf, 2002).

Mud volcanoes (MVs) represent large positive morphological structures at the seafloor, at which fluids along with muddy sediments are being transported. Necessary conditions for the formation of mud volcanoes are high sedimentation rates at passive margins and lateral tectonic compression at active margins, as well as presence of plastic clay layers (Milkov, 2000). Mechanisms of mud volcano formation include the following sequence of events and forces: initial build up of high pore fluid pressures caused by inability of pore fluids to drain from fine-grained sediments; consequential lowering of the bulk density and shear strength; and lastly, density inversion between the undercompacted sediment and the overlying formations can occur, which provides sufficient buoyancy force that initiates diapiric movement of sediment masses (Judd and Hovland, 2007 and references therein). Generation of gas is assumed to provide additional buoyancy, and hence additional driving force for the formation of mud volcanoes (Milkov, 2000; Kopf, 2002). Current estimate of the total number of known and inferred deep-water mud volcanoes is $10^3 – 10^5$, and it is assumed that these correlates to a total gas discharges of 27 Tg y^{-1} (Milkov, 2000, 2003). During the course of this PhD thesis, reduced habitats of two mud volcanoes i.e. the Amon Mud Volcano and the Amsterdam MV in the deep Eastern Mediterranean sea were investigated (Chapter III).

Pockmarks are negative topographical structures at the seafloor, usually only few meters deep and with diameters ranging from less than one meter to couple of hundreds of meters (e.g. the REGAB pockmark off West Africa; Charlou et al., 2004). The processes involved in the formation of pockmarks, are still under debate. One proposed conceptual model for the

formation of pockmark structures (Josenhans et al., 1978; Hovland and Judd, 1988) include: initial accumulation of gas below a sealing layer, which in turn leads to build up of overpressure and upward doming of the seafloor; consequential eruption of fluids due to high pressure, and ejection of gas, water and sediments in the water column; and finally, transport of the fine-grained sediments by bottom water currents resulting in a depression on the seafloor. Alternatively, it has been suggested that pockmarks can be also formed slowly, by the continuous removal of small amounts of sediments by gas bubbles, which may lead to the development of composite, and often very large pockmarks (Sahling et al., 2008). Pockmarks have been described from various regions, such as the North Sea (Judd and Hovland, 2007), the Eastern Mediterranean sea (Dupré et al., 2010) and the West African margin (Ondréas et al., 2005). They can exist individually or form clusters that occupy large areas of the seafloor, such as in the North Sea where up to 30% of the seafloor shows pockmark structures (Judd and Hovland, 2007). The REGAB pockmark (Ondréas et al., 2005) located off the West coast of Africa (Chapter I, II) and the pockmark area in the Central Province of the Nile Deep-sea Fan (Chapter III) were among the main cold seep sites investigated within this PhD thesis.

1.2.1 Origin and importance of methane from marine sources

Methane is the most abundant hydrocarbon gas in sediments and the dominant constituent (90 vol %) of discharging fluids at most cold seep sites (Dimitrov, 2002; Kopf, 2002). Once in the atmosphere, this powerful greenhouse gas can substantially contribute to the global warming, due to its high Global-warming Potential (23 times higher than equal amount of CO_2). However, at cold seep sites where methane is escaping the seafloor this gas nourishes the establishment of highly productive chemosynthetic ecosystems. Seabed methane originate from one of the two sources: i) biogenic methane derived from microbial degradation of organic matter buried in shallower sediments and, ii) abiogenic methane originating from thermocatalytic breakdown of organic matter in deep sedimentary basins (Figure 2). The origin of methane can be distinguished based on the carbon and hydrogen isotope signatures, and the relative proportions of methane and the higher hydrocarbon gases (Whiticar, 1999, 2000). Thus, microbially formed methane is lighter ($\delta^{13}C$ of -50 to -110‰ Pee Dee Belemnite (PDB)) than geothermal methane ($\delta^{13}C$ of -20 to -50‰ PDB) (Whiticar, 1999). Methane production in marine sediments has been estimated at 75 – 320 Tg y^{-1} (Valentine, 2002), of which most is accounted for by methanogenesis (Claypool and Kvenvolden, 1983) – the last step of the microbially mediated anaerobic degradation of organic matter. To date the only

organisms known to perform methanogenesis in anoxic environments belong Euryarchaeota, a subgroup of the archaea domain (Balch et al., 1979). Methanogenesis process accounts for a substantial portion of the remineralization of organic matter in marine environments, with 2 – 5% in coastal marine sediments and approximately 30% in environments with high organic load (Canfield et al., 2005).

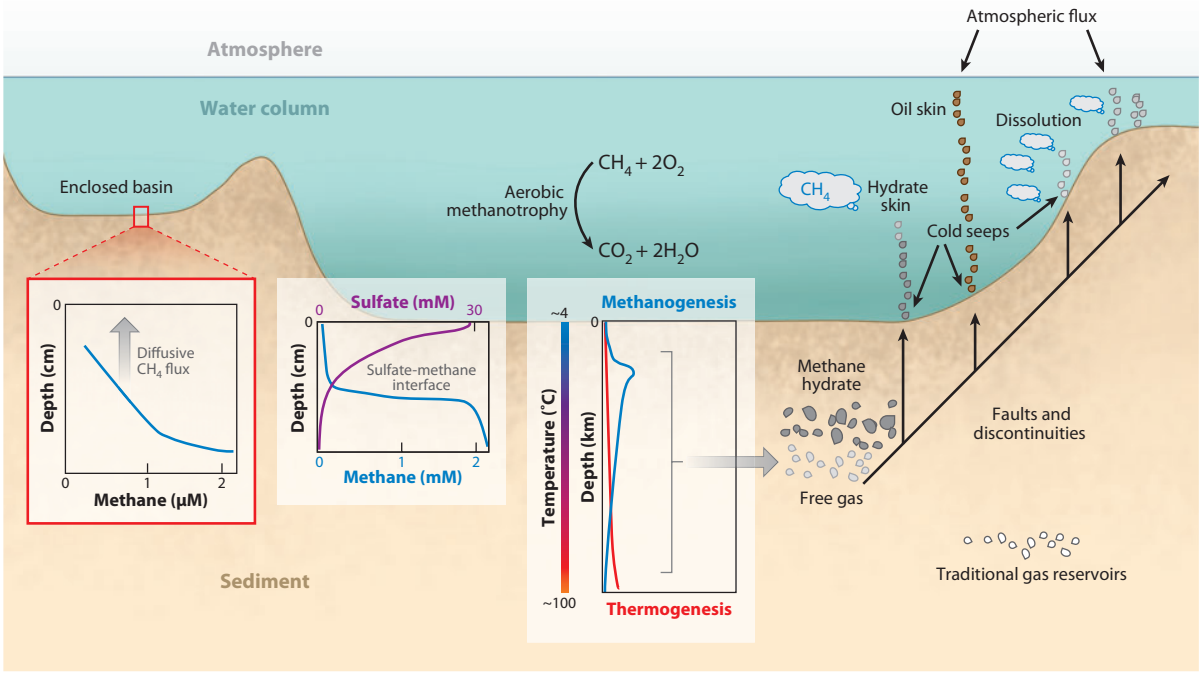

Figure 2 Schematic diagram showing biogeochemical and transport processes affecting marine methane (adapted after Valentine, 2011).

Dissolved methane at cold seep sediments migrate upwards by two possible transport processes, i.e. diffusion and advection. However, if methane concentrations exceed the ambient solubility, it exists as free gas and its buoyancy is the dominant force driving methane gas bubbles towards the surface (Matthews, 1996; Römer, 2011 and references therein) (Figure 2). Although it is commonly regarded that substantially more methane is transported through free gas migration (Clennell et al., 2000), a recent study showed that at the Håkon Mosby Mud Volcano (HMMV) as much methane is transported as dissolved (up to 14 x 10^6 mol CH_4 y^{-1}) as in the form of free gas (Felden et al., 2010). This PhD study contributed towards quantification of dissolved methane effluxes at couple of cold seep sites, covered in Chapter I and III.

In natural systems when methane is present in sufficient concentrations under specific conditions of low temperatures and high pressures, it tends to form gas hydrates (Figure 2). Gas hydrates, or clathrates, are ice-like crystalline structures, in which water molecules form cage-like structures and entrap low molecular weight gasses, such as methane (for a comprehensive review on gas hydrates see Bohrmann and Tores, 2006). Gas hydrates are widely distributed at continental margins including cold seep sites, however, they are not restricted to the marine environment, and have been also found in permafrost regions and lakes with high load of organic matter (Kvenvolden, 1988, 1993). Gas hydrate deposits in marine sediments occur within the so called Gas Hydrate Stability Zone (GHSZ), which is defined predominantly by the factors controlling gas hydrate formation, such as temperature and pressure (Sloan, 1998). Accumulation of gas hydrates within the GHSZ can act as a sealing mechanism (Hovland, 2002) preventing gas to escape the seafloor, and retaining the gas below the GHSZ, which can eventually freeze in additional gas hydrate layers. Nevertheless, seepage of methane gas can also exist in areas within the GHSZ (Suess et al., 1999; Bohrmann et al., 2003). Indeed, within this PhD study, escape of gas bubbles was observed at the REGAB pockmark cold seep site, which lies within the GHSZ. Gas hydrates represent the largest reservoir of methane in the marine realm. Accurate quantification of the global gas hydrate deposits still remains difficult, hence published estimates of hydrate-bound methane range over three order of magnitude (300 – 57 000 Gt C) (Wallmann et al., 2011 and references therein). Latest studies, based on improved models report on global gas hydrate inventory ranging between 400 to 3000 Gt C (Wallmann et al., 2011; Piñero et al., 2012). Estimates on the atmospheric input of methane by oceanic hydrocarbon sources is still controversial, however it is generally viewed that this source is rather small (45 Tg y^{-1}) compared to anthropogenic (e.g. rice agriculture and ruminants, together 225 Tg y^{-1}) and terrigenous natural sources (wetlands, 125 Tg y^{-1} (Kvenvolden and Rogers, 2005). However, different studies agree that the current trend of increasing global temperatures might cause gas hydrate destabilization and sudden release of enormous amounts of methane to the atmosphere (up to 1000 times the amount of methane in the atmosphere, based on the newest estimates of 3000 Gt of methane stored as gas hydrates), that can profoundly affect the global climate by further warming (Bohrmann and Tores, 2006; Reeburgh, 2007).

1.3 Cold seep habitats as biogeochemical hotspots

Methane seepage and its microbial consumption shape the geochemistry of cold seep sediments, and furthermore cause development of extremely steep and often highly variable geochemical gradients over a micrometer to centimeter scale. Hence, cold seep sediments represent **biogeochemical hotspots** at the deep-sea floor (Valentine, 2011) (Figure 3 and 5b).

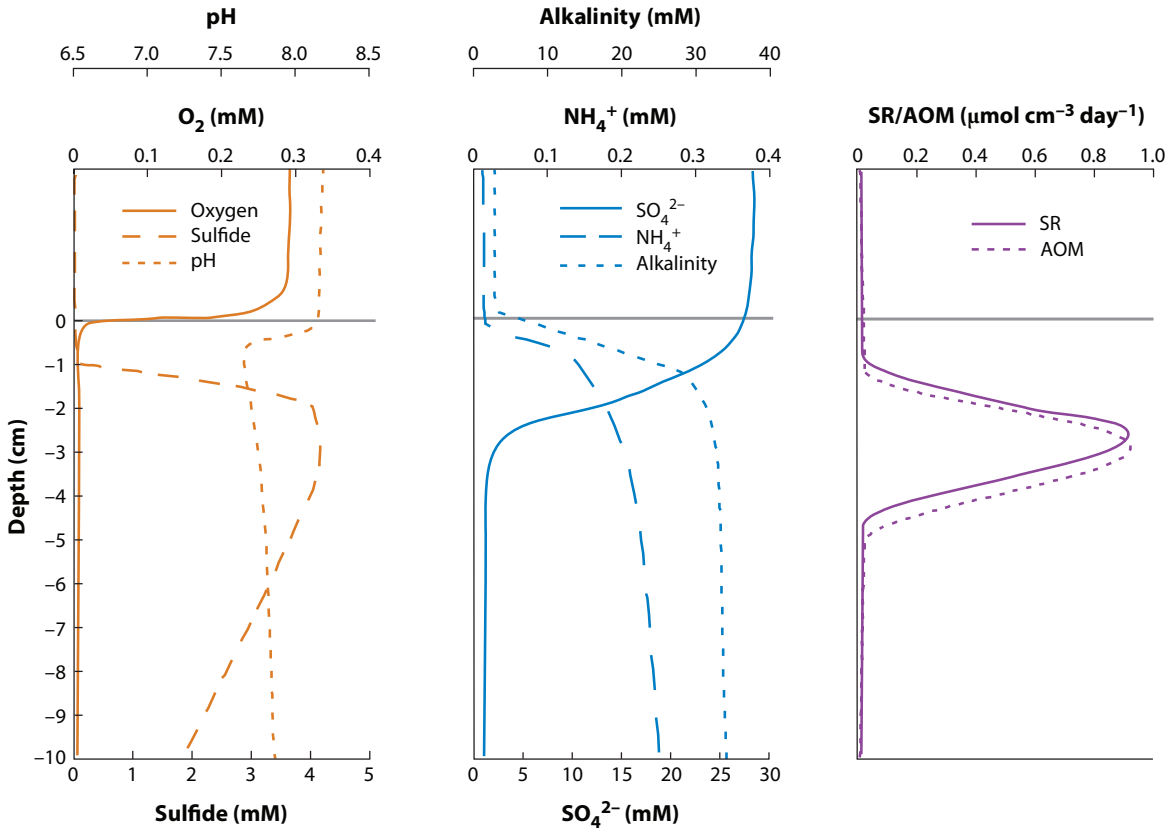

Figure 3 Schema of typical biogeochemical gradients in AOM zone of cold seep sediments (adapted after Knittel and Boetius, 2009). AOM, anaerobic oxidation of methane; SR, sulfate reduction.

Microbially mediated aerobic and anaerobic oxidation of methane are the main geochemical processes that function as major sinks of methane in sediments (Reeburgh, 2007). These processes effectively prevent methane from reaching the water column and the atmosphere. In most seep systems the upward fluid flow limits the oxygen penetration in sediments to often less than one centimeter depth and thus confines the aerobic oxidation of methane to only the topmost surface layers (de Beer et al., 2006; Niemann et al., 2006; Grünke et al., 2011) (Figure 3 and 5b). Exceptions are seep habitats that are impacted by bioirrigation - transport of fluids caused by animals, which causes substantial influx of oxygenated bottom water in the sediments, and hence potentially extend the zone for aerobic oxidation of

methane (Childress and Fisher, 1992; Sahling et al., 2002; Cordes et al., 2005; Fischer et al., 2012). Additionally methane can be oxidized aerobically within the gills of chemosynthetic megafauna that harbor aerobic methane-oxidizing symbionts (Petersen and Dubilier, 2009, 2010).

In anoxic sediments, methane is anaerobically oxidized (AOM) with sulfate as electron acceptor (Figure 3). At most cold seeps this process is predominantly responsible for the removal of methane, and moreover provides the basis for the establishment of high biomass chemosynthetic communities. The discovery of this process has a long history, which dates back to the end of the 1970s, when concurrent depletion of sulfate and methane (sulfate methane transition zones) was observed in pore water profiles (e.g. Martens and Berner, 1974). Furthermore, theoretical approaches (Hoehler et al., 1994), detection of microbial biomarkers (Hinrichs et al., 1999) and finally microscopic approval of the responsible microbial communities (Boetius et al., 2000) helped to reveal the intricacies of the AOM process. Hence, the discovery of AOM represents a nice example how science can prosper by applying a cross-disciplinary and multimethodological approach. Today it is known that a microbial consortium consisting of anaerobic methanotrophic archaea (ANME) and a sulfate reducing bacterial (SRB) partner mediate the low energy-yield reaction of methane oxidation with sulfate in a ratio of approximately 1:1 (Equation 1) (Boetius et al., 2000):

$$\mathbf{CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O} \qquad \text{(Eq.1)}$$

Both products of the AOM coupled to SR reaction, sulfide and bicarbonate, are of high relevance for the biology and chemistry of cold seep ecosystems. The substantial increase in inorganic carbon and alkalinity due to AOM (Eq.1, Figure 3) leads to precipitation of carbonate minerals (e.g. calcite, aragonite) to an extent that often carbonate platforms or carbonate chimneys are formed (Michaelis et al., 2002; Gontharet et al., 2007) (Figure 4a,d,f). These structures provide hard substrates necessary for the settlement of chemosynthetic organisms such as mussels and tubeworms (Olu-Le Roy et al., 2007)(Figure 4c,f).

AOM occurs across a variety of anoxic habitats where overlapping zones of methane and sulfate exist, such as coastal, margin and subsurface Sulfate Methane Transition Zone (SMTZ), cold seeps with surface hydrates, mud volcanoes, marine anoxic waters, lake sediments and waters to name a few, where the right conditions of complete depletion of oxygen are met. Detected AOM rates at these habitats vary by seven orders of magnitude

ranging from few pmol cm^{-3} d^{-1} to µmol cm^{-3} d^{-1}, the latter being measured at SMTZ horizons at cold seeps sites overlying gas hydrates (Knittel and Boetius, 2009 and references therein). Differences in the AOM rates of more than one order of magnitude have been also detected between habitats within a single cold seep, located only few tens to hundreds of meters apart (e.g. Treude et al., 2003). Further information on habitat-specific AOM rates, as well as detailed explanation of the methods applied to quantify AOM activity is presented in Chapter I and III.

The main factor controlling AOM rates at cold seeps is the availability of methane and sulfate, as both electron donor and acceptor are needed for the growth of the AOM consortia. At seep sites transport of sulfate to the sediments is often the critical step limiting AOM, as rates at which methane is advected by porewater fluids upwards usually exceed the diffusive downward flux of sulfate from the bottom water (de Beer et al., 2006). Hence, methane can reach the water column and potentially also the atmosphere, where it acts as strong greenhouse gas (Figure 2). Cold seeps are main methane emission sites in the ocean, where high flow velocity and/or occurrence of gas bubble streams reduces the resident time of methane in the sediment, and hence the potential time for consumption of methane by the AOM consortia (Reeburgh, 2007). Anticipated environmental changes due to global warming might affect the stability of gas hydrates and the efficiency of the biological methane filter. This could result in substantially higher fluxes of methane to the hydrosphere and eventually to the atmosphere. Therefore it is important to quantify the contribution of seeps to the methane budget and identify the areal extent of current and potential methane seepage sites (Knittel and Boetius, 2009; Felden et al., 2010). Studies contributing towards this goal are rare, as the quantification of methane *in situ* still poses a technological challenge (Torres et al., 2002; Treude et al., 2003; Linke et al., 2005; Sommer et al., 2006, 2008, 2010; Felden et al., 2010; Lichtschlag et al., 2010; Grünke et al., 2011) This study contributed towards this goal by quantifying methane effluxes and the efficiency of the microbial filter of individual seep habitats, by using an *in situ* deep-sea benthic chamber module (Figure 5b). Corresponding results are presented in Chapter I and III.

Sulfide, the main product of AOM coupled to SR, represents important energy source at cold seeps. Sulfide is predominantly consumed in surface sediments at oxic-anoxic boundaries, where free-living giant motile sulfide-oxidizing microorganisms have access to both the electron donor (sulfide) and the electron acceptor (oxygen or nitrate) (Figure 3). High concentrations of sulfide detected at cold seeps (reaching up to 20 – 26 mM) can support the

development of extensive thiotrophic bacterial mats, such as ones found at the flank of the Amon Mud Volcano, where they cover areas of app. 200 m^2, (Grünke et al., 2011) (Figure 4b). Sulfide is extremely toxic to most animals even at low concentrations (Somero et al., 1989; Bagarinao, 1992). However, at reduced ecosystems chemosynthetic megafauna have found ways not only to cope with high sulfide concentrations, but also to benefit from it. Sulfide is the main energy source that fuels the highly productive and high biomass benthic faunal communities encountered at seep sites (Sibuet and Olu, 1998; Levin, 2005) (Figure 4c,e,f). Major sink of sulfide in cold seeps sediments represents the complexation with iron to form pyrite that provides the characteristic blackish color of reduced seep sediments (Jørgensen and Kasten, 2006).

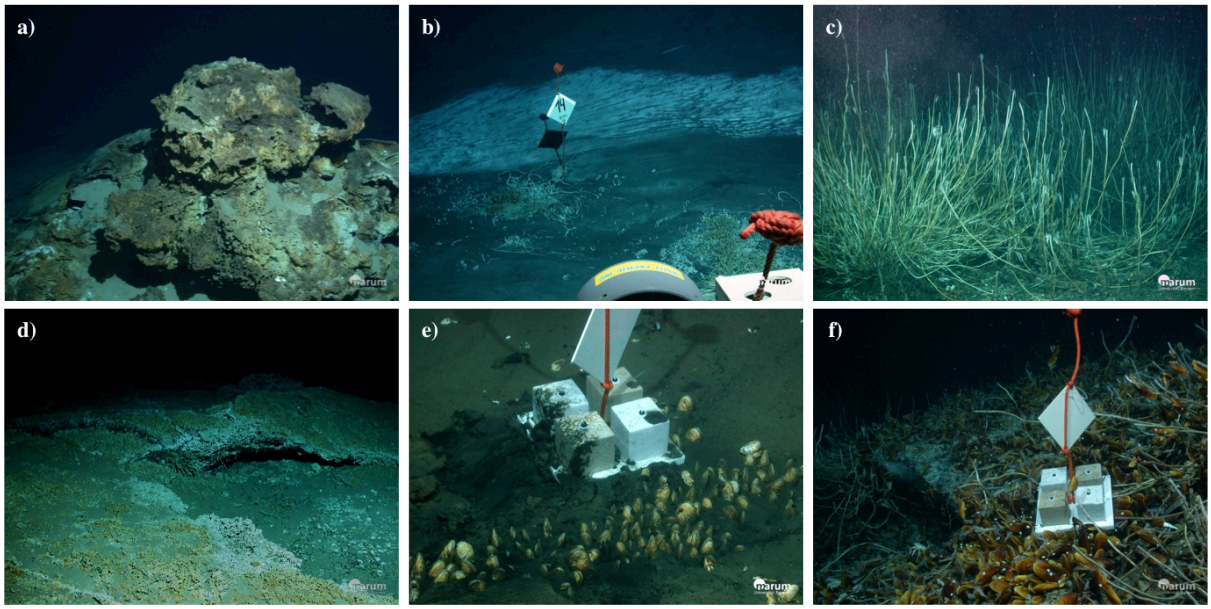

Figure 4 Different type of reduced habitats typically found at cold seep ecosystems: diffrently shaped carbonate crusts (a, d); thiotrophic bacterial mat with small patches of tubeworms (b); extensive bushes of tubeworms (c); clams buried in reduced sediments (e); mussels sitting on top of outcropping hydrates and carbonates. Image source Marum, University of Bremen.

Oxygen is thermodynamically the most favorable electron acceptor, but its concentration even in fully oxic bottom waters is rather low (~ 280 µM). Hence, at highly active seep ecosystems oxygen is usually exhausted within the first couple of millimeters in the sediment (Figure 3 and 5b) and therefore sulfate (found in concentrations 3 orders of magnitude higher than oxygen – 29 mM) represents the more important electron acceptor (Figure 3). Oxygen is predominantly consumed by benthic fauna and aerobic microorganisms, but also for reoxidation of inorganic products released during the process of organic matter degradation

and anaerobic oxidation of methane. Hence, at cold seeps and other natural systems the total benthic oxygen uptake (TOU) has been used to assess benthic respiration rates, as well as to assess total benthic carbon mineralization (Canfield et al., 1993; Wenzhöfer and Glud, 2002; Glud, 2008 and references therein). At continental shelves half of the benthic oxygen consumption is directly or indirectly used for reoxidation of sulfide (Jørgensen and Kasten, 2006), and similar estimates are expected for sulfide produced at seeps. Specialized deep sea-rated instruments, usage of vessels and often ROVs are required for the accurate determination of *in situ* benthic oxygen consumption of targeted, usually quite small deep-sea seep habitats (Figure 5a,b). Therefore to date, only few studies exist dealing with investigation of oxygen consumption at seep sites (Linke et al., 2005; Sommer et al., 2006, 2008, 2010; Felden et al., 2010). However, even such limited dataset as currently available reveal substantial variations in the benthic oxygen uptake, with up to 1 order of magnitude differences between different seeps ecosystems (e.g. 48 and 114 mmol m^{-2} d^{-1} at a *Beggiatoa* mat at the Hydrate Ridge and Håkon Mosby Mud Volcano, respectively (Sommer et al., 2006; Felden et al., 2010)) and between reduced habitats within one cold seep site (e.g. 4 and 48 mmol m^{-2} d^{-1} at a clam patch and bacterial mat at the Hydrate Ridge, respectively (Sommer et al., 2006)).

1.3.1 Methods used in this study to quantify *in situ* fluxes of oxygen and methane

In situ measurements provide substantially higher accuracy of the geochemical status of deep-sea benthic habitats. This is especially important for deep gas-saturated seep sites where degassing of sediments upon retrieval, due to change in pressure and temperature, may profoundly disturb the depth gradients of porewater constituents and even substantially alter their concentrations (Boetius and Wenzhöfer, 2009). Therefore, within this PhD study the *ex situ* methods for porewater analyses were combined with *in situ* detection of oxygen depth gradients, benthic oxygen uptakes and methane effluxes (Figure 5). This allowed accurate cross-comparison and a ranking of various reduced habitats according to their biogeochemical activity, as well as estimation of the faunal contribution to the total benthic oxygen uptake at cold seeps (Chapter I). For this purpose, we used an *in situ* ROV-operated microprofiler (Glud et al., 2009; Lichtschlag et al., 2010a) and a benthic chamber module (Wenzhöfer and Glud, 2002; Felden et al., 2010); Figure 5).

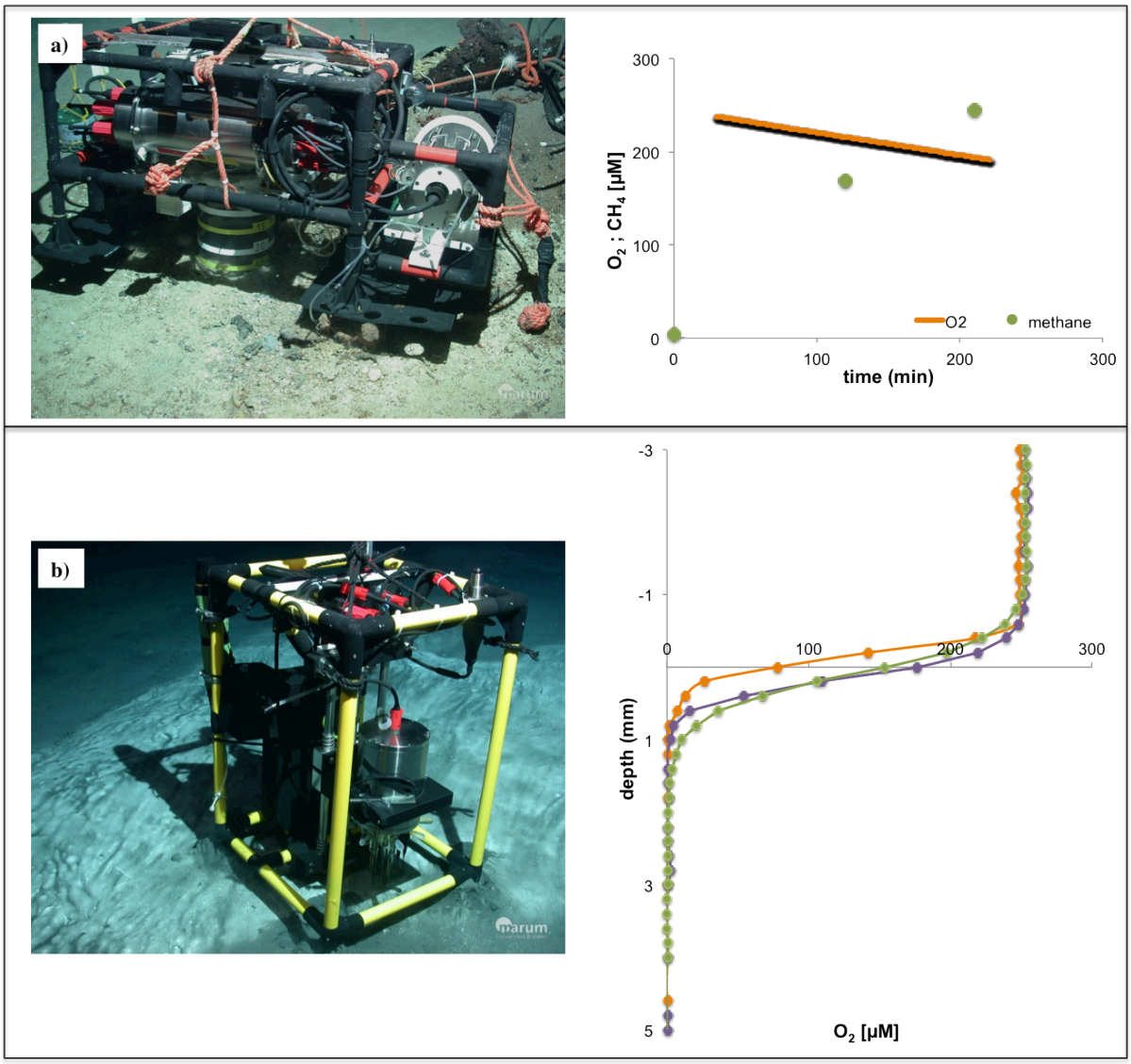

Figure 5 *In situ* benthic chamber (a) and microprofiler (b) modules used for the quantification of oxygen and methane fluxes. Upper panel shows data typically derived from benthic chamber incubations (on sediments populated by mussels), revealing decline in oxygen and increase in methane concentrations with time. Lower panel shows data derived from a microsensor measurements with a microprofiler (on sediment covered with a bacterial mat), revealing very steep oxygen gradients and its disappearance within < 2 mm sediment depth. Image source Marum, University of Bremen.

In short, the microprofiler carries up to three Clark-type oxygen microsensors (Revsbech, 1989) that allow high-resolution detection (every 100 - 200 µm) of oxygen concentrations with sediment depth (Figure 5b). Additional sensors can be mounted to the microprofiler for parallel measurement of sulfide and pH depth gradients. Microsensor based measurements can record steep oxygen concentration gradients that exist over small spatial scales (< 1 cm), which would be missed if gradients were determined by conventional porewater extraction

methods (Figure 5b). Calculation of Diffusive Oxygen Uptakes (DOU) derived from these high-resolution depth gradients allows quantification of microbial respiration rates.

The working principle of the benthic chamber module is based on an enclosure technique, which enables *in situ* detection of the total exchange rates of oxygen, as well as methane across the sediment-bottom water interface (Figure 5a). This measurement integrates all relevant solute transport processes, such as diffusion, advection and fauna-mediated transport. The chamber module used within this PhD study was equipped with an optode for the continuous recording of oxygen concentrations, and a pre-programmed syringe system that enabled retrieval of discrete water samples for the determination of methane concentrations.

1.4 Cold seep habitats as biodiversity hotspots

Biodiversity, or the variety and abundance of species (taxonomical entities in the case of prokaryotes) in a defined area (Rosselló-Mora and Amann, 2001; Magurran, 2003), is assumed to enhance crucial properties of natural systems, such as its functioning, productivity and resilience towards external perturbations (Chapin et al., 2000; Loreau et al., 2001; Danovaro et al., 2008). Rapid destruction of oceanic and deep-sea habitats, due to increased anthropogenic impact and global change, threatens to harm biodiversity. This would in turn affect the ecosystem functioning and its services it provides to the mankind (Berkes et al., 2006; Armstrong et al., 2010; Ramirez-Llodra et al., 2011; Tinch et al., 2012). Therefore, within the last decade, large scientific programs (e.g. Census of Marine Life, EU projects HERMES & HERMIONE) have been established aiming to study ocean biodiversity, and its patterns across space and time, in order to be able to better predict how future changes will affect ecosystem functioning (Jørgensen and Boetius, 2007; Zinger et al., 2011). Although cold seeps represent **biodiversity hotspots** in the deep-sea, because they harbor special biodiversity within a larger area of normal diversity (Jørgensen and Boetius, 2007), the knowledge on the distribution patterns of seep organisms, especially concerning prokaryotes is still very limited.

1.4.1 Microbial diversity at cold seeps

Microorganisms, both free-living and symbiotic, play a pivotal role in the functioning of cold seep ecosystem, as they present the basis of the food chain via the transformation methane

and other hydrocarbons. Moreover, with up to two orders of magnitude higher microbial cell numbers (max. detected 10^{12} cells cm^{-3}; Michaelis et al., 2002; Treude et al., 2007), seep microorganisms contribute relatively more than microorganisms from other deep-sea habitats to the total biomass of their respective ecosystems (Knittel et al., 2003). Based on their metabolic potentials to utilize reduced compounds, methanotrophs, hydrocarbon degraders, sulfate reducers and sulfide oxidizers are the key functional groups at cold seeps (Jørgensen and Boetius, 2007). Currently three distinct clusters of Euryarchaeota, ANME-1, ANME-2, ANME-3 are known to consume methane anaerobically (Orphan et al., 2002; Niemann et al., 2006; Knittel and Boetius, 2009, 2010). Phylogenetically they are related to methanogens, such as *Methanosarcinales* and *Methnomicrobiales* (Knittel et al., 2005). ANME populations can be extremely abundant at cold seep ecosystem, reaching more than 10^{10} cells per cm^{-3} sediment (Knittel and Boetius, 2009 and references therein). Methanotrophic archaea are cosmopolitan and have been found in all locations where methane is present (Knittel and Boetius 2009 and references therein), however, habitats are usually dominated by only one clade, indicating subtle environmental control on their distribution (Girguis et al., 2005; Nauhaus et al., 2005). For example, it has been shown that seep habitat heterogeneity controls the distribution and abundances of ANME-3 at the HMMV (Lösekann et al., 2007). Overall archaeal diversity at cold seeps is low and seep habitats are mainly dominated (> 90 %) by either of the ANME groups. ANME-1 and ANME-2 are usually associated, and form so-called AOM consortia with Sulfate Reducing Bacteria (SRB; Boetius et al. 2000), a functional group that is phylogenetically related to *Desulfosarcina/Desulfococcus* (Deltaproteobacteria). The AOM consortia are predominantly responsible for the removal of methane in anoxic sediments via sulfate reduction. Bacteria are highly diverse at cold seeps (Knittel et al., 2003), with new studies revealing even higher diversity of the bacterial partners associated to ANME-2, and even not necessarily restricted to the class Deltaproteobacteria (Pernthaler et al., 2008).

Sulfide-oxidizers are another important functional group at cold seeps that comprise free-living and symbiotic bacteria, which gain energy by oxidation of sulfide, the end-product of sulfate-dependent AOM. The conspicuous and widely distributed biogenic habitats of cold seep ecosystems - the bacterial mats, are formed by sulfide-oxidizers such as the large vacuolated *Beggiatoa*, *Thiomargarita*, *Thioploca* and *Arcobacter* (Grünke et al., 2011) (Figure 4b). Within the thiotrophic mats sulfide is usually oxidized with oxygen, however some microorganisms use nitrate as an alternative electron acceptor under anoxic conditions

(Fossing et al., 1995; McHatton et al., 1996; Schulz et al., 1999). Main roles attributed to free-living sulfide-oxidizers at cold seeps are the build up of biomass that supports heterotrophic food webs, efficient removal of toxic sulfide and linking of carbon, sulfur and nitrogen cycles (Grünke et al. 2011 and references therein). Distribution of sulfide-oxidizers is influenced by the sediment geochemistry of seep habitats, especially by oxygen and sulfide concentrations, which select for different mat-forming species (Macalady et al., 2008; Grünke et al., 2011).

1.4.2 Diversity of chemosynthetic megafauna at cold seeps

Sulfide-oxidizing bacteria play another crucial role at cold seep ecosystems, as part of chemosynthetic symbiotic associations with various invertebrate animals, where these bacteria harness the energy from the environment and feed their hosts. Typical hosts of sulfur-oxidizers at reduced habitats include bivalves belonging to the subgroups *Vesicomyidae*, *Mytilidae*, *Thyasiridae*, *Lucinidae*, *Solemydae*, as well as polychaetes (for a comprehensive review on chemosynthetic symbioses see (Dubilier et al., 2008) (Figure 6a). Another type of symbiotic association is the one between aerobic methane-oxidizing bacteria and mytilid mussels, as well as polychaete worms (Petersen and Dubilier 2009, 2010 and references therein) (Figure 4c,f). Bathymodiolus mussels and some polychaetes worms can live in dual symbioses with aerobic- and sulfur-oxidizing bacteria, prospering from both sulfide and methane as energy source. Chemosynthetic megafaunal organisms dominate the biomass at seeps, and form dense assemblages of various sizes and shapes that are often used as first indicators of seepage activity at the seafloor during geological surveys (Figure 4). Several chemosynthetic species can live together in a single cold seep structure, owning to their different physiological adaptations and environmental preferences, such as nature of seafloor substrate, intensity of fluid seepage, methane or sulfide affinities and chemical concentrations (Sibuet and Olu, 1998). Vesicomyid clams inhabit soft sediments (Figure c4) and when different species co-occur in a single cold seep, they are usually segregated along sulfide concentration gradients from the seep center to the periphery (Barry et al., 1997), owing most probably to their differential abilities to bind sulfide (Childress and Fisher, 1992). Mytilid mussels are predominantly found on hard substrates (Figure 4e), usually coinciding with high methane bottom water concentrations (Olu-Le Roy et al., 2007).

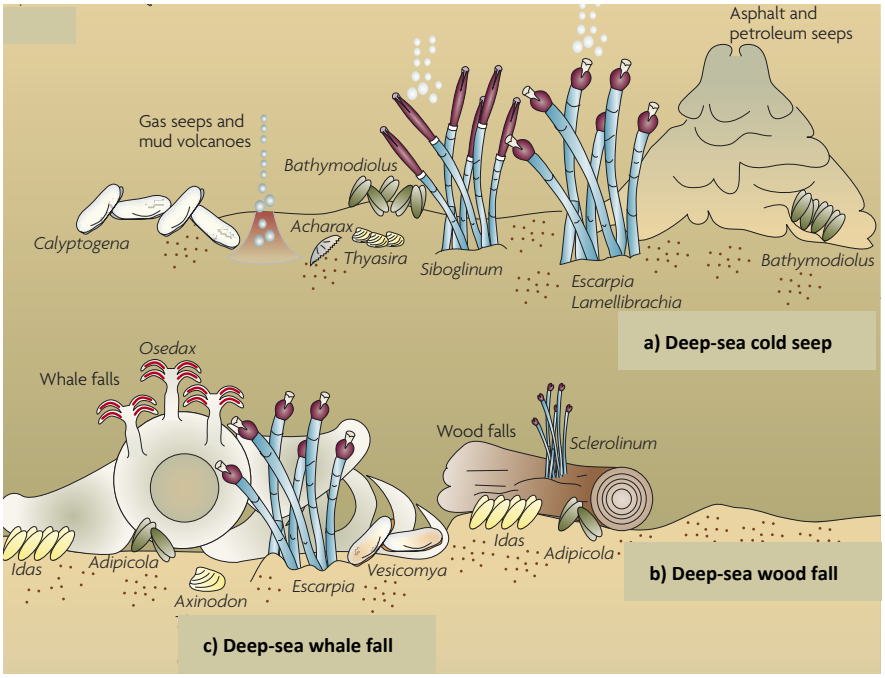

Figure 6 Similar chemosynthetic fauna inhabiting different reduced habitats: deep-sea cold seeps (a), wood (b) and whale falls (c) (adapted after Dubilier, 2008).

Overall variations in bottom water methane and sulfide concentrations, as well as the type of substrate have been identified as the main factors that govern the distribution and densities of symbiont-bearing species at local scales, within individual seep sites (Olu-Le Roy et al., 2007). A comprehensive review based on data available from seeps located in different oceans revealed an exponential relationship between the rate of fluid flow and seep biomass, indicating that high flows support bivalve biomasses proportionally higher than those found at weak flows (Sibuet and Olu-Le Roy 2002; Figure 7b). On local scales, diversity and density also of non-symbiotic megafauna, macro- and meiofauna were found to be influenced by the habitat heterogeneity caused by variations in methane- and sulfide-related geochemistry (Olu et al., 2009; Van Gaever et al., 2009; Menot et al., 2010; Decker et al., 2011; Ritt et al., 2011).

1.4.3 Cold seeps as heterogeneous ecosystems with fragmented global distribution

Cold seeps are highly heterogeneous ecosystems with many distinct habitats, where the tight coupling of geological and geochemical processes, as well as the interaction of microorganisms and fauna leads to high complexity and multilevel heterogeneity (Cordes et al., 2010). In short, geological processes that result in seepage of methane at the deep-sea

floor select for microbial communities, which can utilize methane as energy source. Microorganisms alter the geochemical fluxes, e.g. of methane and sulfide, and with that add a level of heterogeneity by creating habitats suitable for certain types of chemosynthetic megafauna. Finally, chemosynthetic megafauna assemblages add structural complexity that may be perceived by smaller fauna (macro- and meiofauna) as habitat heterogeneity (Cordes et al., 2010), and they alter the geochemistry of the underlying sediments, which might affect the distribution of microorganisms. According to the "habitat heterogeneity hypothesis" structurally complex habitats lead to an increase in species diversity by providing a higher number of distinct niche dimensions and diverse ways of exploiting resources (MacArthur and Wilson, 1967). The habitat heterogeneity concept seems to explain local-scale (within seeps) diversity patterns at cold seeps well. These are thought to harbor high β-diversity (change in community structure and/or composition among sites; Whitaker 1960), due to a fragmentation of habitats. In contrast, less heterogeneous ecosystems, such as the deep-sea floor, are characterized by most probably lower β-diversity, but higher overall species richness (alpha diversity, or total number of different species per unit area; Hesseler and Sanders 1976; Grassle and Maciolek 1992).

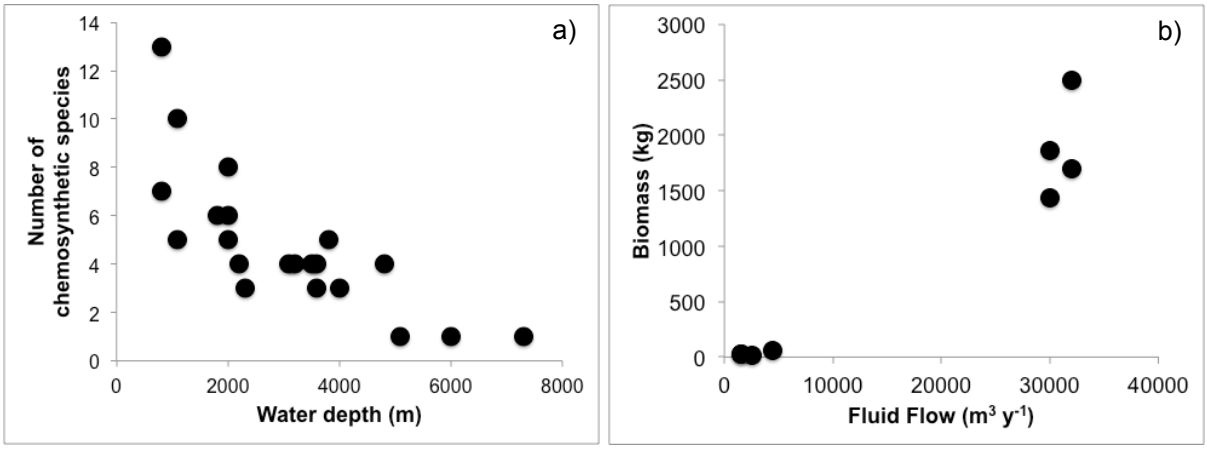

Figure 7 Global trend of decreasing species richness with water depth (a), and increasing biomass (wet weight) with enhanced fluid flow rates (b) at cold seep sites. Symbols depict individual cold seep sites. (data derived and adapted after Sibuet and Olu, 2002).

On larger spatial scales (between seeps) species richness of chemosynthetic organisms is negatively related to ocean water depth, and shallower seeps have more chemosynthetic species than deeper seep sites (Sibuet and Olu 1998; Sibuet and Olu-Le Roy 2002; Figure 7a). The global depth-stratification of the distribution of chemosynthetic species is assumed to be controlled by factors such as the evolutionary history of ocean basins, dispersal with

water-masses and predation pressure. Despite the crucial importance of microbial communities to the functioning of cold seep ecosystems, their diversity patterns on either local or global scales remain largely unknown. Limited knowledge on the microbial distribution patterns exists only for some of the key functional groups, however the factors that shape their diversity are largely unknown as studies including both microbiological and geochemical analyses are rare. To contribute to the understanding of seep diversity, within this PhD thesis we investigated microbial communities and the related environmental factors of different cold seep ecosystems (Chapter I and III).

1.5 Wood fall ecosystems as biogeochemical and biodiversity hotspots

Large organic falls comprising whale carcasses, sunken wood and other plant material, provide locally and temporally restricted large inputs of organic matter to the otherwise oligotrophic deep-sea (Figure 6 and 8c,e,f). Large amounts of organic matter delivered via these falls attract a multitude of organisms and promote the establishment of highly productive and diverse ecosystems in the deep-sea (Smith et al., 1989; Smith and Baco, 2003; Gaudron et al., 2010). Therefore, similar to seeps and vents, organic fall ecosystems are regarded as **biodiversity hotspots** in the deep sea as they harbor special biodiversity within a larger area of normal biodiversity (Jørgensen and Boetius, 2007), including highly endemic species specialized on wood or whale remains as their primary food source. Moreover, with 407 whale-associated faunal species, whale skeletons harbor higher diversity than any other deep-sea hard substrate, and the richness level approaches that of deep-sea soft sediments (Baco and Smith, 2003). To date, comprehensive studies focusing on wood fall associated species richness are still missing. The first observations that whale and wood falls support widespread and opportunistic fauna were made over 100 years ago (Turner, 1973, 2002; Smith and Baco, 2003 and references therein). However, it took until 1989 and the discovery of chemosynthetic assemblages associated with falls (Smith et al., 1989), before the importance of these ecosystems was fully appreciated. Since then substantial advances in understanding whale fall ecology have been made (Smith and Baco, 2003). Wood and whale ecosystems share phylogenetically related and even overlapping chemosynthetic species with cold seeps and hydrothermal vents, such as mussels (*Idas sp., Adipicola sp.*), clams (*Vesicomya sp., Calyptogena sp., Lucinoma sp.*) siboglinid tubeworms, *Beggiatoa* bacterial mats and limpet snails of the genus *Mitrella* (Smith et al., 1989; Deming et al., 1997; Baco

and Smith, 2003; Smith and Baco, 2003; Pailleret et al., 2007; Southward, 2008; Gaudron et al., 2010; Bienhold et al., 2011; Lorion et al., 2012) (Figure 6).

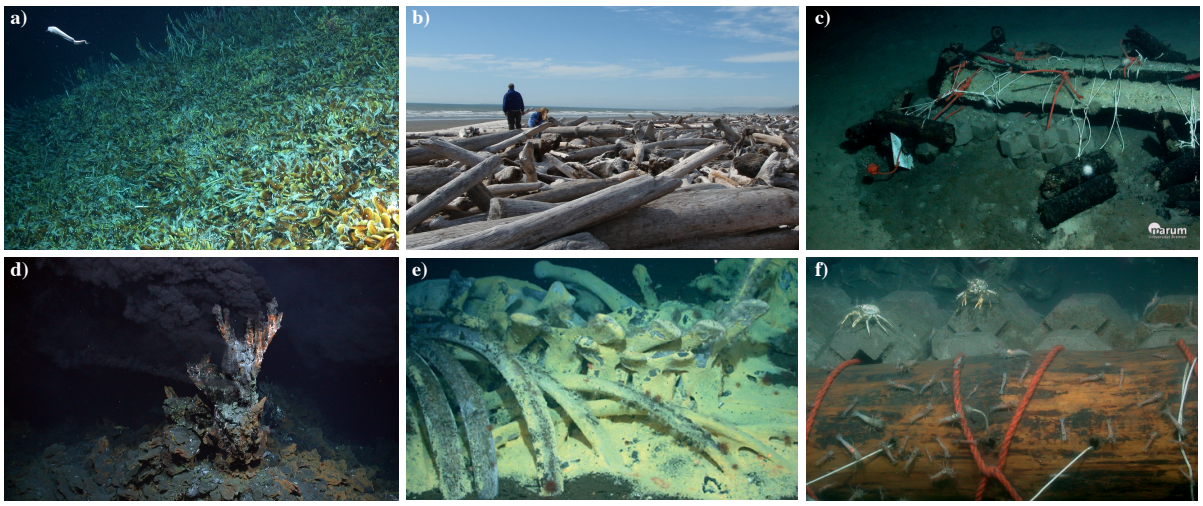

Figure 8 Hotspot ecosystems at the deep-sea floor: cold seep with extensive mussel bed (a); wood experiments populated by numerous shrimps, crabs and echinoderms (c, f); black smoker at a hydrothermal vent; and, a whale fall covered with bacterial mats. Numerous wood logs at a coastal area in Canada (b), illustrating that large amounts of wood can potentially be transported in the oceans (Photo Courtesy of D. deBeer). Image a, c, d, f source Marum, University of Bremen.

This has led to the hypothesis that large food falls may act as stepping stones in the evolution and distribution of chemoautotrophic communities between cold seeps and hydrothermal vents (Smith et al., 1989; Distel et al., 1991). Recently, biogeochemical studies have confirmed that enhanced rates of degradation of organic matter may locally deplete oxygen on the falls as well as in the surrounding sediments, promoting the production of sulfide and the establishment of reduced conditions that attract chemosynthetic organisms (Laurent et al. 2009; Treude et al. 2009; Bienhold et al. 2011). The presence of steep biogeochemical gradients over micrometer to centimeter scales and substantially elevated respiration rates classify organic falls as **biogeochemical hotspots** in the deep sea. Considerable effort has been put into the investigation of spatial and temporal dynamics of whale falls and other chemosynthetic ecosystems (Sibuet and Olu, 1998; Smith et al., 2002; Smith and Baco, 2003; Ramirez-Llodra et al., 2007), however the ecology of sunken wood based ecosystems and their impact on the deep-sea is largely unstudied (Gaudron et al., 2010).

Wood falls are composed of branches and trunks of trees, commonly referred to as coarse woody debris (CWD), that are transported from the terrestrial realm to the ocean by rivers and storm events (Figure 8b). Global estimates of the transport of CWD to the ocean are

difficult, however, two recent studies estimated an annual riverine flux off Japan of 7.31 Gg C y^{-1} (Seo et al., 2008), and a three orders of magnitude higher input of carbon (1.8 – 4 Tg C) due to a single storm event in Taiwan (West et al., 2011). These estimates indicate the importance of CWD as a source, which supplies large quantities of organic carbon to locally restricted areas in the otherwise food-impoverished deep sea. Waterlogged woods have been found in all oceans at all depths, with the highest density near the coastline and at the outlets of large rivers (Wolff 1987; Bernardino et al. 2010 and references therein). Fossil records reveal that wood falls are ancient ecosystems, dating back to the late Cretaceous (Kiel and Goedert, 2006; Kiel et al., 2009). Furthermore, the authors of this study present evidence of fauna associated to the ancient wood falls, which highly resembles communities of modern chemosynthetic ecosystems, including wood and whale falls (Kiel et al., 2009). A typical wood-associated faunal community of a present day wood fall includes animals ranging from wood specialists, opportunists, predators, scavengers and deposit feeders to chemosynthetic organisms (Gaudron et al., 2010). Wood-boring bivalves, mainly from the deep-sea subfamily of *Xylophagaine*, are considered to be keystone species of sunken wood ecosystems, due to their capability to utilize the wood as a food source under deep-sea conditions, and convert the woody material to nutritional sources available for others (Turner, 1973). Moreover, the feeding activity of *Xylophaga sp.* produces wood chips and fecal matter that spread on the surrounding seafloor, enhancing the production of sulfide and development of reduced anoxic zones in the sediments that may attract chemosynthetic organisms (Bienhold et al., 2011). *Xylophaga sp*. are among the few organisms capable of degrading cellulose – the major constituent of wood along with lignin. This ability is ascribed to their endosymbionts contained in their gills, which most probably produce cellulases (Distel and Roberts, 1997) (Distel et al., 1997), as observed in endosymbionts of the shallow water woodborers (*Terredinidae*; Waterbury et al. 1983; Distel et al. 1991; Yang et al. 2009).

Free living and symbiotic bacteria, as well as fungi are the only organisms known to possess the ability to degrade cellulose. Moreover, only a small percentage of bacteria have evolved the metabolic adaptations to utilize hard-to-degrade cellulose, despite the fact that cellulose is the second most abundant hydrocarbon in marine environments (Wilson, 2011). Aerobic degradation of cellulose can be achieved by a single species, however a complex and physiologically diverse microbial community is required for the degradation of cellulose under anaerobic conditions (Leschine, 1995). Only recently, a study for the first time revealed that sunken woods harbor a core bacterial community, distinct from communities in

reference sediments in the same area, and identified members that might play an important role in the degradation of cellulose in the deep sea (Bienhold et al., 2011). Studies focusing on sunken wood ecosystems and specifically on the wood-associated microbial communities are still rare, hence very little is known about the ecology of these ecosystems or the factors shaping their associated communities. Considering the relatively short-term provision of energy by wood falls as well as their patchy worldwide distribution, it is of crucial importance to understand the temporal and spatial dynamics of sunken wood ecosystems in order to elucidate their ecology and role as **biological and biogeochemical hotspots** in the deep sea. These two aspects represent the main aims of the study presented in Chapter IV.

1.6 Objectives of the PhD thesis

As outlined in the previous section, cold seeps and wood falls represent isolated habitats with highly fragmented distribution at the deep-sea floor, where seepage of methane and the degradation of wood promote high local heterogeneity and reduced environmental conditions with steep and variable biogeochemical gradients. The main aim of this PhD study was to develop better understanding of the overall bacterial diversity associated to these ecosystems, test how the unique set of characteristics of these habitats affect the structure of bacteria communities and investigate if general patterns of the distribution of microbial communities of fragmented ecosystems can be revealed. To reach these aims four cold seeps sites i.e. the REGAB pockmark (West African margin), the Amsterdam Mud Volcano, the Amon Mud Volcano and the Pockmark area in the Eastern Mediterranean sea were investigated (Figure 1). Moreover, deployment of wood experiments in the vicinity of cold seep sites in the Eastern Mediterranean and Norwegian seas (Figure 1) were used to investigate bacterial communities associated to wood falls. These objectives were addressed by using a multidisciplinary approach comprising molecular fingerprinting, *in situ* and *ex situ* biogeochemical methods, and by applying multivariate statistics.
The major questions addressed in this thesis were:

1) Do cold seeps and wood falls harbor distinct bacterial diversity and how specific bacterial communities of methane-seeping and cellulose-rich habitats compare to their immediate oxidized vicinity? (Chapter III and IV).
2) Does high heterogeneity, caused by variability in sediment geochemistry and habitat partitioning by large megafauna, influence the bacterial community structure at cold seeps? (Chapter I).
3) Does energy availability at cold seep sites shape the structure and abundances of free living and symbiotic bacteria? (Chapter I – III).
4) Do bacterial and faunal communities of seep and wood falls vary with spatial scales? (Chapter III and IV).
5) Is there temporal succession of wood-associated bacterial and faunal communities? (Chapter IV).

1.7 Publication outline

This thesis includes four manuscripts, prepared for publication in international scientific journals (Chapter I – IV). Additional contributions to publications provided during the thesis works include two further manuscripts of which the abstracts are added to the appendix. One manuscript has already been published (Chapter II), one is submitted (Chapter I), and two are prepared as draft versions close to submission Chapter (III - IV).

Chapter I: Bacterial diversity and biogeochemistry of different chemosynthetic habitats of the REGAB cold seep (West African margin, 3160 m water depth)

Petra Pop Ristova[1,2]*, Frank Wenzhöfer*[1,2]*, Alban Ramette*[1]*, Matthias Zabel*[2]*, David Fischer*[2]*, Sabine Kasten*[3] *and Antje Boetius*[3]

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(22.06.2012 – Manuscript is in review with *Biogeosciences* journal)

Boetius A. initiated the study and planned the field sampling design. Pop Ristova P. and Wenzhöfer F. conducted the *in situ* geochemical measurements, and Pop Ristova P., Boetius A., Wenzöfer F., Fischer D. the sediment sampling. Porewater analyses were done by Fischer D. Geochemical modeling was done by Zabel M. Pop Ristova P. performed all molecular analyses. Statistical analyses were done by Pop Ristova P. with input from Ramette A. The manuscript was written by Pop Ristova P. with support and input from Boetius A. and all coauthors.

Chapter II: Relative abundances of methane- and sulphur-oxidising symbionts in the gills of a cold seep mussel and link to their potential energy sources

Sebastien Duperron[1,2], *Hayat Guezi*[1,2], *Sylvie M. Gaudron*[1,2], *Petra Pop Ristova*[3], *Frank Wenzhöfer*[3,4] *and Antje Boetius*[3,4]

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(The *Geobiology* journal (2011) 10.1111/j.1472-4669.2011.00300.x)

Duperron S. performed the molecular, stable-isotope, image and statistical analyses. *In situ* biological and geochemical observations and measurements were conducted by Pop Ristova P., Wenzhöfer F. and Boetius A. Duperron S. wrote the manuscript with support and input from Pop Ristova P. and all coauthors.

Chapter III: Spatial scaling of bacterial communities associated with cold seep habitats of the Eastern Mediterranean deep sea

Petra Pop Ristova[1,2], *Frank Wenzhöfer*[1,2], *Alban Ramette*[1,2], *Janine Felden*[1,2] *and Antje Boetius*[1,2]

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[2] MARUM - Centre for Marine Environmental Science, University of Bremen, Germany

(22.06.2012 – in preparation for *PloS One* journal)

Boetius A. and Wenzhöfer F. initiated the study and planned the field sampling design. *In situ* measurements were conducted by Pop Ristova P. and F. Wenzhöfer, and Pop Ristova P., Boetius A., Wenzöfer F., Felden J. performed the sediment sampling. Radiotracer incubations were done by Felden J., with help from Pop Ristova P. Molecular analyses were conducted by Pop Ristova P. Corresponding statistical analyses of data were done by Pop Ristova P., with input from Ramette A. Pop Ristova P. wrote the manuscript with support and input from Boetius A. and all coauthors.

Chapter IV: Temporal and spatial variations of bacterial and faunal communities associated with deep-sea wood falls

Petra Pop Ristova[1]*, Christina Bienhold*[1]*, Frank Wenzhöfer*[1] *and Antje Boetius*[1]

[1] HGF-MPG Group for Deep Sea Ecology and Technology, Alfred Wegener Institute for Polar and Marine Research, and Max Planck Institute for Marine Microbiology, Germany

(22.06.2012 – in preparation for *PloS Biology* journal)

Boetius A. initiated the study, and A. Boetius and F. Wenzhöfer devised the experimental design. *In situ* measurements were performed by Pop Ristova P. and F. Wenzhöfer. Sampling and observation on board were done by Bienhold C., Pop Ristova P. and Boetius A. Molecular analyses were conducted by Pop Ristova P. Corresponding statistical analyses of data were done by Pop Ristova P., with input from Bienhold C. The manuscript was written by Pop Ristova P. with input from Boetius A. and all coauthors.

2 Thesis chapters

Chapter I

Bacterial diversity and biogeochemistry of different chemosynthetic habitats of the REGAB cold seep

(West African margin, 3160 m water depth)

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Abstract

The giant pockmark REGAB (West African margin, 3160 m water depth) is an active methane-emitting cold seep ecosystem, where the energy derived from microbially mediated oxidation of methane supports high biomass and diversity of chemosynthetic communities. Bare sediments interspersed with heterogeneous chemosynthetic assemblages of mytilid mussels, vesicomyid clams and siboglinid tubeworms form a complex seep ecosystem. To better understand if benthic bacterial communities reflect the patchy distribution of chemosynthetic fauna, all major chemosynthetic habitats at REGAB were investigated using an interdisciplinary approach combining porewater geochemistry, *in situ* quantification of fluxes and consumption of methane, as well bacterial community fingerprinting. This study revealed that sediments populated by different fauna assemblages show distinct biogeochemical activities and are associated with distinct sediment bacterial communities. The methane consumption and methane effluxes ranged over one to two orders of magnitude across habitats, and reached highest values at the mussel habitat, which hosted a different bacterial community compared to the other habitats. Clam assemblages had a profound impact on the sediment geochemistry, but less so on the bacterial community structure. Moreover, all clam assemblages at REGAB were restricted to sediments characterized by complete methane consumption in the seafloor, and intermediate biogeochemical activity. Overall, variations in the sediment geochemistry were reflected in the distribution of both fauna and microbial communities; and were mostly determined by methane flux.

1 Introduction

Cold seeps belong to the most productive ocean ecosystems, and are hence known as oases of life in the deep sea. They are fuelled by energy provided via microbial transformation of methane and higher hydrocarbons, which supports large biomasses of highly specialized chemosynthetic communities (Sibuet and Olu, 1998; Jørgensen and Boetius, 2007). Cold seep ecosystems are found on passive and active continental margins around the world's oceans, but represent fragmented, isolated habitats of locally small areal coverage, similar to hydrothermal vents. Ever since the discovery of chemosynthetic communities at cold seep ecosystems (Paull et al., 1984; Kennicutt II et al., 1985; Suess et al., 1985) ecologists have been fascinated by the question of their interconnectivity and biogeography on global and regional spatial scales, just as for hydrothermal vent communities (Tunnicliffe, 1991; Sibuet and Olu, 1998; Tunnicliffe et al., 1998; Tyler et al., 2003; Vanreusel et al., 2009). The most conspicuous characteristics of cold seep ecosystems are seafloor emissions of hydrocarbon fluids and gases as primary, locally restricted energy source causing a specific adaptation of species to their habitat. Furthermore, cold seeps show a fragmented distribution along the global continental margins, leading to isolation of populations. Hence, studies dedicated to the geobiology of cold seeps have been seeking to answer the following overarching questions i) how diverse and specific are microbial and faunal communities at cold seeps compared to the background environment ii) to what extent are seep communities indicative of geological processes such as the strength of hydrocarbon seepage, and iii) which factors control the diversity and dispersal rates of seep – associated organisms on both regional and global scales (among and within cold seeps).

These questions were central to this study of the bacterial communities of the giant pockmark cold seep REGAB (Charlou et al., 2004). Studies investigating seep microorganism have predominantly been focusing on the taxonomical identification of the key- microbial players mediating the core-energy producing processes i.e. Anaerobic Oxidation of Methane (AOM) coupled to Sulphate Reduction (SR) and sulphide oxidation, their phylogenetic affiliation, and relation of their abundances and activities to the local geochemistry (Boetius et al., 2000; Knittel et al., 2003; Heijs et al., 2007; Cambon-Bonavita et al., 2009; Knittel and Boetius, 2010; Orcutt et al., 2010). However, high-resolution studies investigating the structure of microbial communities as a whole, over a range of habitats within a single cold seep (m to km scale) are still rare, hence the abiotic and biotic factors that may directly influence the bacterial diversity at cold seeps remain elusive.

Here we tested the hypotheses that the bacterial diversity at cold seeps changes along geochemical gradients with sediment depth, and moreover that the availabilities of methane and sulphide as main energy sources at cold seeps structure the bacterial communities. Secondly, similar to what has been shown for small-size fauna (Van Gaever et al., 2009), we tested if the type and specific activity of chemosynthetic megafauna can exert a selective pressure and therefore influence bacterial diversity patterns at cold seeps. Therefore we combined porewater geochemistry, quantification of fluxes and consumption rates of methane and bacterial community fingerprinting of the major habitat types at REGAB, populated by different chemosynthetic organisms (mytilid mussels, vesicomyid clams or thiotrophic bacterial mats). The main aims were to better understand i) if the bacterial community within a cold seep ecosystem varies according to habitat distribution on a "landscape" scale ii) if the bacterial diversity is shaped by availability of energy, and iii) if habitat partitioning by large symbiotic megafauna influences the structure of bacterial communities.

2 Material and methods

2.1 Description of sampling sites and sampling procedure

The REGAB pockmark, situated at 3160 m water depth on the Congo-Angola margin represents the second largest (800 m ø and 15 – 20 m deep) single cold seep site known to date in the Eastern Atlantic (Sibuet and Olu-Le Roy, 2002; Ondréas et al., 2005). REGAB has unusual sedimentological features resulting from the large amount of terrigenous input by the Congo river and the vicinity of one of the largest submarine canyons, the Congo deep-sea fan (Ondréas et al., 2005 and references therein; Pierre and Fouquet, 2007). The pockmark formation has been related to a sudden release of overpressurized gas followed by a collapse of a sediment dome (Ondréas et al., 2005). The current escape of gas is assumed to occur along a deep pipe (300 m) rooted in a buried paleo-channel (Gay et al., 2003; Ondréas et al., 2005). The seafloor of the REGAB pockmark is characterized by high biomasses of diverse chemosynthetic megafauna which occur clustered as non-overlapping aggregations of mytilid mussels or vesicomyid clams, or siboglinid tubeworms (Ondréas et al., 2005; Olu-Le Roy et al., 2007a).

Video surveys during this study in 2008 (METEOR 76/3b report http://www.dfg-ozean.de/de/berichte/fs_meteor/) revealed that the REGAB pockmark was dominated by the

same three major types of megafauna assemblages already discovered during previous investigation of this cold seep in 1998, 2000 and 2001 (Ondréas et al., 2005; Sibuet and Vangriesheim, 2009; Fig. 2). Extensive carbonate cements and outcropping gas hydrates mark the central part of REGAB, where also strong venting of gas and surface precipitates of gas hydrate were observed. This area was densely populated by mussels and tubeworms attached to the carbonates, and was surrounded by soft sediments littered with clam shells, and patchily distributed beds of living clams. In comparison to the areal coverage of fauna estimated in 2000 (Olu-Le Roy et al., 2007a), the number and sizes of mussel patches associated with soft sediments seemed to have declined with time, as deduced from the Remotely Operated Vehicle (ROV)-video observations performed in 2008. However, the spatial distribution of clam species forming heterogeneous patches (Fig. 2) has remained more or less the same, indicating that relatively stable biogeochemical conditions prevail over time at REGAB.

Sediment sampling, *in situ* geochemical measurement and ROV QUEST (MARUM, Bremen, Germany) video observations at REGAB were performed during the M76/3b cruise in 2008, aboard the R/V Meteor. The eleven investigated sites cluster in three geographical regions of the pockmark (north, south and south-west) and included: 1 bare sediment site where venting of gas bubbles was observed (Gas), 1 bacterial mat (Bacter_N), 3 clam (Clam_N, Clam_S, Clam_SW) and 1 mussel patch (Mussel_S), as well as 6 associated sites devoid of symbiotic megafauna characterized by bare sediment (Bacter_N_Env, Clam_N_Env, Clam_S_Env, Mussel_S_Env, Clam_SW_Env). Push coring and biogeochemical measurements were not possible in the central carbonate-hydrate site, due to the hard substrate at this location. The distances between the megafaunal patches and the adjacent bare sediment were in the range of 3 - 28 m. Approximate distances between the main sampling locations were: 100 – 150 m between N REGAB – S REGAB, 400 – 450 m between S REGAB and SW REGAB and 350 – 400 m between N REGAB and SW REGAB (Fig. 1).

In the northern part of REGAB (REGAB_N; Fig. 2) two main types of habitats, white thiotrophic bacterial mats (not found in the other parts of REGAB) and clam patches were sampled. The two investigated sites (Bacter_N and Clam_N) were found < 36 m apart. The Clam_N patch was the largest of all investigated patches and was dominated by one clam species - *Calyptogena regab* (von Cosel and Olu, 2009). The seafloor at REGAB_N was littered with empty and broken shells of clams. Intermingled among the clam shells, high number of holothurians could be observed. Additional sampling of the bare sediments

surrounding the clam patch (Clam_N_Env) and the bacterial mat (Bacter_N_Env) was also performed.

Two distinct types of megafauna assemblages dominated by either mussels or clams were detected in the Southern part of REGAB (REGAB_S; Fig. 2). The different megafauna patches were found close to each other (20 – 30 m), however in no occasion they overlapped. Uniquely, at this site (Mussel_S) the mussels were surrounded by soft sediment, thus sampling was possible. *Bathymodiolus* sp. aff. *boomerang* (Olu-Le Roy et al., 2007a) was the mussel patch-forming species. Within the mussel patch individuals of the siboglinid polychaetes – *Escarpia southwardae* (Andersen et al., 2004) were visible, as well as numerous shrimps and occasionally galatheid crabs. The investigated clam patch (Clam_S) consisted of *Calyptogena regab* (von Cosel and Olu, 2009) species buried in dark sediment. No shrimps were observed inhabiting the clams' assemblages. The larger area of the surrounding sediment at the mussel patch was overlain with shell and tubeworm debris compared to the sediment in the vicinity of the clam patch. Two additional sites, located in the vicinity of the respective megafauna assemblages (Clam_S_Env and Mussel_S_Env) were investigated during this study.

Clams were the only chemosynthetic organisms forming assemblages in the Soutwestern part of REGAB (REGAB_SW; Fig. 2). Distinctly, the sampled patch (Clam_SW) at this part of REGAB was dominated by two species of vesicomyid clams – *Calyptogena regab* (von Cosel and Olu, 2009) and *Laubiericoncha chuni* (Thiele and Jaeckel, 1931; von Cosel and Olu, 2009) immersed in very dark sediment. No other vagrant megafauna were noticed to dwell in the Clam_SW clam site upon sampling. The sediment adjacent to the Clam_SW_Env was from one side covered by shell debris, and from the other more or less barren.

Sampling locations and sample labels are summarized in Table 1 and Supplement Table 1, and all related data are available in the PANGAEA database.

Push core (Ø 8 cm, sediment height 10 – 20 cm) targeted sampling of the individual habitats was performed using the ROV QUEST camera system and the manipulator arm. Immediately after recovery, the push cores were transferred to a tempered room kept at *in situ* temperature (4 °C) and subsampled for different type of analyses.

2.2 Biogeochemical measurements

2.2.1 Porewater chemistry

Porewater was extracted in one centimetre resolution, from the top 0-20 cm sediment depth, using Rhizon moisture samplers (Seeberg-Elverfeldt et al., 2005; pore size 0.1 µm) inserted into holes of predrilled push core liners at all investigated sites (Table 1). The porewater was immediately subsampled for different types of analysis (total H_2S, SO_4^{2-}, NH_4^+, Fe^{2+}, Mn^{2+}, PO_4^{3-}, Cl^-, and Alkalinity). Description of the measurement procedure for Fe^{2+}, Mn^{2+}, PO_4^{3-} as well as pH can be found in the supplementary material. Alkalinity was calculated from a volumetric analysis by titration of 1 ml of the porewater samples with 0.01 or 0.05 M HCl, performed on board. Concentration of ammonium was determined via the conductivity method on board (modified after Hall and Aller, 1992). Porewater subsamples (1.5 ml) were immediately fixed with 0.6 ml zinc acetate, and the total sulphide ($\Sigma H_2S = H_2S+HS^-+S^{2-}$) concentration was determined photometrically (Cline, 1969) in the home laboratory. Sulphate and chloride subsamples were diluted 1:100 and stored frozen at -20 °C until further processing and determination of the concentration by ion chromatography (Metrohm IC Advanced Compact 861) at a flow rate of 0.7 ml min^{-1}.

To estimate the proportions of bioirrigation and advection on the transport of solutes, the transport-reaction model Explicite was applied (Zabel and Schulz, 2001; Kuester-Heins et al., 2010). For this purpose we used the porewater concentration profiles of sulphate and sulphide. Values for sulphate reduction were adjusted to the integrated rates determined by the radiotracer injection method.

2.2.2 Methane and sulphate consumption rates (AOM and SR)

Rates of AOM and SR were determined *ex situ* at all investigated habitats (Table 1). Immediately after recovery, the sediment push cores were subsampled on board in 3 replicates for each of the methods. Following the whole core injection method (Jørgensen, 1978), subcores (Ø 28 mm) were injected with either $^{14}CH_4$ or $^{35}SO_4$ radiotracers at 1 cm intervals, according to the procedure described in Felden et al. (2010). Methane and sulphate concentrations were measured by gas chromatography (5890A, Hewlett Packard) and anion exchange chromatography (Waters IC-Pak anion exchange column, waters 430 conductivity detector) in the home laboratory, respectively. Turnover rates of methane and sulphate were

determined in the home laboratory by scintillation counting according to Treude et al. (2003) and Kallmeyer et al. (2004), respectively.

2.2.3 Benthic chamber measurements (TOU and CH_4 efflux)

The Total Oxygen Uptake (TOU) and methane efflux through the sediment-water interface were determined *in situ* for most of the investigated habitats (Table 1) using a ROV – operated benthic chamber module (CHAM). Technical description of the benthic chamber module and details on the CH_4 measurement procedure can be found in Felden et al. (2010) and (Duperron et al., 2011). Briefly, 284 cm^2 of sediment with 10 – 15 cm overlying water was incubated *in situ* and changes monitored over time by pre-programmed syringe sampling and optode measurements. Oxygen concentration in the enclosed bottom water was continuously measured with a help of an oxygen optode, and the TOU flux (mmol m^2 d^{-1}) was calculated from the initial linear decrease in O_2 concentration versus time (Wenzhöfer and Glud, 2002; Felden et al., 2010). Methane concentrations were determined on water samples taken from the incubated water column, on board using a gas chromatograph (Agilent 6890N) as described previously (Niemann et al., 2009 and references therein). The methane efflux (mmol m^2 d^{-1}) was calculated as the change of methane concentration in the enclosed bottom water over time (Felden et al., 2010).

2.2.4 Oxygen microsensor measurements

A modified version of a deep – sea microprofiler (MICP) was used to carry out high-resolution microsensor measurements (200 µm) for *in situ* determination of oxygen concentrations (Wenzhöfer et al., 2000; Lichtschlag et al., 2010a). Precise positioning and operation of the MICP was achieved with the ROV. Due to the fragile nature of microelectrodes, measurements of the oxygen inventory were restricted only to sites devoid of hard substrates and shell debris, such as the bacterial mat and the bare sediments surrounding the clam patches (Table 1). For each deployment the MICP carried 3 Clark-type O_2 miroelectrodes with guard cathode and internal reference (Revsbech, 1989). The microsensors were calibrated by applying a two-point calibration (estimated from the O_2 concentration in the bottom water and in the anoxic part of the sediment). Bottom water was sampled by means of ROV – water sampler (KIPS) (Garbe-Schönberg et al., 2006) and the oxygen concentration was determined with the Winkler titration method (Grasshoff et al., 1999). Diffusive Oxygen Uptake (DOU) was calculated from the linear concentration gradient in the DBL (Diffusive Boundary Layer) by applying Fick's first law of diffusion, as

described in (Jørgensen and Des Marais, 1990). The diffusion coefficient of O_2 in seawater was corrected for salinity and temperature (Li and Gregory, 1974).

2.3 Bacterial community characterization

2.3.1 Bacterial cell numbers

The total number of single cells was determined at every station by applying the Acridine Orange Direct Count (AODC) method. Push cores were subsampled vertically with smaller cores (Ø 28 mm), which in turn were sliced into 2 cm sections. Samples were fixed in 4 % formaldehyde/seawater and stored at 4 °C. The AO-staining was done in the home laboratory as previously described (Meyer-Reil, 1983; Boetius and Lochte, 1996). For each sample, a minimum of two replicate filters and 30 grids per filter were randomly counted.

2.3.2 DNA samples

On board, sediment cores for DNA analysis were sliced in 2 cm intervals, down to 18 cm depth and stored at – 20 °C for further analysis in the home laboratory. DNA was extracted from one gram of sediment with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacture's recommendations for maximum yields. DNA was finally eluted in 100 µl 1 x TE buffer (Promega, Madison, WI, USA). DNA quality was confirmed with a gel electrophoresis (1% agarose gel). Extracted DNA was quantified with a microplate spectrometer (Infinite® 200 PRO NanoQuant, TECAN Ltd, Switzerland).

2.3.3 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The bacterial community structure at REGAB was determined by Automated Ribosomal Intergenic Analysis (ARISA; Fisher and Triplett, 1999). Standardized amounts of DNA (10 µl) from each sample were amplified in triplicates using the forward FAM-labelled ITSF and reverse ITSReub primers (Cardinale et al., 2004). The PCR procedure, purification of PCR-products, capillary electrophoresis reaction, as well as data transformation were carried out as described previously (Ramette, 2009). The ARISA peaks were binned, using a bin size of 2 bp, to account for slight peak shifts between runs and for peak size calling imprecision (Interactive Binner function, http://www.ecology-research.com; Ramette, 2009)

2.3.4 Statistical analyses

All statistical analyses were performed with the open-source software R (The R Project for Statistical Computing v.2.9.2 [http://www.r-project.org/]) using the ”vegan” package (Oksanen et al., 2011). All statistical analyses were restricted to the 0 – 10 cm sediment depth. Correlation between differences in geochemical fluxes and β-diversity (calculated as Bray-Curtis and Jaccard distances) was tested applying the Mantel correlation test based on spearman ranking. Mantel p- values were corrected for multiple testing using the Bonferroni correction (Ramette, 2007). A reduced dataset, including the following sites: Clam_N, Clam_S, Clam_S_Env, Mussel_S, Mussel_S_Env, Clam_SW, Clam_SW_Env, and pooled samples within individual habitats (0 – 10 cm merged ARISA peaks) were used for this analysis. All other statistical analyses were performed on individual non-pooled ARISA samples derived from individual sediment depth samples. The bacterial community structure at REGAB was visualized by applying Non-metric MultiDimensional Scaling (NMDS) analysis. Separations of groups identified on the NMDS plot were tested for significance using the Analysis of Similarity (ANOSIM) test.

Forward selection procedure was used to choose the simplest model that can explain most of the variation in the community. The effect of the geochemistry (porewater concentrations of CH_4, total H_2S, SO_4^{2-}, pH, Mn^{2+}, Fe^{2+}, Cl^-, PO_4^{3-} and alkalinity), space (calculated as geographic distances in m), sediment depth and clam presence on the bacterial community structure was assessed by canonical variation partitioning analysis, on a priori Hellinger-transformed response dataset (Legendre and Legendre, 1998; Ramette and Tiedje, 2007). Single and combined effects of factors were tested for significance by performing 999 Monte Carlo permutations. Clam presence/absence was dummy-coded assuming that clams directly influence only the topmost 6 cm of sediment, based on their average shell length and distance reached by their foot.

Distance based test for homogeneity of multivariate dispersions (Anderson et al., 2006) was applied to test for the influence of the clams on the dispersion of bacterial β-diversity on smaller scales. The difference among dispersion groups was tested for significance using the Kruskal Wallis and Mann Whitney U test for unmatched samples. The later test was also used to assess if differences in shared OTUs among sites are statistically significant.

Geochemical data were standardized and normalized using the log-transformation prior to the analyses. Pairwise distances among samples were calculated using the Bray-Curtis

dissimilarity index (Bray and Curtis, 1957) for the ARISA diversity data and Euclidean distances for geochemical data (for both, flux and concentration data) prior to the analyses mentioned above.

3 Results

3.1 Biogeochemistry of different habitats at REGAB

3.1.1 Total sulphide concentration and fluxes

Porewater concentrations of sulphide varied substantially between different habitats of REGAB, both in terms of ranges as well depth profiles (Fig. 3). The sulphide concentration increased with increasing sediment depth at most of the sampling sites. At the bacterial mat and the mussel habitat 0.1 – 2 mM of sulphide was detected already in the surface layers. At all investigated clam habitats, as well as the gas bubble site, sulphide was only detected below 3-9 cm sediment depth (Fig. 3). The maximum sulphide concentrations at the Muss_S and Bacter_N sites reached 9 and 11 mM, which was almost two times higher (3 – 6 mM) compared to the maximum concentrations measured at any of the clam-populated sites. Comparison of the sulphide fluxes revealed similar patterns, with highest values measured at the Muss_S site, followed by slightly lower values at the Bacter_N site (Table 2). The lowest sulphide fluxes (5 – 9 mmol m^2 d^{-1}) were associated with the clam patches (Clam_N, Clam_S, Clam_SW) (Table 2). The bare sediment sites surrounding the mussel, Clam_N and bacterial patches exhibited even higher ranges of sulphide fluxes, but at the Clam_SW_Env and Clam_S_Env sites no sulphide flux could be detected outside the clam bed (Fig. 3, Table 2).

3.1.2 Sulphate concentration and alkalinity

Sulphate concentrations decreased with depth at most of the habitats at REGAB due to the anaerobic oxidation of methane, matching well the increasing concentration of sulphide at the respective sites, as well as the increase in alkalinity (Fig. 3). At the Bacter_N and Mussel_S sites lowest concentrations of only 2 – 3 mM were measured at 15-16 cm sediment depth. In contrast, at all clam populated sites sulphate penetrated deep into the sediment and substantial amounts (19 - 24 mM) were measured up to 17 cm sediment depth. Sulphate was not completely depleted at any of the investigated sites at REGAB (Fig. 3). Most of the sulphate profiles at the bare sediment sites were similar to the ones measured within the megafauna patches. Exceptions were the Bacter_N_Env and Clam_N_Env sites, where minimum sulphate concentrations of 17 and 10 mM were measured. The sulphate depth profile at the Gas site was similar to the clam patches where sulphate concentrations decreased only after 5 cm sediment depth. pH was around 7.6 in the cores which did not show substantial degassing (see Supplementary Fig. 1, also for other porewater constituents not further discussed here).

3.1.3 Ammonium concentrations

Ammonium was measured only at few of the investigated sites, due to restricted availability of porewater volumes (Fig. 3). At both locations (REGAB_S and REGAB_SW), NH_4 concentrations were higher inside the clam patches (Clam_S and Clam_SW) relative to the adjacent bare sediment (Clam_S_Env and Clam_SW_Env). Moreover, within the clam patches ammonium was peaking in shallower depths (3 – 5 cm), while outside the patches ammonium steadily increased with depth. The ammonia content and depth distribution pattern at the Mussel_S_Env was similar to that of the bare sediment sites in the vicinity of the clam patches.

3.1.4 Sulphate consumption rates (SR)

The average integrated (0 – 10 cm) sulphate reduction rates measured at different habitats at REGAB confirmed the trend observed for the sulphide fluxes. The lowest rate of < 1 mmol m^{-2} d^{-1} was observed at Clam_N, and the highest rate of 36 mmol m^{-2} d^{-1} at the Muss_S_Env (Table 2). Of the habitats populated by different chemosynthetic organisms, the Muss_S site exhibited highest average integrated SR rates of 28 mmol m^{-2} d^{-1}, followed by slightly lower rates at the Bacter_N (23 mmol m^{-2} d^{-1}) and lowest integrated SR rates at the clam patches (< 1 – 10 mmol m^{-2} d^{-1}). SR rate depth patterns differed substantially among habitats, ranging from surface peaks (Clam_S and Clam_SW) to deep sediment maxima (Bacter_N and

Mussel_S_Env) (Fig. 3). The bare-sediment sites had lower SR rates than the adjacent fauna-populated sites, except for the conspicuous Clam_N_Env where an average integrated SR rate of 4.5 mmol m^{-2} d^{-1} was detected. Sulphate reduction rates at the Bacter_N_Env were an order of magnitude lower compared to the Bacter_N site.

3.1.5 Methane consumption rates (AOM)

The average integrated (0 – 10 cm) rates of AOM matched well the corresponding SR rates, and for most of the habitats a 1:1 ratio between methane oxidized and sulphate consumed could be observed as predicted by the AOM stoichiometry (Boetius et al., 2000). As already observed when integrated SR rates and sulphide fluxes were compared among fauna-populated habitats, highest integrated AOM average rates were measured at the Muss_S (19 mmol m^{-2} d^{-1}), followed by intermediate rates at the Bacter_N (9 mmol m^{-2} d^{-1}) sites and the lowest rates at the clam patches (< 1 – 6 mmol m^{-2} d^{-1}) (Clam_N, Clam_S, Clam_SW) (Table 2). Highest peaks of AOM activity were measured in the surface layers (2.5 cm) at the Bacter_N site (208 nmol cm^{-3} d^{-1}), mid-sediment peaks (4.5 cm) at the Muss_S site (465 nmol cm^{-3} d^{-1}) and deep-sediment maxima (8.5 cm) at the Clam_SW site (67 nmol cm^{-3} d^{-1}) (Fig. 3). At the Clam_N site the AOM rates remained low (< 11 nmol cm^{-3} d^{-1}) throughout the whole investigated sediment depth. The integrated average AOM rates of fauna-populated sites were similar to their adjacent bare sediment sites, despite the difference in the depth-distribution of AOM rates.

3.1.6 *In situ* CH_4 efflux

CH_4 effluxes were hardly detectable with only < 1 – 3 mmol m^{-2} d^{-1} measured at the investigated clam habitats - both inside and outside the clam patches (Table 2). The opposite was true for the mussel-related site (Mussel_S_Env) where two orders of magnitude higher methane efflux was measured (334 mmol m^{-2} d^{-1}). Within the mussel patch CH_4 efflux varied substantially – from 1 to 81 mmol m^{-2} d^{-1} (the lower value being measured during the beginning of the incubation and the higher towards the end of the same 3h–benthic chamber incubation), most probably reflecting temporal variations in the seepage of methane during the period of incubation.

3.1.7 *In situ* TOU measurements

The REGAB megafauna contributed substantially to the benthic oxygen uptake measured at all fauna-populated sites (Table 2). Within the clam patches TOU was on average one order

of magnitude higher (50 – 590 mmol m^{-2} d^{-1}) relative to the bare sediment sites (12 – 18 mmol m^{-2} d^{-1}). The TOU measured at the bare sediment site (Muss_S_Env) adjacent to the mussel patch was approximately four times higher (70 mmol m^{-2} d^{-1}) than the other bare sediment sites, and was almost as high as the TOU detected at the Muss_S site (94 mmol m^{-2} d^{-1}).

3.1.8 *In situ* oxygen microsensor measurements

The Bacter_N site had the highest Diffusive Oxygen Uptake (DOU; 13 mmol m^{-2} d^{-1}) and shallowest Oxygen Penetration Depth (OPD; 2 mm) compared to other investigated sediment sites (Table 3). The diffusive fluxes of oxygen determined on bare sediments were not uniform, and fluxes changed drastically in dependence on the vicinity of the measurement site to a clam patch. In general measurements performed on bare sediment found more than 1 m away from a clam patch, showed deep OPD and low DOU (35 - 13 mm and 3 mmol m^{-2} d^{-1}, respectively), while similar sites located app. 20 cm from a clam patch exhibited shallower OPD (2 – 3 mm) and much higher DOU (6 – 11 mmol m^{-2} d^{-1}), most probably revealing hotspots of activity in the surrounding of the clam patch.

3.2 Characterization of bacterial communities at REGAB

3.2.1 Bacterial cell numbers

The number of single cells varied substantially among habitats at REGAB. The Muss_S site had up to two times higher (2.7 x 10^{10} cm^{-2} sediment) total integrated (0 – 10 cm) cell counts compared to the other fauna-populated sites (0.7 – 1.4 x 10^{10} cm^{-2} sediment) (Table 2). Unusually low cell numbers were detected below the bacterial mat (0.7 x 10^{10} cm^{-2} sediment). A sharp decrease in cell numbers with depth was detected at both mussel-related sites (Muss_S and Muss_S_Env), while at all other sites the cell numbers remained more or less constant over the entire investigated sediment depth (Fig. 3). No major differences could be observed among the populated sites and their respective adjacent bare sediment sites, except at REGAB_N area where the Clam_N site had a two times lower cell number integrated over depth (0.7 x 10^{10} cm^{-2} sediment) compared to the Clam_N_Env site. The Muss_S_Env was the site with highest cell numbers (3.5 x 10^{10} cm^{-2} sediment) at REGAB.

3.2.2 Bacterial community structure

The analysis of occurrence, abundance and distribution of bacterial types at the REGAB cold seep was based on defining Operational Taxonomic Units (OTU) representing relatively abundant bacterial populations as detected with the ARISA fingerprinting method (Brown and Fuhrman, 2005; Hewson and Fuhrman, 2006; Böer et al., 2009). Within individual habitats, all horizons sampled from the top 10 cm sediment depth shared on average only 30 % of the OTUs, with maximum similarity detected at the Gas site (40 %) and minimum at the Mussel_S (18 %). In general, no depth related pattern in the OTU richness was observed, except for the mussel sites, Clam_SW and Clam_S_Env where a decline in the percentage of shared OTUs with increasing depth was revealed. Overall 450 unique OTUs were detected across all 11 sites investigated here, with the maximum number of unique OTUs per single site (401) in the top 10 cm layer at Bacter_N, and the minimum number (252) at Mussel_S_Env. All OTUs occurred at least at 2 sites, and 24% occurred at all sites. The megafauna and bacterial mat populated sites shared on average 74 % of their OTUs with the adjacent bare sediments (Supplement Table 2). In general the adjacent sites had more OTUs in common compared to more distant sites (Supplement Table 2). The percentage of shared OTUs among samples at the clam-populated sites was not significantly different from the shared OTUs among samples at the non-clam-populated sites (Mann-Whitney U-test for unmatched samples $W = 4$, $p = 1$) (Supplement Table 3). Finally, no clam habitat-specific bacterial signature was revealed, as shown by the comparison of shared OTUs by all clam patches versus the OTUs present at all other investigated sites at REGAB (Supplement Fig. 2 and 3).

The bacterial community structure of samples from two adjacent sites i.e. samples from within a patch and samples from the respective adjacent bare sediment, grouped very close to each other, (Fig. 4), indicating high similarities between the patches and the corresponding adjacent bare sediments (here defined as one habitat). Different habitats had different bacterial community structures (Table 4, Fig. 4). The mussel habitat, characterized by highest fluxes and consumption rates of methane and sulphate (Table 2), had a very distinct bacterial community structure, which was significantly different from all other sites (Fig. 4, Table 4). The bacterial mat habitat, where intermediate levels of geochemical fluxes were detected (Table 2) had a rather similar bacterial community with the low-geochemical flux habitats, the Clam_N and Clam_SW habitats (Fig. 4, Table 4). Although, all clam habitats had similar low fluxes (Table 2), it was shown that the Clam_S habitat had a significantly different

bacterial community structure from the other two clam habitats (Clam_N and Clam_SW) (Table 4, Fig. 4).

Bacterial β -diversity (change in community structure and/or composition among sites calculated using the Bray-Curtis index; Whittaker, 1960) at REGAB was significantly positively correlated to differences in CH_4 effluxes among sites, as revealed by the Mantel correlation test (Table 5). Marginally significant positive relations (the relation was not significant when corrected for multiple comparisons, applying the Bonferroni correction) was also revealed between β-diversity and differences in integrated AOM and SR rates, as well as the alkalinity fluxes, but not to any other single porewater parameter (Table 5).

Variation partitioning analysis performed on the full data set, including all porewater concentrations and ARISA samples from all sediment depths, showed that environmental variables comprised under "sediment geochemistry" (21%, p = 0.001), "space" - geographic distances among sites (7%, p = 0.001), and "sediment depth" (0 to 10 cm, in 1 cm horizons) (2%, p = 0.012) explained most of the variation in the bacterial community structure of REGAB habitats (Fig. 5a). The combined effect of "sediment geochemistry" and "space" accounted for additional 5% of the observed variations (Fig. 5a). Additional variation partitioning analysis aiming to disentangle the individual effects of the geochemical parameters considered above, revealed that methane sediment concentrations (5%, p = 0.001) significantly explained the highest portion of the variability in the bacterial community at REGAB including all sediment depth layers (Fig. 5b). In contrast, sulphide concentrations alone did not significantly account for the variation in the bacterial community, but the confounding effect with the other geochemical parameters explained 6% of the observed diversity shifts (Fig. 5b). A small portion of the variation in the bacterial community structure at REGAB was related to shifts in alkalinity (1%, p = 0.038). Finally, the presence of clams had a very small direct overall effect, though not statistically significant, on the bacterial community structure at REGAB (1%, p = 0.106) (Fig. 5a). Accordingly, the comparison of the community dispersions among the clam-populated sites and the adjacent bare sediment sites revealed no significant differences (Supplement Table 4).

4 Discussion

The giant pockmark REGAB is among the best studied deep-water cold seeps in terms of biogeographical and geobiological processes shaping faunal communities (Sibuet and Olu-Le Roy, 2002; Sibuet and Vangriesheim, 2009; Cordes et al., 2010). REGAB is an endmember

of the Atlantic Equatorial Belt (AEB) - the longitudinal connection from Costa Rica to the continental margin off West Africa comprising many types of reduced, chemosynthetic ecosystems. Comparison of REGAB fauna to that of other cold seep sites from the AEB has shown that water depth, rather than geographic distance shapes the distribution of megafauna, not only within biogeographic provinces (Sahling et al., 2003; Cordes et al., 2007), but also on much larger spatial scales (Sibuet and Olu, 1998; Sibuet and Olu-Le Roy, 2002; Olu et al., 2010). Furthermore, several of the key chemosynthetic species show a broad distribution, such as two amphi-Atlantic *Bathymodiolus* species complexes, indicating high past and/or present dispersal capabilities of these organisms across the AEB within bathymetric zones (Olu-Le Roy et al., 2007b). Evolutionary history of ocean basins, dispersal with water-masses and predation pressure have been assumed to be the broad-scale factors controlling the depth-stratification of seep chemosynthetic communities on a global scale.

On the regional to local scale, previous investigations have matched the distribution of the benthic communities to specific bottom water conditions indicative of methane seepage, and the presence of chemosynthetic megafauna (Olu-Le Roy et al., 2007a; Cambon-Bonavita et al. 2009; Olu et al., 2009; Van Gaever et al., 2009; Menot et al., 2010). This study combines a detailed biogeochemical description of the chemosynthetic megafauna habitats with their bacterial biodiversity, and shows that different biological habitats at REGAB are linked to distinct biogeochemical regimes of the underlying sediment. The main aim was to evaluate the major factors shaping the structure of these communities and to gain a better understanding of the complexity and heterogeneity of cold seep ecosystems caused by the interplay of geochemistry, faunal and microbial distribution.

4.1 Methane flux and its subsurface microbial consumption shapes habitats at the REGAB giant pockmark

Previous studies of the composition of seep fauna communities on local and regional spatial scales revealed the role of energy availability – in the form of methane, sulphide and oxygen fluxes, along with the type of seafloor substrate (Olu-Le Roy et al., 2007a; Sahling et al., 2008; Olu et al., 2009; Van Gaever et al., 2009; Levin et al., 2010; Menot et al., 2010; Decker et al., 2011; Fischer et al., 2011; Ritt et al., 2011). Respective hypotheses have also been tested at the giant REGAB pockmark, which shows a high spatial heterogeneity and non-overlapping habitats dominated by different chemosynthetic megafauna (Olu-Le Roy et al., 2007a). Megafauna distribution and densities could be linked to local variations in energy

availability, in particular to methane concentrations in the bottom water, and most probably underlying gas emissions (Olu-Le Roy et al., 2007a). Also the diversity and density of non-symbiotic megafauna, macro- and meiofauna was found to be influenced by the habitat heterogeneity caused by variations in bottom water geochemistry as well as by the presence and activity of symbiotic megafauna (Olu et al., 2009; Van Gaever et al., 2009; Menot et al., 2010). Here we tested this hypothesis both for the distribution of chemosynthetic megafauna as well as for the associated bacterial community using high resolution *in situ* and *ex situ* measurements of methane fluxes from the seafloor, microbial methane consumption (AOM) and associated biogeochemical activities.

Similar to other cold seeps, the REGAB pockmark comprises highly reduced, patchy habitats where due to the local upward transport of hydrocarbons oxygen is completely consumed within the first millimetres of seafloor (Beer et al., 2006; Girnth et al., 2010; Lichtschlag et al., 2010a; Menot et al., 2010; Grünke et al., 2011). The strongest gas venting in the form of bubble streams and outcropping hydrates were observed at the central carbonate cements (Fig 2a). Previous investigations of deep water cold seeps have shown that free gas may escape from the seafloor within the gas hydrate stability zone, even at such high pressure and cold temperature as at REGAB (Suess et al., 1999; Bohrmann et al., 2003; Greinert et al., 2006; Klaucke et al., 2006 Sauter et al., 2006; Fischer et al., 2011). Methane concentrations in the bottom waters were highest at the Mussel_S (3.6 µM), around 0.4 µM at Clam_S to and decreased to 0.2 µM at the clam habitat (Clam_SW) furthest away from the central gas vents. These values fall into the low range of values detected previously (Duperron et al., 2005; Olu-Le Roy et al., 2007a).

For the first time we measured *in situ* methane fluxes from the sedimentary seafloor at REGAB using benthic chambers. Highest methane efflux and also highest methane consumption rates were found at the mussel habitat of REGAB (Mussel_S and Mussel_M_Env). This *Bathymodiolus* type hosts sulphur- and methane-oxidizing endosymbionts and hence depends mostly on methane (Duperron et al., 2011). The methane and sulphate consumption rates in the sediments, as well as sulphide fluxes and to certain extent TOU were 0.5 – 4 times higher compared to other habitats at REGAB. The high fluxes concomitantly supported up to two times higher numbers of bacterial cells at this site relative to the other habitats, and a dense colony of mussels interspersed with tubeworms (Fig. 2b). In a previous study at REGAB, Cambon-Bonavita et al. (2009) detected highest abundances of ANME/SRB aggregates - a microbial consortium shown to mediate AOM coupled to SR

processes (Boetius et al., 2000), in the sediments inhabited by mussels. Despite the high AOM rates, the mussel habitat was the only site where extensive seepage of gaseous and dissolved methane was observed, indicating substantial transport of methane to the bottom water. The AOM process removed only 6 – 20 % of the total upward diffusing methane. Accordingly, the highest bottom water methane concentrations were also detected previously above mussel patches at REGAB (Charlou et al., 2004).

Sediments covered by bacterial mats, a common feature of many cold seeps (Treude et al., 2003; Niemann et al., 2006; Lessard-Pilon et al., 2010; Fischer et al., 2011; Grünke et al., 2011), were rather scarce and restricted only to the northern part of REGAB. The sediment below the bacterial mat exhibited intermediate levels of AOM, SR rates and H_2S fluxes, approximately 2 times higher than in the clam patches, but lower relative to the mussel patch. At this habitat, SR rates differed by an order of magnitude and SR and AOM rate did not match well, probably reflecting substantial spatial and temporal heterogeneity in the methane transport, as previously observed in relation with bacterial mats and other reduced habitats associated with hydrates (Treude et al., 2003; Lichtschlag et al., 2010b). However, both sites sampled within the bacterial mat habitat were characterized by low single cell counts in comparison to the other habitats at REGAB. In corroboration with our results, Cambon-Bonavita et al. (2009) found that ANME/SRB aggregates were the least abundant in the sediments covered by bacterial mat at the REGAB pockmark, which is a striking contrast to other cold seep settings (Lösekann et al., 2007; Girnth et al., 2010).

The clam patches were the most widely distributed sedimentary chemosynthetic habitats at REGAB. The clam species *Calyptogena regab* and *Laubiericoncha chuni* have sulphide-oxidizing symbionts and are not known to use methane directly (von Cosel and Olu, 2009; Krylova and Sahling, 2010). Vast areas within the REGAB pockmark were littered with shells of dead clams, indicating a long-term association and turnover of this bottom-dwelling, mobile chemosynthetic megafauna. The water depth at REGAB is above the calcite compensation depth, hence bivalve shells are not dissolved, and may accumulate over long times. Independent of their location within REGAB, all clam patches were associated to sediments with similar geochemistry. These sediments were characterized by lowest CH_4 and SO_4 consumption rates, as well as lowest H_2S fluxes. Very little to no methane was escaping the sediment at the clam habitats, and AOM accounted for 50 – 80% of the upward removal of methane. Lowest bottom water methane concentrations were detected above clam patches also during previous studies at REGAB (Olu-Le Roy et al., 2007a). A common feature of all

clam habitats was the absence of sulphide from the topmost surface (up to 5 cm) sediment layers. This appears to be a universal characteristics for clam beds at cold seeps, as it has been also shown for other seeps throughout the world i.e. Northern California Seeps (Levin et al., 2003) Cascadia Convergent margin seeps (Sahling et al., 2002), Monterey Bay cold seeps (Barry et al., 1997), Makran accretionary prism (Fischer et al., 2012). Apparently, the bottom dwelling activity of the clams enables them to populate cold seep habitats with low gas fluxes and hence low microbial activity, so that they dwell the subsurface sediments to exploit rather deep peaks in sulphide production via AOM (Fischer et al., 2012).

The gas bubble site (Gas) was the only other sedimentary site, apart for the mussel habitat, where escape of gas into the water column was observed. Although the AOM and SR rates, as well as sulphide fluxes were in the range of values measured at the clam habitats, the gas bubble site was completely devoid of any visible megafauna. The relatively deep production of sulphide (> 9 cm sediment depth) and the low surface methane concentrations can potentially limit the dispersal of megafaunal organisms, which for their survival need more or less constant supply of energy sources i.e. sulphide and/or methane. It is possible that we had sampled a relatively fresh gas vent, which was not yet populated by the slow growing AOM communities transforming methane to sulphide and fuelling other chemosynthetic megafauna.

In this study we could show that the REGAB habitats differ in their methane efflux and porewater geochemistry, and that they are associated with different types of megafauna. However, methane efflux was relatively similar between the megafauna patches and their direct surroundings. In contrast, oxygen fluxes and oxygen penetration depths were different between the bare sediments and those populated by bacterial mats. Based on the biogeochemical analyses from this study, the sedimentary habitats at REGAB can be grouped in three categories: 1) mussel-associated sediments – characterized by highest methane efflux, methane consumption and bacterial counts; 2) bacterial mat-associated sediments – characterized by intermediate activity and low bacterial counts 3) clam-associated sediments – characterized by no methane efflux, intermediate methane consumption and bacterial counts. Overall, the megafauna distribution reflects the underlying sediment characteristics, thus we propose that the megafauna assemblages can be used as reliable first visual indicator of the sediment geochemistry at cold seeps i.e. of the magnitude of methane and oxygen fluxes, and the depth of sulphide production within the sediments.

In comparison to other cold seeps, the REGAB pockmark is a relatively unique ecosystem due to the co-occurrence of diverse symbiont-bearing megafauna (one species of siboglind tubeworms and three bivalve species) as well as occasional thiotrophic bacterial mats, which form highly specialized non-overlapping assemblages (except for mussels and tubeworms in some areas). The only other seep sites harbouring similar chemosynthetic habitats have been also found in the Congo Fan area, in the vicinity of REGAB (Sahling et al., 2008). However, in general, the magnitude of the measured fluxes and geochemical processes at REGAB fall within the range of values previously reported from other gas hydrate- and carbonate-bearing cold seeps (Table 6). A striking exception is the total oxygen uptake, detected within the dense clam patches at REGAB, which to our knowledge represents the highest oxygen consumption ever measured in a cold seep environment (Table 6). The highest flux of dissolved methane at REGAB, detected at the mussel habitat, was in the range of maximum fluxes reported for bacterial mats at the Hydrate Ridge (Sommer et al., 2006), ampharetid polychaete habitats at the Hikurangi margin (Sommer et al., 2010) and the summit of Dvurechenskii Mud Volcano (DMV; Lichtschlag et al., 2010b; Table 6). AOM and SR rates can vary substantially among different cold seeps, but as well among different habitats within a single cold seep, as it is the case of the REGAB pockmark. The consumption of methane and sulphate at all investigated habitats at REGAB was quite low in comparison to other cold seeps i.e. Hydrate Ridge (Treude et al., 2003), DMV (Lichtschlag et al., 2010b), with respectively ten and five times lower rates relative to maximum reported rates from other cold seeps (Table 6).

4.2 Methane flux influences bacterial community structure at REGAB

In addition to investigating the link between methane fluxes and the distribution of chemosynthetic megafauna habitats, we aimed at testing if a) the bacterial community structure differs between the different habitats, and b) if the underlying patterns in bacterial biodiversity could be linked to methane fluxes. In this regard, we used several independent measures of seepage activity: the magnitude of methane effluxes, AOM coupled to sulphate consumption rates, sulphide fluxes and alkalinity produced by AOM, and other associated geochemical variables (Table 5; Fig. 3; Supplement Fig. 1). In different ways, these biogeochemical processes are indicative of potential energy availability to the seep communities, with methane and sulphide representing the major sources of reduced chemical energy. Of the different indicators of bacterial diversity analysed here, the bacterial β-

diversity - change in species composition and/or structure between habitats (Whittaker, 1960) - was foremost significantly positively correlated to differences in *in situ* methane effluxes. A positive trend existed also between the bacterial β-diversity and the difference in core geochemical processes AOM and SR, as well as alkalinity flux, which respond usually directly to variations in methane supply via AOM or via subsurface fluid advection (Bohrmann et al., 1998; Valentine, 2002; Luff and Wallmann, 2003). These results support the hypothesis that the bacterial community structure at cold seeps is influenced foremost by methane supply, as primary source of energy to anaerobic and aerobic methanotrophs (Cambon-Bonavita et al., 2009), and as a main indicator of the activity of geological processes such as gas overpressure, fluid flow and hydrate formation or dissociation.

Surprisingly, even though a much higher diversity of bacteria and animals could be biologically influenced by sulphide as energy source or as toxin, bacterial β-diversity was not significantly correlated to difference in sulphide fluxes among habitats. Sulphide is a secondary energy source, provided via microbial methane consumption with sulphate, and it is possible that this indirect link to methane blurs relations with diversity indicators. However, generally, habitat types with similar biogeochemistry/energy availability were more similar in terms of bacterial diversity as opposed to habitats with distinct geochemistry.

A further analysis of the links between community structure and concentrations of porewater constituents across all individual depth samples also confirmed a link between β-diversity and sediment geochemistry (Fig. 5). Even when using *ex situ* methane concentrations in the analysis (i.e. after degassing and depressurization of the cores upon retrieval), it explained the highest proportion of the variation in the bacterial community structure. Again, sulphide concentrations could not explain any variation in the bacterial community structure, however, the confounding effect of this variable with the rest of the geochemical parameters accounted for a substantial portion of the variation in the community structure at REGAB, as did sediment depth and geographic distance between samples (Fig. 5). Geographic (spatial) distance among sampling sites and to lesser extent sediment depth of individual samples are two other variables that appeared to play a role in shaping the bacterial communities at REGAB. Of course, both are coupled strongly also to geological and geochemical processes, e.g. distance to gas seepage, or upward transport of highly reduced porewater fluids to the sulphate or oxygen penetrated sediment surface layer, and their role in structuring bacterial diversity cannot be further disentangled in this study.

Although only a small proportion of the total bacterial community can use methane directly as energy source, we propose that methane oxidation, as the primary energy producing process in the seep ecosystem, is a main driver of community structure, from bacteria to megafauna. Previous studies of β - diversity patterns of benthic bacterial communities have shown significant relationships to geobiological indicators of energy availability, such as phytodetritus sedimentation to oligotrophic continental margins (Bienhold et al., 2011), and benthic primary productivity in coastal sands (Böer et al., 2009).

4.3 Link between megafauna, geochemistry and bacterial community structure

Our data indicate that methane fluxes determine sediment geochemistry, which selects for different types of chemosynthetic megafauna at REGAB. A further aim of this study was to test if the distribution of the chemosynthetic megafauna is also influencing bacterial community structure. It has been shown previously that chemosynthetic megafauna influences its local environment by bioturbation, bioirrigation, burrowing and by exudates, altering the local seafloor biogeochemistry (Barry et al., 1997; Levin et al., 2003; Fischer et al., 2011). Clam and mussel respiration accounted for a substantial local increase in the total benthic oxygen uptake rates, as compared to the adjacent bare sediments. Furthermore, the *Bathymodiolus* mussels contain methanotrophic symbionts and consume methane efficiently (Duperron et al., 2009, 2011; Petersen and Dubilier, 2009), causing a reduction of methane efflux within the mussel patch.

Similar to sites populated by clams at other cold seeps, sulphide was absent from the surface sediments (Barry et al., 1997; Sahling et al., 2002; Levin et al., 2003; Fischer et al., 2012) and bottom water sulphate penetrated till 6 cm depth at all clam patches at REGAB – a signature ascribed to the dwelling activity of thiotrophic clams to access sulphide (Childress and Fisher, 1992), leading to a deeper sulphate penetration (Sahling et al., 2002; Cordes et al., 2005, 2010; Fischer et al., 2012). Furthermore, unusual and elevated ammonium concentrations were measured below the clam patches, where the maximum concentrations - indicating local production of ammonium, were observed within the depth range of the clam's foot. Previous studies from the Gulf of Mexico cold seeps also showed that clam-associated sediments had very high ammonium concentrations (Joye et al., 2010). These results indicate a potential link between the clam presence and the NH_4 concentrations in pore water, most probably via local excretions of metabolites by the clams, but further

investigations are needed to decipher this relation. However, when comparing the structure of bacterial communities of adjacent sites at REGAB with and without chemosynthetic megafauna or bacterial mats, both methane fluxes and bacterial community structure were similar (Fig. 4, Table 2). Accordingly, no direct association of unique bacterial types with the different megafauna was detected. Even the dense clam patches had little effect on the bacterial community structure. This indicates that the abundant bacterial types in this cold seep ecosystem as detected by ARISA fingerprinting were directly affected by methane seepage and other geochemical processes, but only indirectly by the presence and absence of megafauna types. This finding may differ with other types of molecular methods, which include rare bacterial types. Furthermore, space played an important role in structuring the distribution and diversity of chemosynthetic megafauna and bacterial communities at spatial scales of meters to hundreds of meters, which needs further investigation, especially when considering the need for conservation, protection and management of cold seeps as unique deep-water ecosystems.

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Tables and Figures

Table 1. Overview of the sampling sites, their geographic position and type of measurements performed within this study. Samples and measurements were taken in July – August 2008. AOM = Anaerobic Oxidation of Methane, SR = Sulphate Reduction, MICP = microprofiler measurement, CHAM = Benthic Chamber Incubation.

Location	Sampling site	Latitude	Longitude	Measurement; Sample
N REGAB	**Bacter_N** (Bacterial mat)	S 5°47.8364'	E 9°42.6364'	DNA; Porewater;AOM; SR; MICP
	Bacter_N_Env (Outside bacterial mat)	S 5°47.835'	E 9°42.6377'	DNA;Porewater; AOM; SR
	Clam_N (Clam patch)	S 5°47.836'	E 9°42.6164'	DNA; Porewater; AOM; SR; CHAM
	Clam_N_Env (Outside clam patch)	S 5°47.842'	E 9°42.6304'	DNA; Porewater;AOM; SR; MICP
S REGAB	**Mussel_S** (Mussel patch)	S 5°47.863'	E 9°42.6929'	DNA; Porewater; AOM; SR; CHAM
	Mussel_S_Env (Outside mussel patch)	S 5°47.861'	E 9°42.685'	DNA; Porewater; AOM; SR; CHAM
	Clam_S (Clam patch)	S 5°47.879'	E 9°42.6844'	DNA; Porewater; AOM; SR; CHAM
	Clam_S_Env (Outside clam patch)	S 5°47.873'	E 9°42.684'	DNA; Porewater; AOM; SR; CHAM; MICP
REGAB	**Gas** (Gas Bubble)	S 5°47.865'	E 9°42.6954'	DNA;Porewater; AOM; SR
SW REGAB	**Clam_SW** (Clam patch)	S 5°47.981'	E 9°42.4803'	DNA; Porewater; AOM; SR; CHAM
	Clam_SW_Env (Outside clam patch)	S 5°47.97'	E 9°42.4821'	DNA; Porewater; AOM; SR; CHAM; MICP

Table 2. Biogeochemical characterization of different habitats at REGAB. Maximum H_2S flux in the sediment, CH_4 efflux, TOU, average integrated (0 – 10 cm sediment depth) AOM and SR rates, methane consumption efficiency calculated as the percentage of methane consumption (AOM) from the total methane flux (AOM + CH_4 efflux), total integrated (0 – 10 cm sediment depth) single cell numbers, as well as alkalinity flux and modelled values of bioirrigation and advective flow.

	H_2S flux	**CH_4 efflux**	**TOU**	**AOM**	**Methane consumption efficiency**	**SR**	**Bioirigation (x 10^{-8})**	**Single cells (x 10^{10})**	**Alkalinity flux**	**Advective flow (x 10^{-8})**
	(mmol m^{-2} d^{-1})	(mmol m^{-2} d^{-1})	(mmol m^{-2} d^{-1})	(mmol m^{-2} d^{-1})	(%)	(mmol m^{-2} d^{-1})	(m s^{-1})	(cm^{-2} sediment)	(mmol m^{-2} d^{-1})	(m s^{-1})
Bacter_N	13	n.d.	n.d.	9	n.d.	23	1	0.7	0.4	0
Bacter_N_Env	16	n.d.	n.d.	9	n.d.	3	2	0.6	0.1	0
Clam_N	5	< 1	50	1	49	<1	4	0.7	0.1	1.4
Clam_N_Env	9	n.d.	n.d.	5	n.d.	5	4	1.5	0.3	1.0
Clam_S	6	1	590	6	81	6	5	0.9	0.1	5.0
Clam_S_Env	0	3	18	3	47	2	2	1.0	0	0
Mussel_S	20	1-81	94	19	19	28	2	2.7	0.4	0
Mussel_S_Env	23	334	77	20	6	36	9	3.5	0.4	6.0
Gas	7	n.d.	n.d.	4	n.d	8	3	1.6	0.2	0
Clam_SW	5	< 1	294	3	81	10	5	1.4	0	0
Clam_SW_Env	0	0	12	2	97	1	1	1.0	0	0

Table 3. Sediment oxygen penetration depth (OPD) and diffusive oxygen uptake (DOU) of sediments in relation to the vicinity of clam patches and bacterial mat at REGAB, as measured *in situ* with a microprofiler.

	Distance to clam patch/bacterial mat	**OPD** (mm)	**DOU** (mmol m^{-2} d^{-1})
Bacter_N_Env	within bacterial mat	2	13
Clam_S_Env	< 1 m from clam patch	2	6
Clam_SW_Env	< 1 m from clam patch	3	11
Clam_N_Env	> 1 m from clam patch	35	3
Clam_SW_Env	> 1 m from clam patch	13	3

Table 4. Analysis of Similarity (ANOSIM; lower triangle), testing for significant differences in bacterial community structure between habitats and percentage of shared OTUs between habitats (upper triangle). ANOSIM R-values (lower triangle) are interpreted as follows: R<0.25 = strongly overlapping, 0.25<R<0.5 = separated but with overlap, 0.5<R<0.75 = separated with only minor overlap, R>0.75 = strongly separated. *p<0.05, **p<0.01, ***<0.001.

	Bacter_N	**Clam_N**	**Mussel_S**	**Clam_S**	**Clam_SW**
Bacter_N		92	77	80	87
Clam_N	0.3		78	78	86
Mussel_S	0.7**	0.6**		70	76
Clam_S	0.8***	0.8***	0.8***		78
Clam_SW	0.5**	0.4*	0.8***	0.7**	

Table 5. Mantel test, checking for correlation between β-diversity (calculated as Bray-Curtis dissimilarities) and difference in CH_4 efflux, Total Oxygen Uptake (TOU), integrated Anaerobic Oxidation of Methane (AOM) and Sulphate Reduction (SR) rate, alkalinity flux, advective flow, integrated sulphide flux and bioirrigation rate. The spearman rank coefficient was used for calculating correlations. * = p <0.01; Bonferroni-correction was applied to correct for multiple testing. (*) only significant without Bonferroni correction. Mantel correlation test was performed on pooled ARISA samples (0 – 10 cm depth) according to habitat. For this analysis data from the following sites was used: Clam_N, Mussel_S, Mussel_S_Env, Clam_S, Clam_S_Env, Clam_SW, Clam_SW_Env.

	CH_4 efflux	**TOU**	**AOM**	**Bioirrigation rate**	**Alkalinity flux**	**Advective flow**	**Integrated H_2S flux**	**SR**
Mantel statistics	0.6**	-0.1	0.5(*)	0.2	0.5(*)	0.2	0.4	0.5(*)
Significance p-value	0.003	0.641	0.048	0.265	0.021	0.222	0.076	0.037
Bonferroni corrected p-value	0.021	1	0.336	1	0.147	1	0.532	0.259

Table 6. Compilation of *in situ* CH_4 effluxes and TOU, *ex situ* average integrated AOM, SR rates, as well as H_2S fluxes from cold seep sites worldwide, including data from REGAB (this study).

Cold seep	Habitat	CH_4 efflux (mmol m^2 d^{-1})	AOM (mmol m^2 d^{-1})	SR (mmol m^2 d^{-1})	H_2S flux (mmol m^2 d^{-1})	TOU (mmol m^2 d^{-1})	Reference
REGAB (3160 m water depth)	**Bacterial mat**	n.d.	9	23	13	n.d.	This study
	Clam patch	1	3 (± 2.5)	8 (± 2.5)	5 (± 0.6)	311 (± 294)	
	Mussel patch	1-81	19	28	20	94	
HMMV[1)] (1250 m water depth)	**Beggiatoa mat**	78	10 (± 6.6)	14 (± 6.2)		101; 114	Felden et al. (2010)
Hydrate Ridge (600 - 1000 m water depth)	**Beggiatoa mat**	5.7; 30; 90	5 (± 4.4); 99 (± 102)	32 (± 34)	23 (± 13)	48	Treude et al. (2003); Sahling et al. (2002); Sommer et al. (2006); Torres et al. (2002)
	Clam patch	0.6; 0.45	56 (± 54)	65 (± 58)	7 (± 2.4)	4	Treude et al. (2003); Sahling et al. (2002); Sommer et al. (2006); Torres et al. (2002)
	Acharax field	n.d.	2 (± 1.4)	0.4 (± 0.3)	0.05 (± 0.05)	n.d.	Treude et al. (2003); Sahling et al. (2002)
DMV[2)] (2060 m water depth; anoxic Black Sea)	**Summit**	458	0.07 (± 0.1)	0.05 (± 0)	n.d.	0	Lichtschlag et al. (2010)
	Geographical centre of DMV	n.d.	9 (± 6)	20 (± 5.7)	n.d.	0	Lichtschlag et al. (2010)
	Western edge	n.d.	11 (± 9.6)	108 (± 38)	n.d.	0	Lichtschlag et al. (2010)
Hikurangi Margin (1050 m water depth)	**Ampharetids**	max. 265	17 (± 9.2)	n.d.	n.d.	max. 118	Sommer et al. (2010)
	Tube worms	max. 5	n.d.	n.d.	n.d.	max. 65	Sommer et al. (2010)

[1)] Håkon Mosby Mud Volcano

[2)] Dvurechenskii Mud Volcano

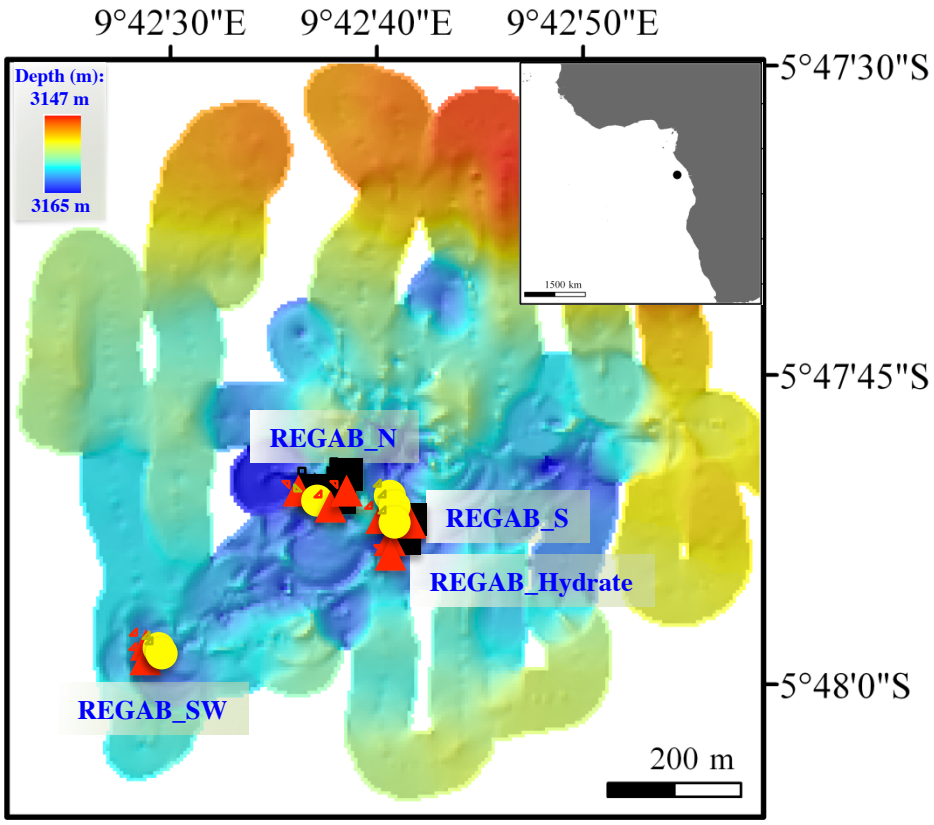


Fig. 1. Map of the REGAB pockmark (adopted from Ondréas et al., 2005), derived from ROV Victor 6000 data (Ifremer, France), with the main sampling locations. Locations of push core sediment sampling are depicted as red triangles, benthic chamber incubations as yellow circles and microprofiler measurements as squares.

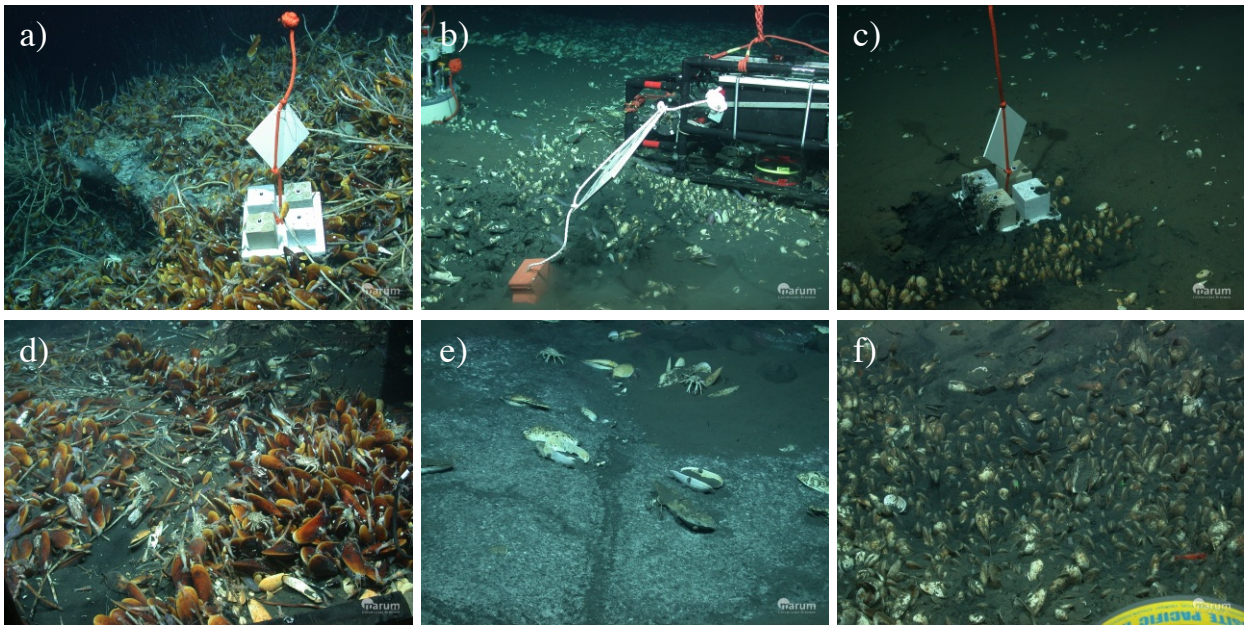


Fig. 2. Typical habitats at the REGAB pockmark: the central area with extensive carbonate crust and outcropping hydrates populated by mussels and tubeworms (a); clam patches overlying blackened reduced sediment (b, c, f); mussel patch on soft sediment (d); white bacterial mat at the REGAB_N (e); Benthic chamber incubation is visible in (b) picture.

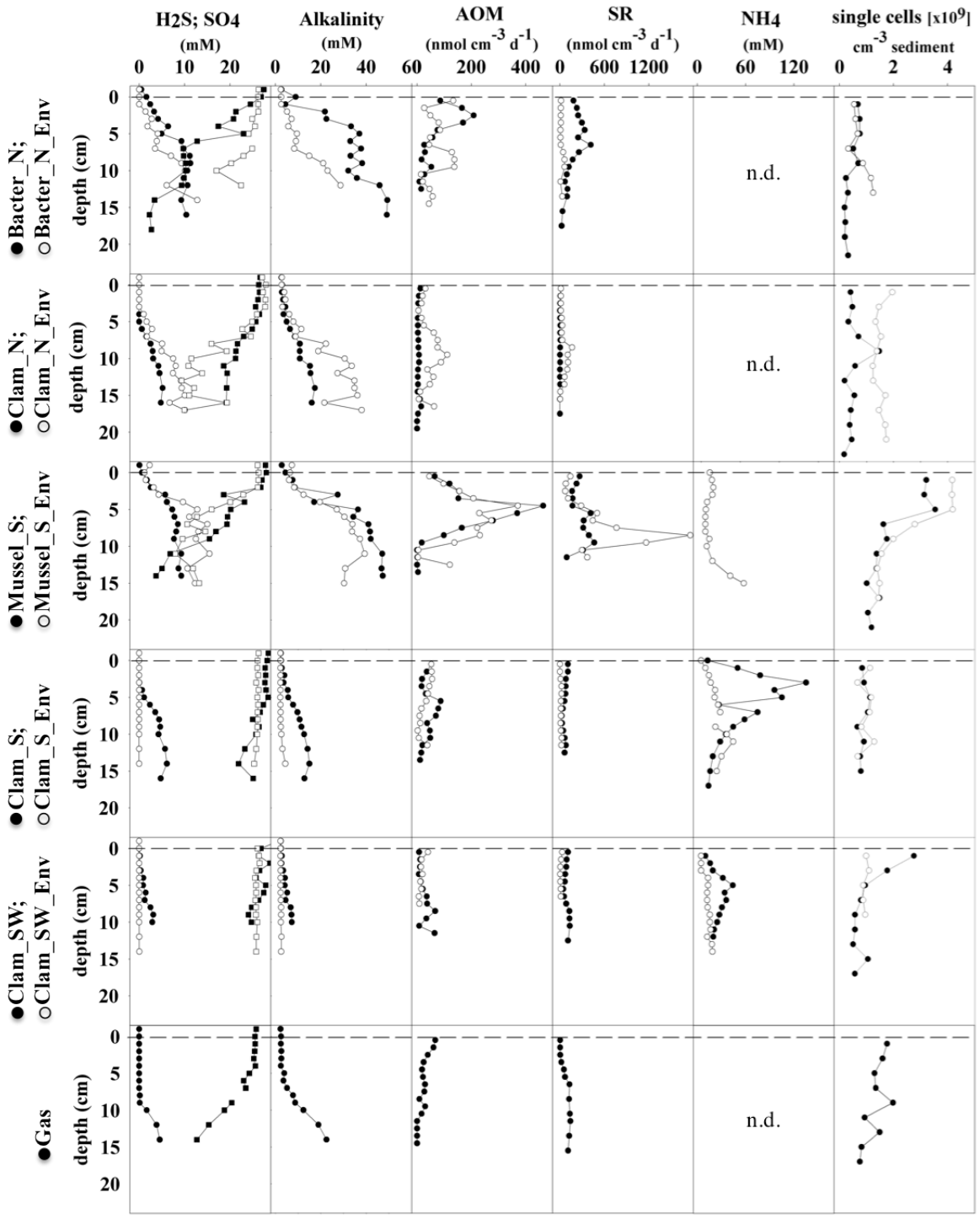


Fig. 3. Geochemical depth profiles of H_2S, SO_4, AOM, SR, NH_4, alkalinity and single cell counts at all investigated sites at REGAB. Closed symbols denote measurements taken within the patches/bacterial mat, and open symbols denote measurements taken at the nearby bare sediments. N.d. = Not Determined.

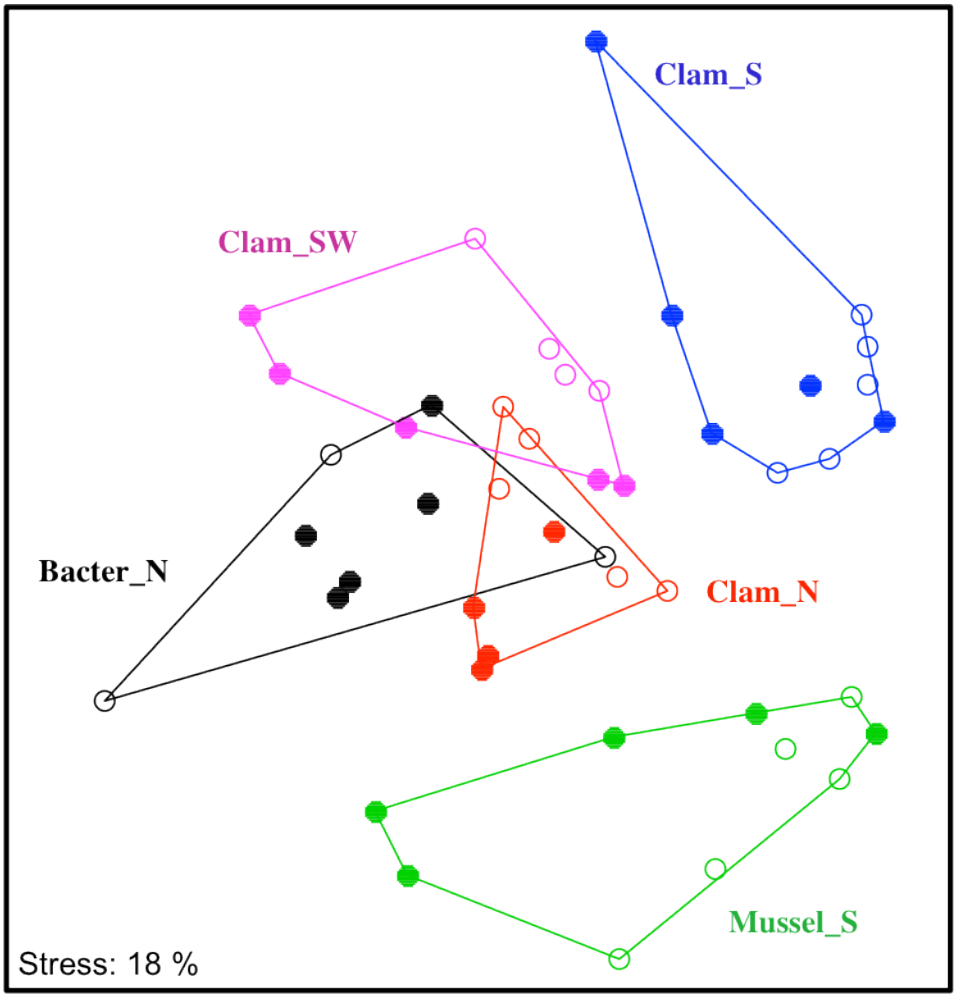


Fig. 4. Nonmetric MultiDimensional Scaling (NMDS) ordination plot (based on Bray Curtis distance matrix) of ARISA merged profiles (2 – 3 PCR replicates were pooled to form a consensus profile). Ordihull grouping of samples (0 – 10 cm) according to sampling location (samples from within the patches and the samples from the respective bare sediment were grouped together). NMDS stress 18%. Open symbols denote samples from the bare sediment sites - outside of the respective patch; closed symbols denote samples from within the respective patch.

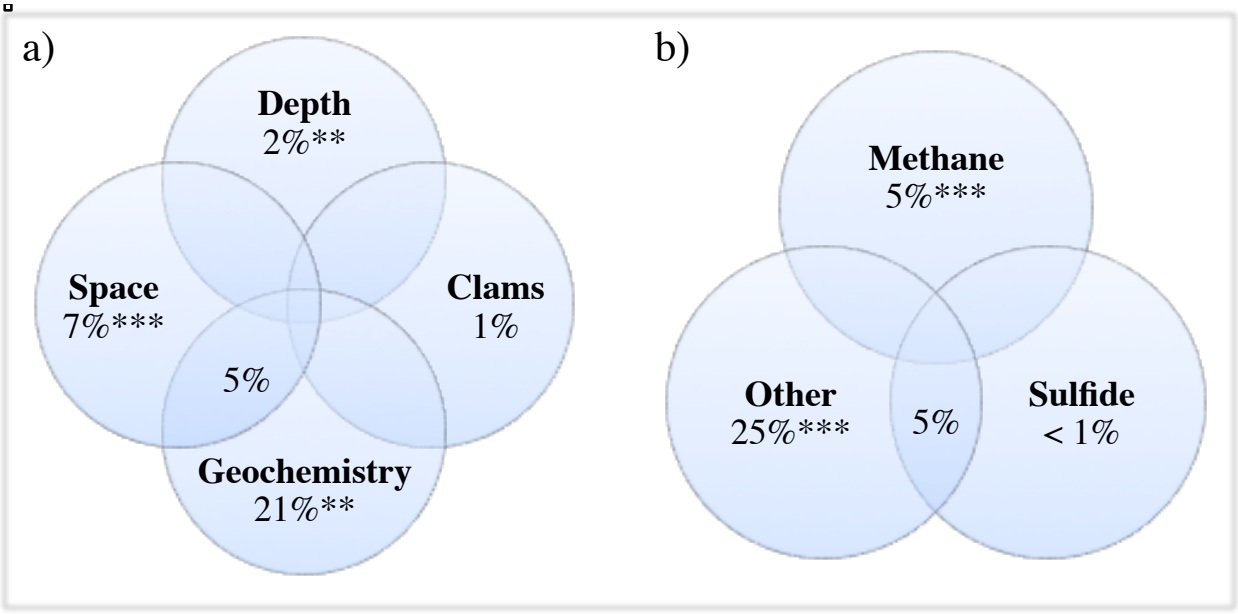


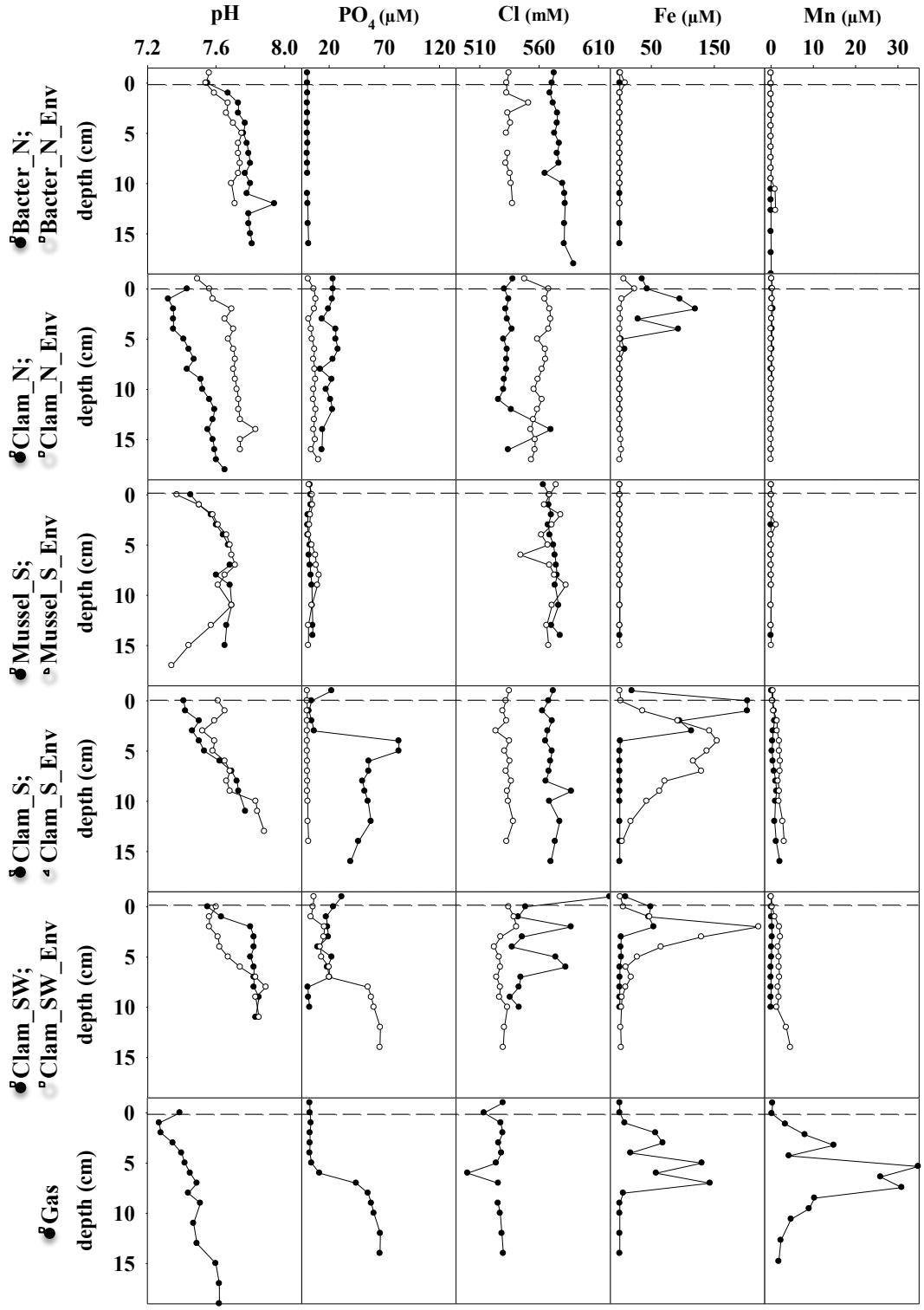
Fig. 5. Variation partitioning analysis. Effect (%) of the geochemistry (concentrations of CH_4, H_2S, Cl, Fe, Mn, PO_4, SO_4, pH), clams presence (clams directly influence the top 6 cm sediment), space (geographic distance among sampling sites) and depth (sediment depth) on the bacterial diversity at REGAB (a). Geochemistry, followed by space and depth significantly shape the diversity at REGAB. Visualization of the individual influence of CH_4 and H_2S on the bacterial community structure (b). Other = all parameters included in (a), excluding CH_4 and H_2S Individually CH_4 ($p < 0.001$) significantly explains the variation in the ARISA dataset. *** $p<0.001$, **$p<0.002$, *$p<0.03$. The complete model explained 36% of the total variation in the bacterial diversity at REGAB.

Supplement text 1:

Porewater geochemistry

Immediately after recovery and transfer of the push cores to *in situ* temperature of 4 °C, pH of every centimetre sediment depth was determined with punch-in electrodes on undisturbed sediment cores. Porewater analyses of phosphate and iron (Fe^{2+}) were carried out on board. For the analyses of dissolved iron (Fe^{2+}) porewater subsamples of 1 ml were immediately complexed with 50 μl of "Ferrospectral" and determined photometrically. A photometric procedure was used to determine the concentration of phosphate in extracted porewater subsamples. Aliquots of the porewater were diluted 1:10 and acidified with $HNO_{3\ (suprapure)}$ for the determination of manganese concentration by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES) and Atomic absorption Spectroscopy (AAS) in the home laboratory.

Elevated alkalinity values were detected at all sites where also enhanced AOM activity was measured, indicating potential variation in the seepage between sites (Fig. 3). At these sites alkalinity increased with depth to reach maximum values of 47 and 49 mM in the deepest investigated horizons at the Mussel_S and Bacter_N sites, respectively (Fig.3). Highest alkalinity flux of 0.4 mmol m^{-2} d^{-1} was detected at the Mussel_S and the Bacter_N sites. Near background (2.5 mM) values were detected at the Clam_S_Env, Clam_SW, Clam_SW_Env. The iron profiles matched well the sulphide depth pattern, with elevated concentrations detected at the clam habitats where free sulphide was absent from the topmost surface layers (Supplement Fig. 1).



Supplement Fig. 1 Geochemical depth profiles of pH, PO_4, Cl, Fe and Mn at all investigated sites at REGAB. Closed symbols denote measurements taken within the patches/bacterial mat, and open symbols denote measurements taken at the respective bare sediments.

Supplement Table 1 Overview of the samples and measurements acquired at REGAB during M76/3b, with their PANGAEA reference numbers. All data has been deposited and is available online in the PANGAEA database (www.pangaea.de).

Location	Sampling site	Measurement; Sample	Pangaea Event Label
N REGAB	**Bacter_N** (Bacterial mat)	DNA	M76/3b_310_PUC13; M76/3b_310_PUC27
		Porewater	M76/3b_310_PUC28; M76/3b_310_PUC32
		pH	M76/3b_310_PUC8
		AOM; SR	M76/3b_310_PUC27; M76/3b_310_PUC13; M76/3b_310_PUC12
		MICP	M76/3b_312_MICP1
	Bacter_N_Env (Outside bacterial mat)	DNA	M76/3b_312_PUC7
		Porewater	M76/3b_312_PUC15
		pH	M76/3b_312_PUC34
		AOM; SR	M76/3b_312_PUC22; M76/3b_312_PUC23; M76/3b_312_PUC7;
	Clam_N (Clam patch)	DNA	M76/3b_323_PUC14
		Porewater	M76/3b_323_PUC15
		pH	M76/3b_323_PUC30
		AOM; SR	M76/3b_323_PUC28; M76/3b_323_PUC31; M76/3b_323_PUC14; M76/3b_323_PUC12
		CHAM	M76/3b_325_CHAM1
	Clam_N_Env (Outside clam patch)	DNA	M76/3b_332_PUC29
		Porewater	M76/3b_332_PUC31
		pH	M76/3b_332_PUC20
		AOM; SR	M76/3b_332_PUC23; M76/3b_332_PUC29; M76/3b_332_PUC34
		MICP	M76/3b_335_MICP1
S REGAB	**Mussel_S** (Mussel patch)	DNA	M76/3b_344_PUC23
		Porewater	M76/3b_344_PUC30
		pH	M76/3b_344_PUC29
		AOM; SR	M76/3b_344_PUC23; M76/3b_344_PUC28; M76/3b_344_PUC15
		CHAM	M76/3b_364_CHAM1
	Mussel_S_Env (Outside mussel patch)	DNA	M76/3b_361_PUC36
		Porewater	M76/3b_361_PUC14
		pH	M76/3b_361_PUC13
		AOM; SR	M76/3b_361_PUC36; M76/3b_361_PUC15
		CHAM	M76/3b_364_CHAM2
	Clam_S (Clam patch)	DNA	M76/3b_361_PUC24
		Porewater	M76/3b_361_PUC31
		pH	M76/3b_355_PUC9
		AOM; SR	M76/3b_361_PUC10; M76/3b_361_PUC21; M76/3b_361_PUC24
		CHAM	M76/3b_355_CHAM1
	Clam_S_Env (Outside clam patch)	DNA	M76/3b_355_PUC29
		Porewater	M76/3b_355_PUC35
		pH	M76/3b_355_PUC20
		AOM; SR	M76/3b_355_PUC11; M76/3b_355_PUC7; M76/3b_355_PUC29
		MICP	M76/3b_361_MICP1; M76/3b_361_MICP2
		CHAM	M76/3b_355_CHAM2
REGAB	**Gas** (Gas bubble)	DNA	M76/3b_364_PUC7
		Porewater	M76/3b_364_PUC21
		pH	M76/3b_364_PUC28
		AOM; SR	M76/3b_364_PUC7; M76/3b_364_PUC29; M76/3b_364_PUC9
SW REGAB	**Clam_SW** (Clam patch)	DNA	M76/3b_379_PUC28
		Porewater	M76/3b_379_PUC29
		pH	M76/3b_379_PUC34
		AOM; SR	M76/3b_379_PUC28; M76/3b_379_PUC14; M76/3b_379_PUC15
		CHAM	M76/3b_379_CHAM1
	Clam_SW_Env (Outside clam patch)	DNA	M76/3b_379_PUC10
		Porewater	M76/3b_379_PUC9
		pH	M76/3b_379_PUC5
		AOM; SR	M76/3b_379_PUC10; M76/3b_379_PUC13
		MICP	M76/3b_385_MICP1; M76/3b_385_MICP2
		CHAM	M76/3b_379_CHAM2

Supplement Table 2 Percentage of shared OTUs between all sites investigated at REGAB. Prior to this analysis, the depth samples within individual sites were merged.

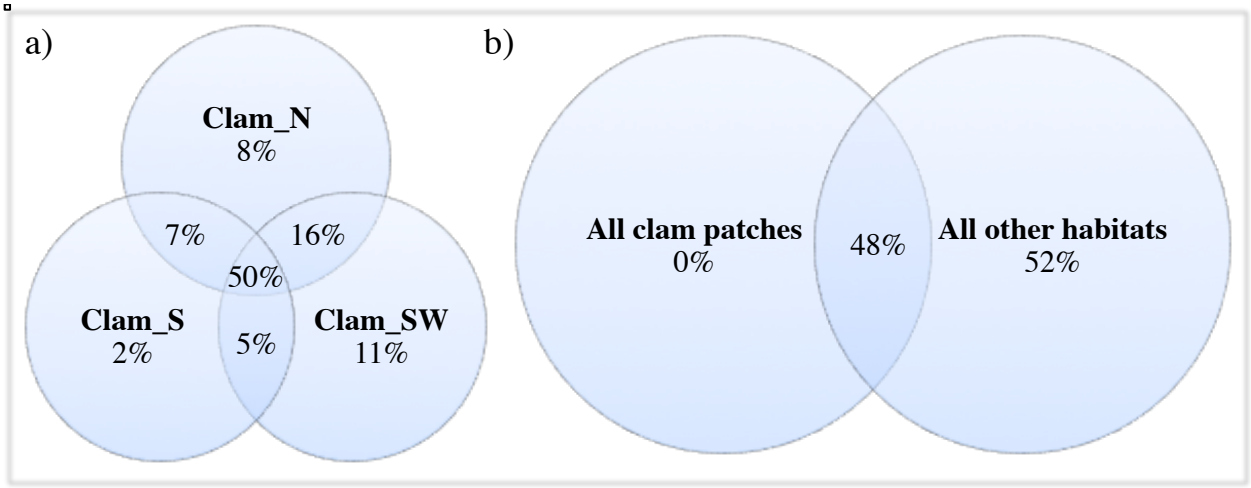
	Bacter_N	Bacter_N_Env	Clam_N	Clam_N_Env	Mussel_S	Mussel_S_Env	Clam_S_Env	Clam_S	Gas	Clam_SW
Bacter_N_Env	79									
Clam_N	83	73								
Clam_N_Env	84	77	82							
Mussel_S	69	65	67	71						
Mussel_S_Env	58	56	61	57	64					
Clam_S_Env	64	60	62	65	61	55				
Clam_S	68	64	69	68	66	57	67			
Gas	69	63	67	65	65	56	61	65		
Clam_SW	80	71	72	78	70	54	64	66	70	
Clam_SW_Env	73	65	69	72	68	53	60	63	65	79

Supplement Table 3 Comparison of the shared OTUs (given as percentage) among all depth samples, between bare sediment sites and clam populated sites. An OTU was regarded as shared only if it was present in all samples (0 – 10 cm or 0 – 5 cm). The percentage of shared OTUs was calculated as the fraction of the total OTUs at the individual habitat.

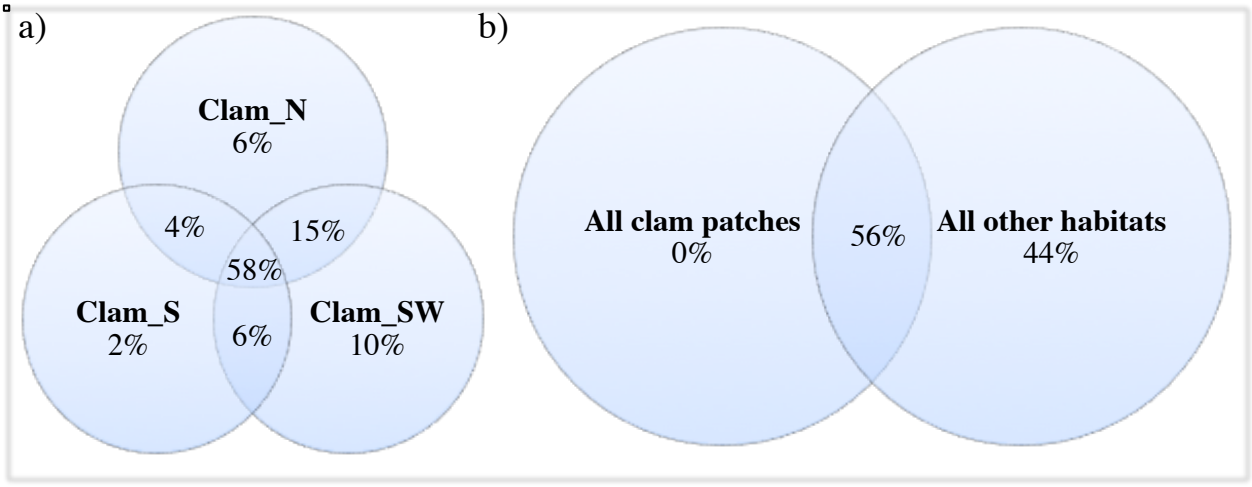
	Clam_N (0 - 10 cm)	**Clam_S** (0 - 10 cm)	**SW_Clam** (0 - 10 cm)	**Clam_N** (0 - 5 cm)	**Clam_S** (0 - 5 cm)	**SW_Clam** (0 - 5 cm)
Inside	36	30	22	47	40	47
Outside	34	30	36	42	44	44

Supplement Table 4 Distance-based test for homogeneity of multivariate dispersions. Table comprises average distances to the centroids, calculated based on Jaccard and Bray-Curtis dissimilarity indices. The higher the value of the average distance to the centroid, the higher the dispersion (variance) within the respective group. The test was performed incorporating only the surface samples (0 – 5 cm), or samples from all depths (0 – 10 cm).

	Clam_N	**Clam_N_Env**	**Clam_S**	**Clam_S_Env**	**Clam_SW**	**Clam_SW_Env**
0 - 5 cm (Jaccard)	0.3	0.3	0.4	0.3	0.3	0.3
0 - 5 cm (Bray-Curtis)	0.2	0.2	0.3	0.2	0.2	0.2
0 - 10 cm (Jaccard)	0.4	0.4	0.4	0.4	0.4	0.4
0 - 10 cm (Bray-Curtis)	0.2	0.2	0.3	0.3	0.3	0.3



Supplement Fig. 2 OTU partitioning analysis taking into account all sediment depth samples (0 – 10 cm). 50% of the total OTUs were found to be shared by all three clam patches (a). The clam patches had no unique OTUs relative to the other investigated habitats at REGAB (b).



Supplement Fig. 3 OTU partitioning analysis taking into account the topmost 5 cm sediment depth samples. 58% of the total OTUs were found to be shared by all three clam patches (a). The clam patches had no unique OTUs relative to the other investigated habitats at REGAB (b).

Chapter II
Relative abundances of methane- and sulphur-oxidising symbionts in the gills of a cold seep mussel and link to their potential energy sources

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Relative abundances of methane- and sulphur-oxidising symbionts in the gills of a cold seep mussel and link to their potential energy sources

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ABSTRACT

Bathymodiolus mussels are key species in many deep-sea chemosynthetic ecosystems. They often harbour two types of endosymbiotic bacteria in their gills, sulphur- and methane oxidisers. These bacteria take up sulphide and methane from the environment and provide energy to their hosts, supporting some of the most prolific ecosystems in the sea. In this study, we tested whether symbiont relative abundances in *Bathymodiolus* gills reflect variations in the highly spatially dynamic chemical environment of cold seep mussels. Samples of *Bathymodiolus* aff. *boomerang* were obtained from two cold seeps of the deep Gulf of Guinea, REGAB (5°47.86S, 9°42.69E, 3170 m depth) and DIAPIR (6°41.58S, 10°20.94E, 2700 m depth). Relative abundances of both symbiont types were measured by means of 3D fluorescence *in situ* hybridisation and image analysis and compared considering the local sulphide and methane concentrations and fluxes assessed via benthic chamber incubations. Specimens inhabiting areas with highest methane content displayed higher relative abundances of methane oxidisers. The bacterial abundances correlated also with carbon stable isotope signatures in the mussel tissue, suggesting a higher contribution of methane-derived carbon to the biomass of mussels harbouring higher densities of methane-oxidising symbionts. A dynamic adaptation of abundances of methanotrophs and thiotrophs in the gill could be a key factor optimising the energy yield for the symbiotic system and could explain the success of dual symbiotic mussels at many cold seeps and hydrothermal vents of the Atlantic and Gulf of Mexico.

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INTRODUCTION

Symbioses involving both methane- and sulphur-oxidising bacteria occur in at least six deep-sea mytilid species from hydrothermal vents along the mid-Atlantic Ridge and from cold seeps in the Gulf of Mexico, Gulf of Guinea and Eastern Mediterranean (Fisher *et al.*, 1993; Distel *et al.*, 1995; Duperron *et al.*, 2009). The co-occurrence of several types of symbionts with different types of metabolism within mussel gill epithelial cells (bacteriocytes) allows hosts to cope with environments that are highly variable, in both time and space (Distel *et al.*, 1995; Le Bris & Duperron, 2010). Contribution of the different symbionts to mussel host nutrition is likely influenced by the availability of methane and reduced sulphur and their respective substrates (Colaco *et al.*, 2002; Dattagupta *et al.*, 2004). Hence, a key factor ensuring adaptation of the associations with fluctuating environmental conditions may be variation in symbiont abundances. Mussel symbionts are initially most likely acquired from the environment during early life stages, but once they actively divide in host cells, allowing potential variations in relative abundances to occur (Duperron *et al.*, 2005; Duperron, 2010). As quantification of endosymbionts by microscopy is very difficult owing to their high density in bacteriocytes, this variability remains poorly documented in wild-type mussel symbioses as well as in mussels kept in aquaria (Kadar *et al.*, 2005; Salerno *et al.*, 2005). Recently, symbiont abundances were shown to vary significantly among specimens of *Bathymodiolus azoricus*

from different hydrothermal vent sites, different aggregates of animals within a single site and different sampling times at the Mid-Atlantic Ridge using a newly developed fluorescence *in situ* hybridisation protocol coupled with three-dimensional reconstruction of bacteriocyte sections and image analysis (3D FISH) (Halary *et al.*, 2008; Riou *et al.*, 2008, 2010). Experimental evidence using a simulated sulphide pulse confirmed that sulphur-oxidising bacteria increased in relative abundance quickly in response to the pulse, potentially explaining the source of differences observed among recovered specimens (Halary *et al.*, 2008).

Cold seeps are often assumed to be more stable environments compared with hydrothermal vents, lasting thousands to millions of years (Sibuet & Olu, 1998). However, the local spatial and temporal variation of methane and sulphide fluxes at cold seeps can also be high, because of the underlying geological dynamics at continental margins (Felden *et al.*, 2010). First studies from hydrothermal vents suggest that shifts in endosymbiont abundances can be linked to the variability of their environment. As seep mussels are closely related to vent mussels, a similar interplay is expected, but has not yet been tested properly. In a previous attempt to estimate symbiont densities, 16S rRNA from the different symbionts present in *Bathymodiolus brooksi* and *Bathymodiolus heckerae* from the Gulf of Mexico was quantified (Duperron *et al.*, 2007). Although methanotrophs dominated fluorescent *in situ* hybridisation (FISH) images, the method chosen to quantify their contribution might have introduced bias in the abundance estimates because it is based on the usage of extracted RNA, which is difficult to preserve onboard and whose expression depends upon symbiont activities as well as densities (Stahl *et al.*, 1988; Amann *et al.*, 1995). Besides, no *in situ* chemical data were presented for discussion.

In this study, we investigated the variability of relative abundances of symbionts in bacteriocytes of *Bathymodiolus* aff. *boomerang* from two cold seep sites in the south-east Atlantic. This species harbours both sulphur- and methane-oxidising endosymbionts and forms locally restricted dense aggregates associated with tubeworms (*Escarpia southwardae*), shrimps (*Alvinocaris muricola*) and sea cucumbers (*Chiridota* sp.) at the REGAB site, a 800-m-wide pockmark located north of the deep Congo Canyon in the Gulf of Guinea (eastern Atlantic) (Duperron *et al.*, 2005; Olu-Leroy *et al.*, 2007a). We tested whether specimens collected from different mussel aggregates at REGAB (3170 m depth) and the shallower site DIAPIR (2700 m depth) harboured different relative symbiont abundances in relation to the availability of dissolved methane in their respective habitat. The fraction of bacterial volume occupied by methane- and sulphur oxidisers was measured in mussel bacteriocytes using 3D FISH and compared within and between sites using statistical tools. Because relative abundances of symbionts may vary with time, this approach was supplemented by the measurements of carbon stable isotopic signatures in mussel tissues, which supposedly reflect the relative contribution of food sources over a longer period of the mussel's life (Nix *et al.*, 1995; Dattagupta *et al.*, 2004; Becker *et al.*, 2010). Indeed, carbon sources used by the two types of symbionts display distinct $\delta^{13}C$ signatures, and as a result, mussels harbouring only sulphur oxidisers often show less negative tissue $\delta^{13}C$ values (in the range of −20‰ to −40‰) than mussels harbouring methane oxidisers or dual symbioses (−21‰ to −94‰) (Duperron, 2010). Roughly, one could expect mussels exposed to higher methane to display higher ratios of methanotrophs to thiotrophs, and if methane is higher over a long period of time, more negative $\delta^{13}C$ values relative to mussels exposed to lower methane. Nitrogen and sulphur stable isotope composition were also measured. Results are discussed in the light of measured ambient methane concentrations at the mussel habitat and compared with previous data obtained from hydrothermal vent mussels.

MATERIALS AND METHODS

Sampling locations

Mussels were collected using the ROV *Quest* (MARUM, Bremen, Germany) from two cold seep areas in the Gulf of Guinea during the cruise M76/3b with the RV *Meteor* (2008, chief scientist: A. Boetius, web page: http://www.ifm.zmaw.de/fileadmin/files/leitstelle/meteor/M76/M76-3b-SCR.pdf). A set of five mussels was collected by a net haul from a dense mussel bed of several tenths of metres dimension at the DIAPIR site (6°41.58S, 10°20.94E; 2708 m depth) during ROV dive 207. This site, characterised by a large mussel bed, was found by the ROV sonar as a result of intense bubbling, releasing high amounts of methane into the water column (Fig. 1A). Only one exploratory dive could be carried at this site during mission M76/3b. Three sets of mussels were collected from the REGAB site (5°47.86S; 9°42.69E, 3170 m depth). During *Quest* dive, 217, five mussels were collected from a central site with a large mussel bed on carbonate crust and strong emission of free gas (Fig. 1B). During dives 222 and 223, two sets of five mussels were collected from patchy mussel aggregates overlying soft sediments (Fig. 1C). All mussel specimens were collected from dense mussel aggregates, which were also inhabited by visible escarpid tubeworms, gastropods and crustaceans. Sampling locations 222 and 223 were separated by 3.4 m and were approximately 27 m away from the aggregate sampled during dive 217 (see Fig. 1D). Sampling locations 222 and 223 correspond to site M3 described previously (Duperron *et al.*, 2005; Olu-Leroy *et al.*, 2007a). Initially, no methane bubbles were observed at both sites (222 and 223); however, after strong disturbance with the net, some gas escaped the sediment. Sampled sets of mussels each consisted of five adult specimens, representing five distinct size classes ranging from the largest mean shell length of 186.8 mm (SD = 13.3 mm, M1) to the smallest mean shell length of 39.4 mm (SD = 12.2 mm, M5, Table 1). Mussels

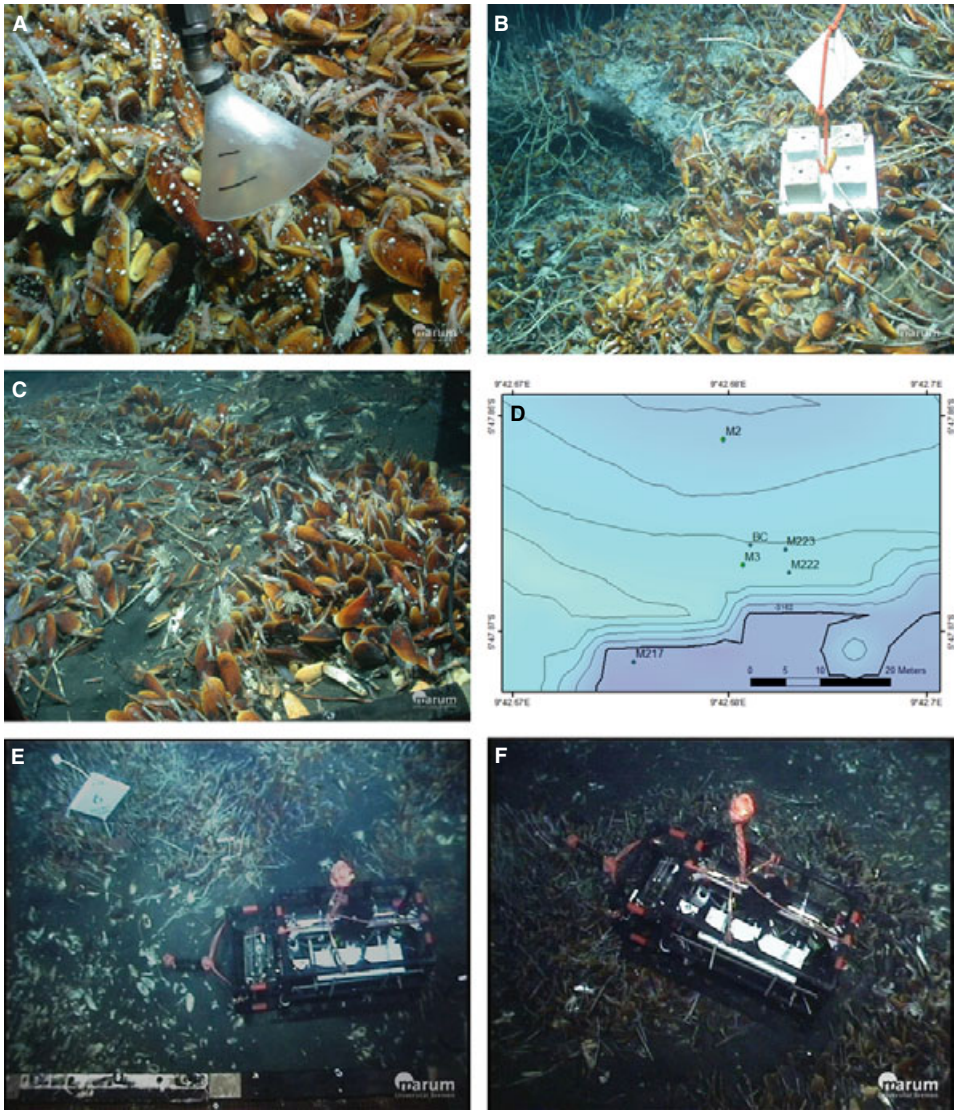

Fig. 1 Sampling sites. (A) DIAPIR, dive 207, methane is bubbling and hydrates accumulate on the funnel sampler, indicative of high degassing. (B) REGAB, dive 217, mussels were sampled above a hydrate accumulation. (C) REGAB, dive 222, mussels were sampled on sediment, and methane bubbles were observed only upon disturbance. (D) Map displaying the sites sampled for mussels (M) at REGAB during dives 217, 222 and 223, the benthic chamber on mussels (BC), and for reference, the localisation of sites M2 and M3 described in Duperron *et al.* (2005) and Olu-Leroy *et al.* (2007a,b). (E) Benthic chamber incubation during dive 223 outside of mussel aggregate. (F) Benthic chamber incubation on the mussel aggregate during dive 223. Image Source: MARUM University Bremen.

were kept in closed boxes filled with ambient seawater at 4 °C until processing onboard. Shells sizes were measured (maximum length, height and width), and gills were dissected. The most anterior part of the gill was stored for FISH as described elsewhere (Duperron *et al.*, 2005), and two fragments were frozen for stable isotope characterisation.

Methane measurements

The dissolved methane efflux at a mussel aggregate and just next to it (<0.5 m distance, Fig. 1E,F) was determined *in situ* using a benthic chamber module (Boetius & Wenzhöfer, 2009; Treude *et al.*, 2009), during ROV dive 223 at the REGAB site (Pangaea Event Labels: M76/3b_364_CHAM1; M76/3b_364_CHAM2). The benthic chamber module was gently lowered by the ROV to ensure a targeted measurement without major disturbances of the mussel habitat at the selected location. The chamber encloses 987 cm^2 of sediment with 10–25 cm overlaying bottom water visually determined using the ROV camera system. The enclosed bottom water was gently stirred, and a one-way valve ensured release of excess water during placement of the chamber. To assess the change in the methane concentration over time, a pre-programmed syringe system integrated in the benthic chamber module was used to take samples from the enclosed bottom water at defined time intervals. Back on board, the

Table 1 Number of gill bacteriocytes and stacks analysed, mean percentage and standard deviation of volume occupied by methane oxidisers (MOX) in gill bacteriocytes sections, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values (in ‰ vs. standards), and shell length in mm

Dive	Methane (bw)	Specimen	Bacteriocytes (stacks)	Mean % MOX	Standard deviation	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{34}S$ (‰)	Shell length (mm)
217	0.6 μM	M1	21 (4)	69.0	21.8	−62.6	1.6	15.8	193
REGAB		M2	30 (5)	79.1	6.3	−85.6	3.2	8.2	129.5
		M3	15 (4)	85.6	13.9	−85.9	2.9	14.7	89.1
		M4	14 (4)	72.1	8.6	−71.6	1.8	13.0	62.4
		M5	10 (3)	71.5	8.1	−66.3	2.3	12.8	38.9
222	–	M1	16 (3)	70.1	10.4	−58.6	−0.5	11.8	171.6
REGAB		M2	28 (4)	67.5	8.7	−59.7	−1.9	5.7	151
		M3	25 (4)	70.9	7.6	−61.5	−2.1	13.5	118.4
		M4	29 (4)	66.4	9.7	−59.0	−3.8	7.5	102.7
		M5	18 (3)	75.1	7.9	−56.8	−2.6	–	51.8
223	2.1 μM	M1	22 (4)	64.7	6.6	−62.9	−1.9	11.8	192.3
REGAB		M2	27 (4)	79.2	6.6	−62.5	−1.0	8.4	138.5
		M3	18 (3)	57.5	18.3	−61.9	−2.7	11.6	98.1
		M4	27 (6)	71.1	6.9	−62.3	−1.0	10.1	76
		M5	26 (5)	80.9	6.3	−64.1	−1.6	–	44.1
207	24.1 μM	M1	21 (4)	88.4	10.9	−69.9	3.2	22.3	196.5
DIAPIR		M2	28 (3)	79.6	8.6	−81.3	3.0	16.5	165.2
		M3	30 (5)	87.6	5.8	−83.6	3.6	–	127.1
		M4	16 (3)	86.1	7.4	−82.9	4.1	–	77.4
		M5	8 (2)	85.4	7.4	−79.0	2.1	–	22.9

See text for sampling sites. Mean methane concentration in bottom water (bw) is given for each measured site. All metadata is stored in the PANGAEA database (http://www.pangaea.de) under labels M76/3b_302_NET1 (dive 207), M76/3b_344_NET3 (dive 217), M76/3b_361_NET1 (dive 222) and M76/3b_364_NET1 (dive 223).

methane water samples were immediately transferred into vacuumed vials and fixed using 2–3 pellets of solid NaOH. Filled vials were thoroughly shaken to release the dissolved methane into the headspace. The methane concentrations were measured on board by injecting 100 μL of headspace into a two-channel 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph. The methane efflux was calculated according to Felden *et al.* (2010).

The bottom water methane concentration was determined on samples taken with the ROV-based KIPS water sampler in the vicinity of the sampling sites (Garbe-Schönberg *et al.*, 2006). Methane samples near mussel aggregates were acquired during ROV dives 207 (Pangaea Event Labels: M76/3b_302_KIPS2 to M76/3b_302_KIPS9), 217 (Pangaea Event Label: M76/3b_344_KIPS7) and 223 (Pangaea Event Labels: M76/3b_364_KIPS9; M76/3b_364_KIPS6; M76/3b_364_KIPS5) prior to mussel sampling. Methane concentration was measured on board as described earlier.

Turnover rates of methane

Ex situ turnover rates of methane anaerobic oxidation of methane (AOM) below (Pangaea Event Labels: M76/3b_344_PUC28; M76/3b_344_PUC23; M76/3b_344_PUC15) and right next to a mussel aggregate (Pangaea Event Label: M76/3b_361_PUC15) were determined near the collection sites 222/223 at REGAB, according to Felden *et al.* (2010) and references therein. According to the whole-core injection method (Jorgensen, 1978), triplicate push core subsamples (diameter 2.8 cm) were injected at every centimetre with 25 μL of $^{14}CH_4$ tracer (activity 2.5 kBq μL^{-1}). Subcores were incubated for 12 h at *in situ* temperature (4 °C) in the dark and afterwards sliced into 1 cm sections. Samples were immediately transferred into gas-tight glass vials filled with NaOH (2.5% w/v), and the vials were shaken thoroughly. Applying the headspace method, the methane turnover rates were determined according to Treude *et al.* (2003). The methane concentration and the radioactivity of the samples were determined in the home laboratory using gas chromatograph (5890A; Hewlett Packard, Palo Alto, CA, USA) and a liquid scintillation counter, respectively. AOM rates were calculated using the following formula:

$$\mathrm{AOM} = \frac{^{14}CO_2}{(^{14}CH_4 + ^{14}CO_2)} \times \frac{CH_4}{(V \times t)}$$

where CH_4 represents the methane concentration, $^{14}CO_2$ and $^{14}CH_4$ the radioactivity of the produced carbon dioxide and labelled methane, respectively, t the incubation time and V the sample volume.

H_2S concentration

Bottom water and porewater from sediments below and next to the mussel aggregates at collection sites 222 and 223 at REGAB, as well as at DIAPIR (Pangaea Event Label:

M76/3b_344_PUC30; M76/3b_361_PUC14; M76/3b_302_PUC18), were extracted immediately after recovery of the push cores, by means of Rhizon moisture samplers (Seeberg-Elverfeldt *et al.*, 2005; pore size 0.1 μm) at *in situ* temperature. Subsamples for the determination of the H_2S concentration were fixed and measured spectrophotometrically (detection limit 20 μM) in the home laboratory (Cline, 1969). Bottom water sulphide concentration could be detected *ex situ* only in the core taken below the mussel aggregate (site 223). Unfortunately, H_2S bottom water content was not determined *in situ* because of failure of the microsensor measurements.

Stable isotopes values

Frozen gill tissue from each specimen was acidified (0.1 N HCl for 6 h), dehydrated (60 °C, 48 h), grounded into powder with mortar and pestle and sent to Iso-Analytical (Crewe, UK) for the determination of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotopic ratios. Stable isotope data are expressed as the relative per mil (‰) differences between the samples and the conventional standard Pee Dee Belemnite (PDB) for carbon, air N_2 for nitrogen and Canyon Diablo Troilite for sulphur, according to the following equation:

$$\delta(X) = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right] * 1000$$

Where X (‰) is ^{13}C, ^{15}N, ^{34}S abundance and R is the ^{13}C: ^{12}C, ^{15}N: ^{14}N, ^{34}S: ^{33}S ratio.

3D FISH acquisition

Gill tissue was dehydrated in ethanol series and embedded in polyethylene glycol distearate: 1-hexadecanol (9:1) wax. Transverse sections of gill filaments, 10 μm thick, were cut using a microtome (Jung, Heidelberg, Germany) and deposited on SuperFrost Plus slides (Roth, Karlsrühe, Germany). Wax was removed using ethanol, and sections were rehydrated. Hybridisation was performed with 30% formamide as described previously using the eubacteria-specific probe Eub-338 (5′-GCTGCCTCCCGTAGGAGT-3′), the methanotroph-specific probe ImedM-138 (5′-ACCATGTTGTC CCCCACTAA-3′) and the thiotroph-specific probe BangT-642 (5′-CCTATACTCTAGCTTGCCAG-3′) (Amann *et al.*, 1990; Duperron *et al.*, 2005, 2008). Probes were labelled with FITC, Cy3 and Cy5 fluorochromes. Hybridised sections were mounted using SlowFade medium (Invitrogen, Carlsbad, CA, USA) and a coverslip. Image stacks, consisting of series of consecutive images taken every 0.3 μm over the whole thickness of the gill filament section, were obtained for each fluorochrome using an Olympus BX61 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with Optigrid™ (QiOptic, Rochester, NY, USA).

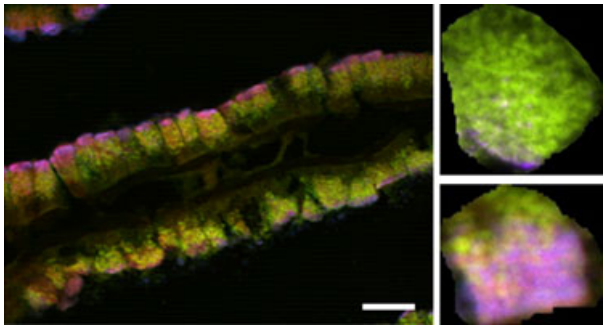

Fig. 2 Examples of flattened image stacks of FISH images from a gill filament (left, scale bar = 30 μm) and two individual bacteriocytes (right). Volumes occupied by sulphur oxidisers (labelled in pink) and methane oxidisers (in green) were computed using SymbiontJ (see text). Upper right: a bacteriocyte with 89% volume occupied by methane oxidisers. Lower right: a bacteriocyte with 47% volume occupied by methane oxidisers.

Image analysis

Image stacks from the three fluorochromes were merged into a red green blue (RGB) image, using the red channel for the Eub-338 signal, the green channel for ImedM-138 and the blue channel for BangT-642 (Fig. 2). Individual bacteriocytes were randomly selected and isolated manually using the ImageJ software (Abramoff *et al.*, 2004). Relative volumes occupied by methanotrophic and thiotrophic symbionts were measured as percentages of the total volume occupied by bacteria in each bacteriocyte section using the plugin SymbiontJ [(Halary *et al.*, 2008) http://www.snv.jussieu.fr/%7Ewboudier/softs/symbiontj.html].

Statistical analyses

Percentages of methanotrophic bacteria were analysed using Minitab (State College, PA, USA) (version 15). Data were transformed using the arcsine function and tested for normality using the Anderson–Darling test, and heterogeneity of variance was tested by both the Bartlett and Levene tests. A general linear model (GLM) and ANOVA were run to test the effect of sampling locations (dive) and size classes (M1–M5) on the relative abundance of methanotrophic bacteria. *Post hoc t*-tests were performed applying the Bonferroni correction, in which the threshold *P*-value to reject null hypothesis is corrected for multiple testing.

RESULTS

Habitat geochemistry

The initial video survey, using the ROV camera system, revealed intensive bubbling at the sampling locations 207 (DIAPIR) and 217 (REGAB), indicating high *in situ* methane concentrations at these sites (Fig. 1A,B). *Ex situ* bottom water methane concentrations at the two sampled mussel

aggregates of REGAB, dive 217 and 223, were only 0.6 and 2.1 μM (±0.2 SD, $n = 3$), respectively. At the intensely bubbling DIAPIR site (dive 207), *ex situ* bottom water methane concentrations were higher than at REGAB, ranging between 7 and 86.7 μM (mean = 24.1 μM; ±26.5 SD, $n = 8$).

Detailed investigation of the pore water chemistry and benthic fluxes was only available at the REGAB pockmark, at mussel sampling locations 222 and 223 (Fig. 1C,D). Benthic chamber measurement at one of the mussel aggregates revealed a dissolved methane efflux between 1 and 81 mmol m^{-2} day^{-1} within a period of 3-h incubation (Fig. 1F). The observed variation in methane efflux most likely reflects temporal variations in the fluid flow conditions. The incubation performed on sediments next to the mussel aggregate (Fig. 1E) showed a much higher dissolved methane flux (334 mmol m^{-2} day^{-1}). The depth (0–10 cm bsf) integrated average microbial oxidation rate of methane (aerobic and anaerobic oxidation of methane) was in the range of 19 mmol m^{-2} day^{-1} ($n = 3$) both outside and below the mussel aggregate. *Ex situ* determination of pore water hydrogen sulphide in the sediment below the mussel aggregate and next to it revealed increasing sulphide concentration with depth, where in the deepest investigated layer, it reached 9 mM (16 cm depth) and 15.5 mM (12 cm depth), respectively. At both the sites, sulphide could be detected in the topmost surface layers, ranging between 0.6 and 1.2 mM. The only *ex situ* bottom water sulphide measurement revealed a concentration of 0.1 mM at the mussel patch (site 223). Although sulphide was not measured *in situ*, occasional degassing events observed *in situ*, as well as *ex situ* after core retrieval, indicated that potentially gas ebullitions might be responsible for temporarily highly variable transport of subsurface sulphide to the bottom water at certain sites at REGAB. Sulphide could not be detected in the first 12-cm sediment depth at DIAPIR (dive 207). Deeper, the sulphide concentration increased slightly and reached only 0.9 mM at 18 cm depth.

Stable isotopes analyses

Carbon stable isotope values in mussel gill tissue varied between −85.9‰ and −56.8‰ and are summarised in Table 1. Mussel samples from the locations 207 and 217, characterised by free gas emission, were more depleted in ^{13}C ($\delta^{13}C$ values between −85.9‰ and −62.6‰) than samples collected on locations not showing active gas bubble escape (values between −64.1‰ and −56.8‰). Nitrogen values ranged between −3.8‰ and 4.1‰ and were significantly negatively correlated with $\delta^{13}C$ values ($r = -0.85$, $P < 0.0001$, Fig. 3A). Mussels from locations 222 and 223 displayed negative $\delta^{15}N$ values (between −3.8‰ and −0.5‰), while mussels from locations 207 and 217 displayed positive values (between 1.6‰ and 4.1‰). Values for sulphur ($\delta^{34}S$) were measured in 15 specimens from all dives and ranged between 5.7‰ and 22.3 ‰ (Table 1, Fig. 3C).

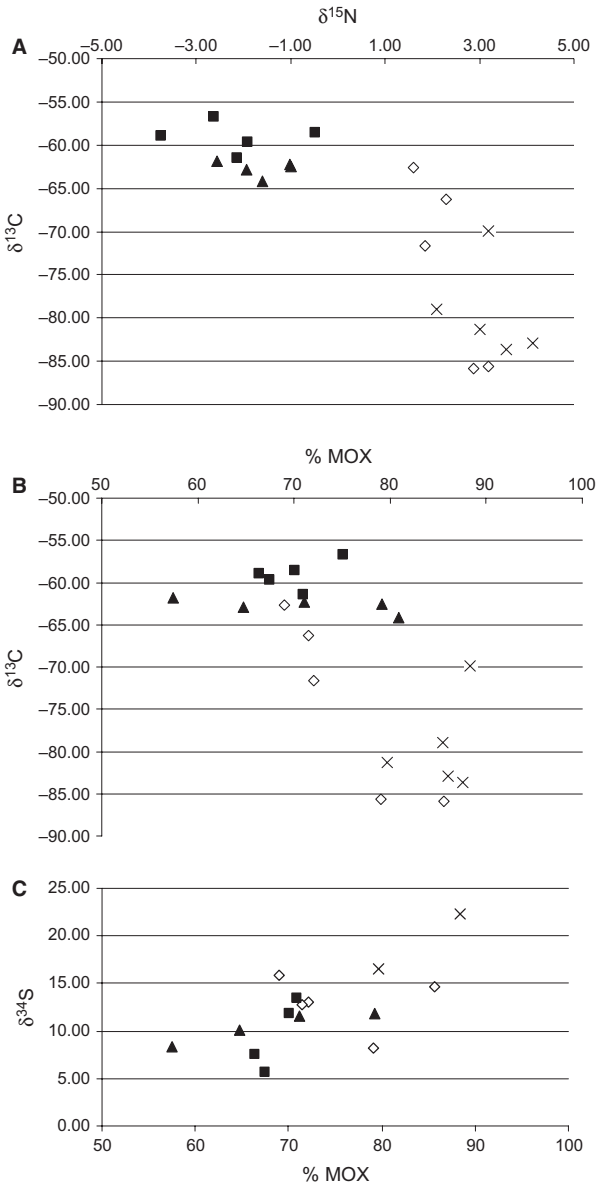

Fig. 3 (A) Relationship between gill $\delta^{13}C$ and $\delta^{15}N$ values in ‰ vs. standards ($r = -0.85$, $P < 0.0001$). (B) Relationship between $\delta^{13}C$ values and the percentage of volume occupied by methane oxidisers (% MOX) ($r = -0.712$, $P < 0.001$). (C) Relationship between $\delta^{34}S$ values and the percentage of volume occupied by methane oxidisers (% MOX) ($r = 0.663$, $P = 0.007$). Sampling points: 207 (cross), 217 (white diamond), 222 (black square) and 223 (black triangle).

3D FISH

Methanotrophic and thiotrophic symbionts co-occurred in gill bacteriocytes of all investigated specimens (Fig. 2). Bacteriocytes were densely populated, and as the sum of methanotroph (probe ImedM-138) and thiotroph (probe BangT-642) FISH signals fully overlaid the bacterial (probe Eub-338) signal, it can be assumed that no abundant bacterial symbiont was missed. Taking this into account, and the fact

that methanotrophs clearly dominated the gills of all specimens, further analysis mainly focused on the relative abundances of methanotrophs. Thiotrophs tended to be mainly located in the most apical part of bacteriocytes, closer to circulating fluids (Fig. 2). Two to six RGB stacks were obtained from each of the 20 studied specimens (77 useable acquisitions in total), and 3–10 bacteriocytes were analysed per RGB stack, resulting in 8–30 bacteriocytes analysed per individual (overall 429 bacteriocytes, Table 1). The arcsine-transformed percentages of methanotrophs measured for dives 207 and 222 were normally distributed ($P > 0.63$ for both), contrary to the percentages of methanotrophs measured for 223 and 217 dives ($P < 0.005$). Even though variances were unequal, the GLM tests are known to be relatively robust even if variances are unequal (Campbell, 1967). The GLM indicated that significant differences existed among the sets of mussel samples from REGAB and DIAPIR ($F_{1,3} = 51.13$, $P < 0.001$). *Post hoc* tests with a Bonferroni correction indicated that at REGAB, percentages of methanotrophic bacteria measured from the closeby sedimentary mussel aggregates sampled during dives 222 and 223 were not significantly different (69.5% and 71.8% methanotrophs in volume, $P = 0.072$), while bacteriocytes from specimens collected from the central carbonates (dive 217) had a significantly higher percentage (75.9%, $P < 0.008$). Specimens from DIAPIR (dive 207) showed a markedly higher percentage of methanotrophs (85.2%, $P < 0.008$) than those from specimens collected at the three locations in REGAB. Regarding size classes, mean percentages varied between 72.5% (M4) and 78.3% methanotrophs (M5), and GLM indicated overall non-significant differences with size ($F_{1,4} = 2.09$, $P = 0.081$). Using *post hoc t*-tests with a Bonferroni correction, the only significant difference was found between size classes M4 and M5 (see Table 1, $P < 0.005$), supporting that if a size effect does exist, it is very moderate in the range of sizes considered here.

DISCUSSION

Dominance of methanotrophs

Flexibility of dual symbioses to adapt to varying environmental conditions has been suggested to be the major factor explaining the success of dual symbiotic mussels in the Atlantic area (Distel *et al.*, 1995; Halary *et al.*, 2008; Duperron, 2010). Several previous studies indicate that methanotrophs tend to predominate in gills of dual symbiotic mussels at many cold seep habitats, in accordance with generally higher fluxes of methane from the seafloor compared with sulphide. However, attempts to quantify the natural spatial variation in symbiont densities in comparison with habitat conditions remain scarce. The 3D FISH-based approach used here was recently developed and applied on *B. azoricus*, a dual symbiotic mussel from the Mid-Atlantic Ridge. Thiotrophs were shown to dominate over methanotrophs, occupying between 53.1% and 68.5% of the bacterial volume in gill bacteriocytes of specimens from the Menez Gwen and Lucky Strike hydrothermal vent sites (Halary *et al.*, 2008). The volume occupied by thiotrophs was also shown to increase quickly following a simulated sulphide pulse of 35 μM for 6 h (Halary *et al.*, 2008). In the present study, we provide the first 3D FISH data regarding *Bathymodiolus* mussels from two cold seep sites, REGAB and DIAPIR (Gulf of Guinea). As previously suggested based on qualitative estimates, methanotrophs dominated in all of the investigated specimens (Duperron *et al.*, 2005). They represented between 57.5% and 88.4% of the volume occupied by bacteria in bacteriocytes. Within the range of shell lengths considered here, variation in methanotroph percentages with mussel size class was not significant. Previous studies have already shown that both methanotrophic and thiotrophic symbionts were present in very small specimens of dual symbiotic mussels and that symbiont contribution to host carbon nutrition increased with time during early mussel growth (Salerno *et al.*, 2005; Martins *et al.*, 2008). Smallest specimen investigated here measured 22.9-mm shell length and was already an adult. Thus, to potentially reveal size-linked variations not seen in larger mussels, smaller specimens should be included in future studies.

Variations among and within sites and link with methane concentrations

AOM (one of the main processes responsible for methane consumption and sulphide production at cold seeps) rates detected at REGAB were relatively high and comparable to fauna-inhabited habitats from other cold seeps [e.g. 20 mmol m^{-2} day^{-1} at siboglinid tubeworm sites at the Haakon Mosby Mud Volcano (Felden *et al.*, 2010)]. The AOM process did not account for the complete removal of the ascending methane at REGAB, and relatively high methane concentrations and fluxes could be detected in the bottom water where it could become available to the symbiotic mussels. Close to the mussel beds at the REGAB seep, some of the highest methane fluxes with up to 334 mmol m^{-2} day^{-1} were measured – a flux comparable to those of microbial mat habitats at other seeps (Felden *et al.*, 2010). Mussels containing methanotrophic symbionts, such as ones found at REGAB, consume methane from the surrounding bottom water and thus act as an additional biofilter that decreases the transport of methane to the water column. Indeed, similarity in the AOM rates and discrepancy in the methane effluxes measured between the mussel aggregate and the adjacent bare sediment (1–81 and 334 mmol m^{-2} day^{-1}, respectively) could potentially indicate partial removal of the ascending methane by the mussel consumption, resulting in lower methane fluxes detected right above the mussel aggregate. It has to be noted that the observed discrepancy in the methane effluxes among the two sites can be

a consequence, in addition or solely, of the high spatial heterogeneity existing at REGAB.

Methane concentrations in the surrounding bottom water at REGAB ranged from 0.6 to 2.1 μM. These values fall into in the low range of values measured in 2001 in the water column around mussels [0.7 and 33.4 μM; (Duperron *et al.*, 2005; Olu-Leroy *et al.*, 2007a)]. This may indicate to a steady, but temporarily fluctuating, availability of methane for the mussels or results from spatial heterogeneity.

Sulphide could be detected throughout the complete core length, as well as in the topmost surface sediment at the mussel aggregate and next to it (REGAB site 223). Although our data suggest that sulphide can be present in the bottom water at REGAB, we strongly believe that the elevated sulphide content detected in the bottom water is an artefact of the *ex situ* sampling procedure, as it could be observed that the respective core was strongly degassing on board, potentially transporting subsurface sulphide to the bottom water. This is supported by the finding from Olu-Leroy *et al.* (2007a) and Duperron *et al.* (2005), whose targeted sampling could show very low H_2S availability (<0.1 μM) in the bottom water surrounding mussel aggregates at REGAB in 2001. Nevertheless, *in situ* gas ebullitions occurring at localised areas at REGAB probably transport variable amounts of sulphide to the bottom water over temporarily limited periods of time. However, it remains unknown whether mussel symbionts can use such short pulses of sulphide. Although the DIAPIR site was not characterised as detailed, methane measurements in bottom water yielded higher values than at REGAB, with an average value of 24.1 μM, and sulphide was below the detection limit in the bottom water and in the surface sediment. Methane is clearly available to mussels at all investigated locations, and sulphide content is most likely low in the bottom water. This could explain the predominance of methanotrophs in the gills of all REGAB/DIAPIR mussels.

Mussels from the two sampling locations characterised by the visible emission of free gas in the form of bubble streams through the mussel beds (DIAPIR dive 207 and REGAB dive 217) showed highest mean abundances in methanotrophs (75.9–85.2%). The specimens collected from the two other, not actively degassing, mussel aggregates sampled at REGAB during dives 222 and 223 displayed significantly lower mean values (69.5–71.8% methanotrophs). The highest methanotroph relative abundance was measured in specimens from DIAPIR, the site with the highest bottom water methane concentration and the most vigorous bubbling of gas a few metres away from the sampling location. Symbiont relative abundances have been previously documented to vary and adapt depending on local characteristics at hydrothermal vents as well as in experimental settings in *B. azoricus* (Kadar *et al.*, 2005; Duperron *et al.*, 2006; Halary *et al.*, 2008; Riou *et al.*, 2008, 2010). Detailed characterisation of mussel habitats at Mid-Atlantic Ridge hydrothermal vent sites revealed *in situ* sulphide concentrations of up to 40 and 100 μM around mussels at the Lucky Strike and Menez Gwen sites, respectively. *Ex situ* methane concentrations of up to 10, 18 and 30 μM were measured around mussels at Menez Gwen, Lucky Strike and Rainbow, respectively (Le Bris & Duperron, 2010). Using these values and geochemical modelling approaches, the authors compared the energy gradient along the mixing zone and related this with FISH-based estimations of symbiont relative abundances. Results indicated that sulphide- and methane oxidisers each occupied half of the total bacterial volume in mussels at Menez Gwen (53.1% sulphur oxidisers), a site where sulphide and methane represented comparable energy pools in the mussels local habitat. Sulphur oxidisers dominated in the gills of mussels from the 'Tour Eiffel' edifice at Lucky Strike (56.2–70.1% sulphur oxidisers) where sulphide was the most abundant source of energy in the mixing zone. On the other hand, methanotrophs dominated in the gills of mussels at Rainbow (61.6% methane oxidisers), a site rich in methane, resulting from serpentinisation, and almost devoid of free sulphide (Le Bris & Duperron, 2010). Based on these previous observations, it is reasonable to assume that the higher proportions of methanotrophs observed in the gills of *B.* aff. *boomerang* specimens sampled at the two actively degassing locations from the present study result from the higher methane availability in the ascending fluids, illustrating a direct link between symbiont abundances and the chemical characteristics of the environment.

Relationship between methanotrophs' percentages and carbon and sulphur stable isotope signatures

Mean carbon stable isotope values of mussels from the four sampling points in this study were between −79.4‰ and −59.1‰. These values extend the range previously reported for gills of REGAB mussels collected in 2001 (mean values between −62.7‰ and −67.0‰ from three sampling points at REGAB) (Olu-Leroy *et al.*, 2007a). Calculated volumes percentages of methanotrophic symbionts were significantly correlated with carbon stable isotope signatures ($r = -0.712$, Fig. 3B; $r = -0.719$, $P < 0.001$ using arcsine-transformed percentages). However, when looking at specimens from the different sampling points separately, this correlation became less evident, and two distinct groups appeared. Percentages of methanotrophs in specimens from dives 222 and 223 were indeed spread between 57.5% and 80.9%. Their range of $\delta^{13}C$ values from −64.1‰ to −56.8‰ was relatively narrow (Table 1). Correlation in this group was not significant ($P = 0.68$), suggesting that long-term exploitation of carbon sources could be similar for all specimens although symbiont densities differed at the time of sampling, possibly as a result of short-term dynamics. The second group included specimens from dives 217 and 207, for which methanotrophs represented between 69.0% and 88.4% of bacterial volume, and $\delta^{13}C$ values were between −85.9‰ and −62.6‰. Correlation was significant in this group ($P < 0.05$). Because methane at

REGAB has a quite negative signature of −70‰ to −67‰, with a value of −69.3‰ published for gas hydrates (Charlou *et al.*, 2004; Olu-Leroy *et al.*, 2007a), this suggests greater contribution of methane to the host carbon pool in specimens with higher percentages of methanotrophs. These results overall indicate that mussels collected from sampling locations displaying bubble emission and higher concentrations of dissolved gas tend to display higher percentages of methanotrophs and a more negative carbon isotope signature. It has to be noted that the distinct behaviour observed in the two groups could underline significant biological differences, which will need to be addressed using more specimens.

Values between 5.7‰ and 22.3‰ measured for $\delta^{34}S$ in gills of *B.* aff. *boomerang* are higher than values previously reported for *Bathymodiolus childressi*, a cold seep methanotrophic mussel from the Gulf of Mexico, which displayed values between −1.5‰ and 13.5‰ (Dattagupta *et al.*, 2004; Cordes *et al.*, 2010). These values were significantly positively correlated with arcsine-transformed percentages of methanotrophs ($r = 0.68$, $P < 0.006$, Fig. 3C). This correlation was driven by the same two groups as above and became non-significant if we considered only specimens from dives 207 and 217 ($P = 0.27$) or specimens from dives 222 and 223 ($P = 0.17$). Previous studies mentioned $\delta^{34}S$ values of 21‰ for sulphates in the water column (20.3‰ in bottom seawater), resulting in particulate organic matter with signatures between 17‰ and 21‰ (Carlier *et al.*, 2010; Cordes *et al.*, 2010). Sulphate reduction by bacteria results in values between −25‰ and 5‰. With a mean value of 12.5‰, data reported here suggest acquisition of sulphur from bottom water or organic matter rather than from sulphide emitted by the underlying sediment. The trend towards higher $\delta^{34}S$ values in *B.* aff. *boomerang* specimens harbouring higher percentages of methane oxidisers supports that specimens with lower proportion of sulphur oxidisers derive more of their sulphur from the bottom water sulphate or organic matter. Specimens with lower relative abundances of methane oxidisers, and thus higher abundances of sulphur oxidisers, likely derive a higher fraction of their sulphur from sulphide, probably through symbiont metabolism. It is important to notice that sulphur-oxidising symbionts of *B.* aff. *boomerang* do not store sulphur granules, so values are probably not influenced by the presence of such structures (Duperron *et al.*, 2005). However, sulphur metabolism remains poorly understood in deep-sea mussels.

Relationship between carbon and nitrogen signatures

A significant correlation between gill $\delta^{15}N$ and $\delta^{13}C$ signatures was previously found in the cold seep species *B. heckerae*, a close relative of the REGAB mussel occurring in the deep Gulf of Mexico, but not in *B. brooksi* and *B. childressi* (Duperron *et al.*, 2007; Olu-Leroy *et al.*, 2007b). This suggested coupled dynamics between carbon and nitrogen uptake in *B. heckerae*. Interestingly, such a correlation also occurs in *B.* aff. *boomerang*, but while the correlation was positive in *B. heckerae*, it is negative here (Fig. 3A). This correlation is driven by the same two groups of mussels as aforementioned. Specimens from dives 222 and 223 displayed negative $\delta^{15}N$ values and correlation was not significant ($P = 0.39$); meanwhile, specimens from dives 217 and 207 displayed positive $\delta^{15}N$ values correlated with $\delta^{13}C$ ($P < 0.05$). The distinct behaviour, along with non-overlapping ranges of $\delta^{15}N$ values, suggests that specimens from the two groups have different coupling mechanisms. They could be using different nitrogen sources. Indeed, ammonium and nitrate can both be used by *B. childressi* (Lee & Childress, 1994) and thus possibly by other bathymodiolin mussels. Signatures in mussel tissue are also dependant on the signature of local nitrogen sources, which were not measured at REGAB and DIAPIR.

CONCLUSION

Dissolved methane diffusing from the seafloor appears to be the dominant energy source to cold seep mussels collected from the deep Gulf of Guinea. Generally, sulphide was retained in the seafloor, besides a few hotspots where degassing of methane could transport sulphide into the overlying bottom waters. As a result, methanotrophs dominate in the gills of the dual symbiotic mussel *B.* aff. *boomerang*. Mussels from the two sampling locations characterised by ebullition of free gas showed significantly higher relative abundances of methanotrophs in their gills compared with those from two other sampling locations not emitting free gas. The relationships between percentages of methanotrophs, carbon, sulphur and nitrogen signatures also differed between these two groups, possibly revealing habitat-linked biological differences that remain to be investigated. Following this study, time series sampling of mussels coupled with geochemical measurements should be undertaken to better understand the interplay between temporal variations of environmental parameters and symbiont dynamics at cold seeps.

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Chapter III

Spatial scaling of bacterial communities associated with cold seep habitats of the Eastern Mediterranean deep sea

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Abstract

Cold seep ecosystems are highly productive, fragmented ecosystems of the vast deep-sea floor. They form worldwide where methane and other hydrocarbons reach the surface seafloor, and are characterized by rich chemosynthetic communities fueled by the microbial utilization of hydrocarbons. Here we investigated the spatial scaling of sediment bacterial communities at 14 different habitats associated with cold seeps in the Eastern Mediterranean deep-sea, using an interdisciplinary approach combining microbial fingerprinting and *in situ* and *ex situ* geochemical techniques. Bacterial communities showed a high dissimilarity on the scale of meters between methane-seeping habitats and their surroundings, matching differences in geochemical settings. Highest bacterial turnover with more than 50 % of replacement with new bacterial types was detected between individual seep habitats, on intermediate spatial scales ranging from tens to hundreds of meters. Variations among the seep bacterial community structure were not directly correlated to geographic distance, and occasionally higher similarities were detected between distant (> 300 km separated) than proximate sites (< 1 km separated). However, number of bacterial types increased substantially with increasing number of sampled seep habitats. The patchy structure of bacterial communities reflected well the differences in sediment geochemistry, and specifically seep bacterial β-diversity was positively correlated to variability in sulfide, DIC and sulfate concentrations as indicators of seepage activity and microbial hydrocarbon consumption. The results of this study show that patches of reduced seep habitats, often not more than few meters in diameter, can be regarded as biodiversity hotspots and contribute significantly to the biodiversity of deep-sea ecosystems. Hence, reduced habitats play an important role for the interconnectivity and resilience of cold seep ecosystems and should be integrated into conservation planning aiming to protect deep-sea biodiversity.

1 Introduction

Biodiversity, here defined as richness and variety of taxonomic entities (Magurran, 2003) is assumed to enhance crucial functions of natural ecosystems such as productivity and resilience (Chapin et al., 2000; Loreau et al., 2001; Danovaro et al., 2008). Increasing anthropogenic impact on the deep sea may affect its biodiversity and function, as well as the current and future services and goods this ecosystem provides for the well-being of the humankind (van den Hove, 2007; Benn et al., 2010). However, only since recently the focus on biodiversity conservation and establishment of marine protected areas (MPAs) has been expanded to include deep-sea ecosystems (Nandan et al., 2002; Gjerde, 2006; Davies et al., 2007). In order to establish effective deep-sea MPAs that would protect deep-sea biodiversity and function, as well as to anticipate responses of the deep-sea ecosystem to future environmental changes, it is important to gain knowledge on the biodiversity patterns of deep-sea organisms across space and time. Here we investigated the spatial scaling of bacterial community structure between cold seep habitats and their immediate surroundings to assess the turnover of bacterial types on distances of meters to hundreds of kilometers within an ocean basin.

Microorganisms represent the most numerous and abundant life form in deep-sea sediments (Rex and Etter, 2010; Whitman et al., 1998) and possess enormous catalytic potential and ability to couple multiple redox reactions of organic or inorganic compounds (Jørgensen and Boetius, 2007). Despite their crucial role in the functioning of the deep-sea ecosystem, still very little is known about the microbial biodiversity or the factors controlling their distribution (Jørgensen and Boetius, 2007).

Cold seeps ecosystems, fueled with energy via microbial transformation of methane and higher hydrocarbons, are known to possess the highest biomasses and productivity of all deep-sea ecosystems (Jørgensen and Boetius, 2007). However, due to the sporadic, localized and dynamic supply of their main energy source – methane and other hydrocarbons, cold seeps have a highly fragmented distribution along passive and active continental margins, and represent isolated habitats on the vast deep-sea floor (Sibuet and Olu, 1998). Hence, their associated communities could be particularly vulnerable to potential habitat losses due to increased exploitation of deep-sea floor resources (Ramirez-Llodra et al., 2011; Van Dover et al., 2011). Main questions regarding the biodiversity patterns, dispersal capabilities and life history of

fauna and microbes restricted to these chemosynthetic habitats need to be answered in order to understand the interconnectivity and resilience of these dynamic ecosystems (Tyler and Young, 1999; Pradillon et al., 2005, 2007). Moreover, as biodiversity changes over multiple spatial scales (Levin, 1992; Green and Bohannan, 2006; Ramette and Tiedje, 2007; Martiny et al., 2011), a better understanding of the interconnectivity of these fragmented ecosystems needs to be based on quantitative assessments of spatial patterns of species diversity. Although members of the domains Bacteria and Archaea contribute the main biomass and functions in deep-sea ecosystems, such as organic matter and hydrocarbon degradation, sulfide production and consumption, to date virtually nothing is known about their spatial variation at the deep-sea floor (Huber et al., 2007; Bienhold, 2011).

In this study we investigated bacterial diversity patterns of seep ecosystems, with the specific aim to understand how bacterial communities of fragmented, highly reduced sediment habitats vary with spatial scales. Bacterial communities of 14 different habitats distributed over three cold seep ecosystems in the Eastern Mediterranean were studied in their environmental context, using a combined approach including bacterial community fingerprinting, porewater geochemistry, quantification of element fluxes and microbial consumption rates. This allowed comparison of microbial communities on small to intermediate geographical scales, ranging from tens of centimeters to hundreds of kilometers. The main aims were to better understand i) how specific bacterial communities of reduced methane-seeping habitats compare to their immediate oxidized vicinity ii) which factors shape the bacterial diversity at cold seep ecosystems iii) if bacterial communities of deep-sea seep ecosystems vary with spatial scales and iv) on which spatial scale highest variation in the bacterial diversity occurs.

2 Material and Methods

2.1 Description of sampling sites

During two expeditions in 2009, MSM13/3 & 4 on board the RV Maria S. Merian, different types of reduced sedimentary habitats were studied within the realm of three cold seeps in the deep Eastern Mediterranean sea i.e. the Amon Mud Volcano (AMV), the Central Pockmark (Pock) and the Amsterdam Mud Volcano (AmsMV;

Table 1). Reduced sites investigated in this study represented typical methane seeping habitats, characterized by highly reduced, sulfidic sediments. Some of the reduced habitats were covered with white thiotrophic bacterial mats (Table 1; Fig. 2b-c and e-f). Additional samples were retrieved from sediments not impacted by seepage activity (non-seep sites) located in the immediate vicinity of the cold seep sites (Table 1).

The Amsterdam Mud Volcano (AmsMV) is located at water depth of 2030 m, within the Anaximander Mountains region, on the northeast flank of the Mediterranean Ridge (Fig. 1b). The mud volcano has a "mud pie-like" structure (Ø 3 km), comprising of a 2 km wide flat-topped central dome elevated app. 100 m above the seafloor. Geological surveys revealed structures related to recent mud extrusions in the crater and outflow downslope to the south (Woodside et al., 1998; Zitter et al., 2005; Lykousis et al., 2008). Moreover, Haese et al. (2006) determined moderate advective flow rates (4 – 5 cm y^{-1}) of low salinity porewater. Elevated concentrations of methane have been detected in the sediment and in the bottom water above the mud volcano, along with elevated temperature (Woodside et al., 1998; Charlou et al., 2003). Finally, shallow gas hydrates (0.3 – 1.5 mbsf) accompanied with gas bubble release are common at the summit and the eastern central part (Zitter et al., 2005; Lykousis et al., 2008). All sampled habitats at the AmsMV had blackened sediments and were not overlaid by bacterial mats (Fig. 2d; Table 1).

The dome-shaped Amon Mud Volcano (AMV) has a diameter of 2 km and is located in the Nile Deep-Sea Fan (NDSF) area, southeast of the Mediterranean Ridge at water depths of 1120 m (Loncke et al., 2004; Dupre et al., 2007) (Fig. 1c - d). The center of the AMV is associated to gassy sediments laden with methane, elevated bottom water methane concentrations, relatively steep temperature gradients and a patchy distribution of bacterial mats at the outer rim of the center (Dupre et al., 2007; Dupré et al., 2008; Felden, 2009; Feseker et al., 2009). A conspicuous briny mudflow at the southwestern flank of the AMV, named the "sulfur band", is characterized by highly reduced sediment covered with extensive bacterial mats as well as patchy distributed carbonate crusts (Girnth et al., 2010; Fig. 2b-c). At the AMV, two types of reduced habitats were sampled i.e. typical hydrocarbon-vented sediments from the center of the AMV (Fig. 2a) and sediments associated to briny mud and bacterial mats from the "sulfur band" (Fig. 2b-c; Table 1).

The Central Province area of the NDSF lies at 1690 m and is characterized by many small circular pockmark-like depressions of few meters in diameter and about 1m deep, associated with active methane seepage and the occurrence of large flat authigenic carbonate crusts above reduced sediments, as well as white thiotrophic bacterial mats (Loncke et al., 2004; Dupre et al., 2007; Gontharet et al., 2007; Foucher et al., 2009; Grünke et al., 2011). Thiotrophic mats overlaid reduced blackish sediments (Fig. 2e-f; Table 1) at both investigated habitats at this cold seep site. An overview of all samples and geochemical measurements performed within this study, as well as the link to corresponding contextual data are given in Supplement Table 1.

2.2 Sediment sampling

Sediments (0.5 – 25 cm depth) from targeted sampling sites were recovered with ROV-based push cores (Ø 8 cm) and TV-guided Multiple Corer (MUCs; Ø 9.5 cm) (Table 1). After retrieval, sediments were immediately subsampled in 1 cm steps at *in situ* temperature (14 °C), and fixed for different types of analyses (Supplement Table 1).

2.3 Biogeochemical measurements

2.3.1 Porewater chemistry

Porewater from every centimetre sediment depth was extracted using Rhizon moisture samplers (Seeberg-Elverfeldt et al., 2005; pore size 0.1 μm) inserted into holes of predrilled cores liners at all investigated sites (Supplement Table 1). The porewater was immediately subsampled for different types of analysis (H_2S, SO_4, NH_4, NO_3, NO_2, Si, PO_4, DIC, alkalinity). Total sulphide ($H_2S + HS^- + S_2^-$) and sulphate concentrations were determined on subsamples (1 ml) a priori fixed with zinc acetate (0.5 ml), using the diamine complexation method (Cline, 1969) and non-suppressed anion exchange chromatography (Waters IC-Pak anion exchange column, waters 430 conductivity detector), respectively. On board nutrient subsamples (10 – 15 ml) were frozen at – 20 °C and their concentration was measured in home laboratory with a Skalar Continous-Flow Analyzer according to the method of (Grasshoff et al., 1999). Subsamples (2 ml) stored headspace-free in gas-tight glass vials were used for the determination of DIC and alkalinity with the flow injection method (conductivity detector: VWR scientific model 1054) according to Hall and Aller (1992).

2.3.2 Sulfate consumption rates (SR)

Turnover rates of sulfate were determined *ex situ*, by incubating sediments with $^{35}SO_4$ radiotracer (5 µl; dissolved in water, 50 kBq; Felden et al., 2010; Treude et al., 2003). Following the whole-core injection technique (Jørgensen, 1978) triplicate subcores (Ø 2.8 cm) were injected with radiotracer at 1 cm resolution at all investigated stations (Supplement Table 1). After incubation of appoximately 8h in the dark and at *in situ* temperature of 14 °C, the cores were subsectioned and samples were fixed with 20 ml zinc acetate solution (20%, w/v) for SR analyses. Sulphate turnover rates were determined in the home laboratory by scintillation counting according to Kallmeyer et al. (2004).

2.3.3 Benthic chamber incubations

In situ Total Oxygen Uptake (TOU) and methane efflux of targeted reduced sediments were measured with a deep-sea benthic chamber module (Felden et al., 2010). Once deployed at the seafloor, the benthic chamber was operated with a ROV, which ensured precise positioning of the module at the targeted sampling locations (Supplement Table 1). The module consisted of a chamber that enclosed 284 cm^2 of sediment with 10 – 15 cm high overlying bottom water. TOU was determined from the initial decline of oxygen concentration in the enclosed bottom water, which was continuously measured with an optode (Aanderaa, Norway) during the course of the incubation. A pre-programmed syringe system connected to the chamber was used to retrieve samples from the enclosed water body at certain time intervals during the incubation. On board syringe samples were fixed with NaOH, for the determination of dissolved methane concentrations. Methane concentrations were determined in the home laboratory by injecting 100 µl of headspace into a gas chromatograph (5890A Hewlett Packard) as described previously (Niemann et al., 2009 and references therein). Methane efflux and TOU (mmol m^{-2} d^{-1}) were calculated according to (Wenzhöfer and Glud, 2002), using the formula:

$$TOU; methane\ efflux = \frac{dC}{dt}\ x \frac{V\ chmaber}{A\ chamber}$$

where, dC/dt (µM L-1 h-1) is the change of concentration over incubation time, $V_{chamber}$ (cm^3) is the volume of the overlying enclosed bottom water, and $A_{chamber}$ is the area of sediment enclosed by the chamber.

2.3.4 Oxygen microsensor measurements

Diffusive Oxygen Uptake (DOU) and Oxygen Penetration Depth (OPD) at seep habitats at the Amon MV were determined *in situ* using a deep-sea modular microprofiler (Supplement Table 1). Detailed technical description of the ROV-operated MICP, oxygen microsensors, as well as calibration method, can be found elsewhere (de Beer et al., 2006; Glud et al., 2009; Treude et al., 2009). Three Clark-type microsensors (Revsbech, 1989) were mounted at the MICP in order to determine high-resolution oxygen concentration profiles (200 µm) at each investigated site. Oxygen fluxes were determined according to the Fick's first law of diffusion, from the linear decrease in oxygen concentration within the Diffusive Boundary Layer (DBL; Jørgensen and Des Marais, 1990):

$$J = D * \frac{\delta C}{\delta z}$$

where, J is the diffusive flux in mmol m^{-2} d^{-1}, D is the diffusion coefficient of oxygen in water (D = 1.6 x 10^{-9} m^2 s^{-1}), corrected for the temperature and salinity (Li and Gregory, 1974) and the $\delta C/\delta z$ is the concentration gradient (mmol m^{-2}) through the DBL.

2.4 Characterization of bacterial communities

2.4.1 Bacterial cell numbers

Sediment cores from every sampling station were subsampled in 1 cm resolution down to 25 cm depth for the determination of total number of single cells by applying the AODC (Acridine Orange Direct Count) method. On board samples were fixed in 4 % formaldehyde and stored at 4 °C. The AO-staining was done in the home laboratory as previously described (Boetius and Lochte, 1994; Meyer-Reil, 1983). For each sample, a minimum of two replicate filters and 30 grids per filter were randomly counted.

2.4.2 DNA extraction

At each investigated site (Supplement Table 1), the topmost 5 cm of sediment were sectioned in 1 cm steps, and from each depth two replicate sediment samples, separated by less then 10 cm, were obtained for DNA analyses. The DNA samples

were immediately frozen at –20 °C for further processing in the home laboratory. DNA was extracted from 0.5 g of sediment using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, USA) and finally eluted in 100 µl 1 x Tris-EDTA buffer (Promega, Madison, WI, USA).

2.4.3 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

To investigate changes in bacterial community structure, standardized amounts (10 ng) of DNA from each sample were amplified in triplicates using ARISA specific ITSR and ITSF primers, the latter labeled with the phosphoramidite dye HEX (Cardinale et al., 2004; Fisher and Triplett, 1999). The quality of the PCR products were checked on a 1 % agarose gel and the concentrations were determined with a microplate spectrometer (Infinite 200 PRO NanoQuant, TECAN Ltd, Switzerland). Standardized amount of PCR product (100 ng DNA) was used for separation of fragments by capillary electrophoresis with the internal size standard MapMarker 1000 ROX (BioVentures Inc., Wahsington DC, USA). The ARISA peaks were binned, using a bin size of 2 bp, to account for slight peak shifts between runs and for peak size calling imprecision (Interactive Binner function, http://www.ecology-research.com; Ramette, 2009). The ARISA PCR triplicates were merged into a consensus profile by keeping only Operational Taxonomic Unit (OTU) that appeared at least twice out of the three PCR replicates and by averaging the corresponding relative peak intensities.

2.4.4 Statistical analyses

All statistical analyses were performed in the R software (The R Foundation for Statistical Computing v. 2.14.2; http://www.r-project.org/), using custom based scripts and the community ecology *vegan* package (Oksanen et al., 2011). Differences in the bacterial community composition and structure between seep and non-seep sites, as well as between individual seep sites, were visualized using Nonmetric MultiDimensional Scaling analyses (NMDS), based on Bray-Curtis and Jaccard (Bray and Curtis, 1957) dissimilarities. Prior to all NMDS analyses, replicate sampled from individual habitats were merged (Fig. 4, 5). Analysis of similarity (ANOSIM; Bray-Curtis dissimilarities) was used to assess significant differences between *a posteriori* groupings of samples. Correlation between β-diversity (calculated as Bray-Curtis and Jaccard distances) differences in geochemical concentrations (incl. DIC, H_2S and

SO_4), as well as geographic distances among samples was tested applying the Mantel correlation test based on Pearson linear and Spearman monotonic correlations, respectively. Mantel P values were corrected for multiple testing using the Bonferroni correction as described elsewhere (Ramette, 2007). For the Mantel test, DNA samples and porewater concentrations derived from individual depth layers were used. For all other analyses the 0 – 5 cm profiles from individual depth samples derived from one habitat, were merged to form one consensus profile per habitat. DNA replicate samples of individual reduced habitats were compared to assess variations at the spatial scale of centimeters (within habitats variations). Reduced habitats within a cold seep structure were compared to assess differences in bacterial diversity at the spatial scale of meters to hundreds of meters (within cold seep variations). Prior to this analysis, DNA replicate samples were merged (Table 3). To compare differences in the bacterial diversity between cold seeps (between the Amon MV, Amsterdam MV and the Pockmark), merged samples (including all reduced habitats within the seep structure) were compared (Table 3).

3 Results

3.1 Geochemical characterization of seep versus non-seeps habitats

At all three cold seep ecosystems, the Amon Mud Volcano (AMV), Amsterdam Mud Volcano (AmsMV) and the NSDF Pockmark area (Pock), patches of highly reduced, methane-seeping sulfidic sediments were distributed among non-reduced oxygenated seafloor areas, separated by tens to hundreds of meters. The non-seep sediments were predominantly beige, indicating aerobic conditions throughout the complete core depth (Table 2). The non-seep sites were fully oxygenated till the penetration depth of the sensors, or at least > 10 - 40 mm sediment depth, with typical bottom water sulfate concentrations detected throughout the core length (27 – 28 mM; Table 2). Correspondingly, total oxygen uptake (TOU), Diffusive Oxygen Uptake (DOU) and the average sulfate reduction (SR) rates (integrated over 0 – 10 cm sediment depth) were low (1 – 12 mmol m^{-2} d^{-1}) at all investigated non-seep sites.

All investigated methane seeping habitats within the three cold seep ecosystems were characterized by gassy, sulfidic sediments of blackish color, some of which were overgrown with bacterial mats, indicating reduced conditions. However, geochemical

parameters investigated within this study varied between individual seep habitats. Seepage of dissolved methane to the bottom water was detected at all seep habitats, with maximum efflux of 1169 and 1175 mmol m^{-2} d^{-1} measured at two of the Amsterdam MV habitats (Table 2). Methane efflux measured at AMV_seep_2 habitat at the different days varied substantially, from 1 to 70 mmol m^{-2} d^{-1}, most probably reflecting high temporal and/or spatial variations in the seepage of methane at these location. At most of the seep habitats oxygen was limited to the topmost 4 mm sediment depth, except at the AMV_seep_4 where oxygen was detected down to 4 cm depth (Fig. 3d and Table 2). Diffusive oxygen flux was elevated at all seep habitats and reached maximum value of 47 mmol m^{-2} d^{-1} at the AMV_seep_1 site (Table 2). Both Pockmark sites had the highest benthic total oxygen uptake (153 and 228 mmol m^{-2} d^{-1}) detected within this study. All other investigated cold seeps had more variable TOU at the other cold seep, ranging between 5 – 119 mmol m^{-2} d^{-1} (Table 2).

Integrated (0 – 10 cm) sulphate reduction rates showed similar patterns as TOU, in that highest rates were detected at the Pockmark habitats (20 and 50 mmol m^{-2} d^{-1}), while the other cold seeps had more variable sulfate consumption, ranging from 1 to 35 mmol m^{-2} d^{-1} (Table 2). Concentrations of total sulphide were elevated (max. 24 mM) at all seep habitats and increased with sediment depth (Fig. 3a), except at two of the Amon MV habitats (AMV_seep_2 and AMV_seep_4) where no free sulphide was detected throughout the complete investigated sediment depth (data now shown). At all other seep habitats, sulphate concentration profiles steeply decreased with depth, and at AMV_seep_1, AmsMV_seep_1, Ams_MV_seep_3 and Pock_seep_1 sulphate was completely consumed (Fig. 3a, Table 2). At most of the seep habitats alkalinity and DIC concentrations were substantially elevated (max. 33 - 37 mM) and increased with depth (Fig. 3b), except at the AMV_seep_2 and AMV_seep_4 habitats where background values (2.4 mM) were measured through the complete core depth. The relatively high methane emission at these geochemically relatively inactive seep habitats indicates that these spots may have newly formed, and not yet developed active hydrocarbon consuming communities.

3.2 Depth related variations in the bacterial communities

Sediment depth (i.e. 1 cm horizons from 0-5 cm sediment depth) related variations in the bacterial community composition were detected for all investigated seep and non-

seep habitats. Community turnover analyses with sediment depth revealed that on average 81±5 SD % (n = 9) of the surface (0 – 1 cm) OTUs were also found at the adjacent 1 – 2 cm depth layer, while only 58±12 SD % (n = 9) at the deepest investigated depth layer (5 cm), across all seep habitats (Fig. 3e). Moreover, 28±9 SD % (n= 9) of the total OTUs were on average common to all depth layers within individual seep habitat. Similar depth related patterns was detected at the non-seep sites, where on average 70±15 SD % (n = 4) of the surface OTUs could also be retrieved from the adjacent depth layer (1- 2 cm) and only 58±7 SD % (n = 4) at 5 cm sediment depth. On average 27±3 SD % (n = 4) of the total OTUs were shared by all sediment depth layers within a given non-seep habitat. No clear trend between OTU richness and sediment depth was detected for the different types of habitats investigated in this study.

3.3 Comparison of bacterial communities at seep and non-seep habitats

At the Amsterdam MV and Pockmark area, bacterial communities of the non-seep sites had higher OTU numbers (156 and 144, respectively) compared to the nearby seep habitats (89±8 SD (n = 3) and 95±4 SD (n = 2), respectively. At the Amon MV this difference was not so obvious and on average 138±19 SD (n = 4) OTUs were detected at the seep and 142 ± 34 SD (n = 4) OTUs at the non-seep habitats.

Pairwise comparison of seep habitats and the corresponding nearby non-seep sites revealed that on average 36 % of the OTUs were shared (Table 3). Highest similarity in terms of shared OTUs (45 %) was detected between seep habitats at the Amon MV (AMV_seep_1 – 4) and the nearby AMV_non-seep_1, while lowest percentage was detected amongst the Amsterdam MV seep habitats and the neighboring AmsMV_non-seep_1 – 3 non-seep sites (22 – 27 %; Table 3). Overall, seep and non-seep sites shared only 18 % of the total 158 OTUs, as revealed by comparing common OTUs found at all seeps versus the common OTUs found at all non-seep sites. Differences between seep and non-seep sites were even more apparent when also the relative abundance of OTUs was taken into account. Namely, substantial difference in the bacterial community structure between seep and non-seep habitats were revealed with NMDS analysis, based on Bray-Curtis dissimilarity (Fig. 4). The ANOSIM test showed that the difference between the two groups (seep vs. non-seep) was

statistically significant (Bonferroni corrected p-value = 0.001; ANOSIM R-value = 0.8). This result has to be taken with caution, as the number of samples for each group varied two-fold.

3.4 Bacterial communities of seep habitats and factors shaping their structure

NMDS analysis revealed that neighboring seep habitats belonging to the same cold seep had in general more similar bacterial community structure and composition compared to distant habitats belonging to different seeps (Fig. 5). This was not the case for the Amon MV where high variation in the bacterial community structures between different habitats was detected. Namely, AMV_seep_1 and AMV_seep_2 grouped away from the other two Amon MV habitats (AMV_seep_3 and AMV_seep_4), and the latter were more similar to the habitats of the Amsterdam MV (Fig. 5). It was not possible to test for significance among different groups (cold seeps), due to the small number of samples (habitats) within each group. The Mantel test, based on Spearman rank correlation, revealed significant positive correlation of ß-diversity (difference in the bacterial community structure between habitats, based on Bray-Curtis dissimilarity) to overall differences in the sediment geochemistry between seep habitats ($R = 0.5$, $p < 0.001$). In particular, increase in ß-diversity was significantly correlated to increasing differences in sulfide, sulfate and DIC sediment concentrations between seep habitats (Fig. 6; Supplement Table 2).

3.5 Spatial variations of seep-influenced bacterial communities

Pairwise analyses based on shared OTUs revealed a subtle trend of decreasing similarity between samples with increasing geographic distance for seep and to certain extent for non-seep samples within the scale of 0 - 350 km investigated here. The sharpest decrease in the percentage of shared OTUs was evident between habitats within one seep, after which on larger spatial scales (between cold seep ecosystems) the trend more or less leveled off. On the smallest investigated spatial scale (< 10 cm; within habitat comparison), a pair of samples shared on average 76 % (76±4 SD (n = 9) and 76±8 SD (n = 5) for non-seep habitat) of the OTUs. At intermediate spatial scales (20 – 630 m; between habitats within one cold seep comparison) only 54±8 SD % (n = 3) of the OTUs were shared between a pair of seep habitats (Table 3). Habitats

within the Pockmark cold seep had the highest percentage of common OTUs (65 %), while lowest number of shared OTUs (44 %) was detected for the habitats within the Amon MV (Table 3). When taking into consideration the relative abundance of OTUs, this corresponded to β-diversity values ranging from 0.2 – 0.4, indicating that habitats within one seep have separated bacterial community structures, but with substantial overlap (Table 3). Similarly, at the largest investigated spatial scale (100 – 300 km; between cold seeps comparison) cold seeps shared on average 52±3 SD % (n = 3) of the OTUs. When taking into account the relative abundance of OTUs, the average Bray-Curtis ß-diversity was 0.3. At the same large spatial scale, non-seep habitats shared 51 % of the OTUs, corresponding to a Bray-Curtis ß-diversity of 0.3 (Table 3). However, the Mantel test based on Spearman rank correlation revealed no significant correlation between ß-diversity (Mantel statistics r = -0.5, p-value = 1) and the percentage of shared OTUs (Mantel statistics r = -0.07, p – value = 0.154) with geographic distance (Fig. 7a). Nevertheless, species accumulation analyses showed a substantial increase in OTU diversity with increasing number of seep habitats (Fig. 7b). Based on ARISA fingerprinting which detects rather abundant bacterial types of a community, approximately the same number of rare OTUs (occurring only at one habitat), and ubiquitous OTUs (occurring at all investigated habitats) were found, indicating that the rare types endemic to one seep habitat contribute substantially to the overall diversity of a cold seep ecosystem (Fig. 8).

4 Discussion

4.1 Differences in diversity patterns between seeping and non-seeping habitats

Cold seep ecosystems are characterized by a highly patchy habitat structure with distinct, but variable geochemical conditions (Table 2). This has been reported for many other cold seep systems throughout the world's oceans (Sahling et al., 2002; Levin et al., 2003; de Beer et al., 2006; Sommer et al., 2006; Lichtschlag et al., 2010; Menot et al., 2010;). Previous studies have analyzed habitat variation of Eastern Mediterranean cold seep ecosystems and their associations with different microbial and faunal communities (Grünke et al., 2011; Heijs et al., 2008; Omoregie et al., 2009; Ritt et al., 2011). Here we compare for the first time effects of small to

intermediate spatial scales and biogeochemical variations on bacterial community richness and structure associated with seeping and nearby non-seeping deep-sea sediments. We used the ARISA fingerprinting method to uncover general patterns in abundant types of bacterial populations, without focusing on particular functional or taxonomic groups. This method can detect up to 450 different taxonomic types of bacteria for a given study, but does not include numerically rare phylotypes (Bent and Forney, 2008; Ramette, 2009 and references therein).

The faunal community structure of different size classes of organisms (meio-, macro and megafauna) inhabiting cold seeps has been shown to differ significantly from background communities colonizing deep-sea sediments (Olu et al., 2009; Van Gaever et al., 2009; Levin et al., 2010, 2003; Menot et al., 2010; Ritt et al., 2011; Sahling et al., 2002). On a broad spatial scale, across the Pacific Ocean, hydrate-bearing sediments bore distinct, diverse bacterial communities compared to hydrate-free sites (Inagaki et al., 2006). Significant differences in the bacterial community structure between seep and non-seep sediments have been reported previously for cold seeps sites in the Eastern Mediterranean (Fig. 4, Heijs et al., 2008). High adaptation of communities to their respective environments and different type of energy sources, as well as the variable tolerance of toxic conditions caused by sulfide production at cold seeps, are regarded as main factors selecting for different communities at seep and non-seep sites. Here we found that seep and non-seep sites shared less than 20 % of the overall bacterial diversity in the whole dataset. In a pairwise comparison, the percentage of shared OTUs between seep and the nearby non-seep habitats varied substantially for different cold seeps structures (22 – 45 %). Clearly, even within very small spatial scales of a few meters, the effect of seeping hydrocarbons, an energy source available only to a few functional types, has a significant impact on the entire community structure of bacteria. Interestingly, this effect was not leading to differences in the turnover of bacterial communities with sediment depth. Sediment depth-related patterns in the bacterial community structure have been previously detected for variety of benthic habitats (Böer et al., 2009 and references therein). In accordance to previous investigations (Inagaki et al., 2002; Luna et al., 2004), high depth-related OTU turnover with >50 % of replacement of OTUs within only 5 cm depth was detected for both seep (Fig. 3e) and non-seep sites in this study.

Although it is commonly viewed that seeps as extreme environments host less diverse macro- and meiofauna communities than the deep-sea (Sahling et al., 2002; Levin et al., 2010; Menot et al., 2010;), an opposite trend was revealed for seep sites in the Eastern Mediterranean and Eel River (Levin et al., 2010, 2003; Ritt et al., 2011). In this study, variable numbers of bacterial OTUs were detected between seeping and non-seeping habitats, but no clear difference in OTU richness. A recent global study of marine bacterial diversity revealed that bacterial vent communities displayed highest intra-variability of all oceanic ecosystem types (Zinger et al., 2011). We expected to reveal a similar variation for cold seep ecosystems, because of their similar fragmentation and environmental variability (Sibuet and Olu, 1998). Accordingly, we found that the fragmented methane-seeping habitats added a substantial diversity to the overall cold seep ecosystem.

4.2 Factors shaping the bacterial diversity at cold seep ecosystems

Cold seeps are highly heterogeneous ecosystems (Cordes et al., 2010), with unique patchy distribution of the main sources of energy i.e. methane and sulfide produced by anaerobic oxidation of methane or other hydrocarbons (Barry et al., 1997; de Beer et al., 2006; Omoregie et al., 2009; Treude et al., 2009). This can result in contrasting geochemical conditions at locations only few meters apart, and conversely similar environmental settings at sites hundreds of meters or kilometers apart. High patchiness in the geochemical settings was revealed for the seep sites investigated within this study. Namely, within the Amon MV, in a radius of < 1 km, we could detect highly contrasting geochemical conditions, ranging from extremely low to substantially elevated SR rates and benthic oxygen consumptions (1 - 28 and 5 – 119 mmol m^2 d^{-1}, respectively) (Table 2). On the other hand, seep habitats at the Amsterdam MV and Pockmark, separated by more than 300 km, had similarly high biogeochemical activity (Table 2). In accordance, bacterial communities of seep habitats at the Amon MV had highest variability, while habitats at the Amsterdam MV and the Pockmark harbored more similar bacterial communities. All in all, bacterial communities of seep sites in the Eastern Mediterranean exhibited a patchy distribution, and their structure seemed to reflect well the heterogeneity of the main energy sources of cold seep ecosystems. Moreover, within this study the structure of seep bacterial communities was found to be significantly influenced by seep sediment

geochemistry, and specifically by the variations in methane-related DIC porewater concentrations, as well as by sulfide produced and sulfate consumed during the anaerobic oxidation of hydrocarbons (Fig. 6). Interestingly, only few bacterial types are known to be directly involved in anaerobic hydrocarbon degradation (Knittel and Boetius, 2010). These types will directly respond to variations in methane seepage, and also influence the geochemistry of the sediments. But it remains an interesting question how variations in methane geochemistry will affect all other types making up the bacterial community as assessed by ARISA fingerprinting. Our results indicate that variations in methane seepage and consequently the geochemical activity of the methanotrophic microorganisms present in the sediments shapes the structure of the entire bacterial community of cold seep habitats.

Variations in bacterial diversity patterns due to contrasting geochemical conditions have been detected not only amongst cold seeps but also between sediments underlying bacterial mats located less then 2 m apart (Omoregie et al., 2008). Although seep habitats can differ considerably in terms of availability of energy, magnitude of geochemical fluxes and presence of diverse fauna, it is striking that half of the bacterial OTUs at each seep habitat investigated in this study was unique and did not appear at other sites. The results of this study show that not only the large seep structures, but also individual reduced seep habitats, usually not more than few meters in diameter, contribute significantly to the biodiversity of deep-sea. A main question remains as to the dispersal of bacteria associated to the patchy, highly reduced, gassy and sulfidic sediments, and not represented in aerobic habitats. Investigation of the dispersal mechanisms of seep microbial communities was out of the scope of this study, however if we are to understand the biodiversity and interconnectivity of cold seeps, future studies focusing on this topic are needed. As proposed for deep-sea sediment surface bacteria, resuspension of sediments by bottom water currents and feeding fauna (Bienhold, 2011) might play an important role also in the dispersal of seep bacterial communities if they can withstand temporarily aerobic conditions. However, also the anoxic subsurface biosphere may be considered as a seed reservoir to surface near cold seep habitats (Inagaki et al., 2006).

4.3 Effect of spatial scales on bacterial diversity of cold seep ecosystems

The taxa-area relationship, or increase in species richness with size of area, has been reported for many different organisms (across domains and different size classes) and hence is thought to be one of the few universal laws in ecology (Rosenzweig, 1995; Lawton, 1999). A general trend of decreasing similarities in the bacterial communities with geographic distance i.e. more similar bacterial communities are found at proximate locations as compared to distant sites, has been identified across various ecosystems (Papke et al., 2003; Whitaker et al., 2003; Martiny et al., 2006; Ramette and Tiedje, 2007; Bienhold et al., 2011). Increase in environmental heterogeneity with area together with the specificity of taxa for different habitats is the most common explanation of taxa–area patterns (Rosenzweig, 1995).

In contrast, within this studies no significant correlation was detected between β-diversity of seep bacterial communities and geographic distance on spatial scales ranging from tens of meters to hundreds of kilometers (Fig. 7a). Moreover, a turnover in bacterial communities of approximately 50 % was detected between both proximate and distant seep habitats (Table 3). This high turnover on small spatial scales appears to cause a low effect of geographic distance on the bacterial community structure over all, for the scales investigated here. It should be noted that this study was confined to one type of ecosystem in one ocean basin (Eastern Mediterranean), and covered only a relatively small overall distance compared to other spatial scaling studies on sediment and soil bacteria (Schauer et al., 2010; Bienhold et al., 2011; Martiny et al., 2011).

Although, the bacterial community structure and composition did not significantly vary with geographic distance, a decreasing trend in the proportion of shared bacterial types between two sites with increasing spatial scale was detected. The percentage of shared bacterial types was the highest within habitats (76 %), declined substantially between habitats (54 %) and remained at the same level between cold seep structures (52 %). Similar pattern of higher inter- than intra-habitat variability was detected for macrofauna inhabiting cold seeps in the Eastern Mediterranean (Ritt et al., 2011). In corroboration to our results, previous microbiological studies detected significant differences in the bacterial diversity of different mud volcanoes in the Eastern Mediterranean (Omoregie et al., 2009) , and moreover revealed only 17 % of common

bacterial phylotypes between neighboring mud volcanoes located within the same geological region (Pachiadaki et al., 2011).

A general pattern of increasing taxon richness with increasing number of seep habitats has identified for different size classes of organisms ranging from microbes (Fig. 7b), meio- and megafauna across many different cold seep ecosystems (this study; Cordes et al., 2010). These observations support the fact that seep habitats are major sources of diversity at cold seep ecosystems, and represent biodiversity hotspots in the deep-sea.

A substantial proportion of the total ARISA OTUs (around 25 %) occurred at only one or two habitats (Fig. 8). When analyses were performed on the level of cold seep ecosystems, still a high number of endemic OTUs representative of only one system (20 %) was found compared to ubiquitous OTUs (40%), occupying all three cold seeps. In contrast, continuous marine habitats, such as the deep-sea floor and the oceanic photic zone are largely dominated by endemic OTUs on spatial scales of kilometers to tens of thousands of kilometers, as defined at 3 % sequence similarity cutoff level (Pommier et al., 2007; Bienhold, 2011). Further studies are needed to confirm if fragmented as opposed to continuous habitats have distinct patterns of cosmopolitan versus endemic species, and identified the respective spatial scales. Discrepancies can arise between studies due to the technical nature of the sampling design or different molecular methodologies applied. Application of next-generation-sequencing-technologies might help resolve this question, and furthermore taxonomically identify members unique to individual seep sites and cosmopolitan to all cold seep ecosystems.

In conclusion, our results corroborate that cold seeps, along with other chemosynthetic ecosystems i.e. hydrothermal vents, whale and wood falls, represent “island” type of ecosystems in the deep-sea characterized by a high patchiness and variability in community structure (Laubier, 1993; Carney, 1994; Sibuet and Olu, 1998; Tyler et al., 2003; Fisher et al., 2007; Jørgensen and Boetius, 2007; Zinger et al., 2011). Moreover, seep bacterial diversity is strongly influenced by the availability of sulfide, which results in highly patchy distribution of the bacterial communities between individual seep habitats and cold seep ecosystems. This is in contrast to the diversity patterns detected at large continuous habitats, where monotonic decline in community similarity has been detected with increasing geographic distances

(Bienhold, 2011; Schauer et al., 2010). Highest variation in the bacterial diversity occurs at the level of seep habitat, among locations spatially separated by few meters to couple of hundreds of meters. With approximately half of the diversity being unique to individual seep habitats, these localities contribute significantly to the biodiversity of deep-sea and can be regarded as biodiversity hotspots. Moreover, our results suggest that these small reduced habitats might play a critical role for the connectivity of seep communities and resilience of cold seep ecosystems to perturbations caused by increased anthropogenic impact. Therefore, we propose that cold seeps ecosystems, especially those harboring highly diverse seep habitats, should be incorporated in the conservation schemas aiming to protect deep-sea biodiversity.

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Tables and Figures

Table 1 Overview of the main sampling sites investigated within this study. Detailed list of all samples and measurements performed at each sampling sites are shown in Supplement Table 1.

Cold seep	Location	Habitat	Sampling site ID	Longitude; Latitude	Sampling time
Amon MV (AMV)	Centre	reduced sediment with bacterial mat	AMV_seep_1	32°22.132272'N 31°42.654951'E	Oct-Nov 2009
		reduced sediment	AMV_seep_2	32°22.174029'N 31°42.627174'E	Oct-Nov 2009
	Sulphur Band	reduced sediment with bacterial mat (M14 marker)	AMV_seep_3	32°22.045089'N 31°42.276642'E	Oct-Nov 2009
		reduced sediment with bacterial mat (M16 marker)	AMV_seep_4	32°22.045956'N 31°42.265083'E	Oct-Nov 2009
		beige non-seep sediment	AMV_non-seep_1	32°22.3002'N 31°41.9598'E	Oct-Nov 2009
Amsterdam MV (AmsMV)	Centre	reduced sediment (M5 marker)	AmsMV_seep_1	35°20.034018'N 30°16.167342'E	Nov-Dec 2009
		reduced sediment (M6 marker)	AmsMV_seep_2	35°20.079390'N 30°16.129803'E	Nov-Dec 2009
		reduced sediment (M10 marker)	AmsMV_seep_3	35°19.945893'N 30°16.098681'E	Nov-Dec 2009
	E Rim	beige non-seep sediment	AmsMV_non-seep_1	35°19.9602'N 30°16.8198'E	Nov-Dec 2009
	NE-E Rim	beige non-seep sediment	AmsMV_non-seep_2	35°20.2398'N 30°17.2902'E	Nov-Dec 2009
	Centre	beige seep-influenced non-seep sediment	AmsMV_non-seep_3	35°20.0598'N 30°16.1202'E	Nov-Dec 2009
Pockmark (Pock)		bacterial mat at marker M10	Pock_seep_1	32°31.99'N 30°21.12'E	Nov-Dec 2009
		bacterial mat at marker M9	Pock_seep_2	32°31.98'N 30°21.15'E	Nov-Dec 2009
		beige seep-influenced non-seep sediment	Pock_non-seep_1	32°32.07'N 30°21.39'E	Nov-Dec 2009

Table 2 Biogeochemical characterization of seep and close-by non-seep habitats, including average integrated (0 – 10 cm) Sulphate Reduction (SR) rates, Total Oxygen Uptake (TOU), methane efflux, Diffusive Oxygen Uptake (DOU), Oxygen Penetration Depth (OPD), minimum detected concentration of sulphate, and sulphate penetration depth.

Cold seep	Habitat	SR mmol m^{-2} d^{-1}	TOU mmol m^{-2} d^{-1}	CH_4 efflux mmol m^{-2} d^{-1}	DOU mmol m^{-2} d^{-1}	OPD mm	min. SO_4 mM	SO_4 depth cm
Amon MV	**AMV_seep_1**	16	115	83; 85; 49	47	2	< 1	10
	AMV_seep_2	2	5	1; 70; 24	11	3	29	> 19
	AMV_seep_3	28	119	<1	3; 6	1; 3.9	< 1	5
	AMV_seep_4	1	13	n.d.	5	45	29	> 18
Amsterdam MV	**AmsMV_seep_1**	35	101	1175	n.d.	n.d.	< 1	15
	AmsMV_seep_2	8	23	196	n.d.	n.d.	4	> 15
	AmsMV_seep_3	13	89	1169	n.d.	n.d.	< 1	8
Pockmarks	**Pock_seep_1**	20	153	688	n.d.	n.d.	< 1	12
	Pock_seep_2	50	228	36	n.d.	n.d.	1	20
Amon MV	**AMV_non-seep_1**	0[1)]	5[1)]	n.d.	12[1)]	> 40[1)]	29[2)]	> 13[2)]
Amsterdam MV	**AmsMV_non-seep_1**	0	n.d.	n.d.	n.d.	n.d.	27	> 30
	AmsMV_non-seep_2	1	n.d.	n.d.	n.d.	n.d.	28	> 25
	AmsMV_non-seep_3	n.d.	n.d.	n.d.	n.d.	n.d.	27	> 17
Pockmark	**Pock_non-seep_1**	0	1[1)]	n.d.	0.5[1)]	>10[1)]	27	> 29

[1)]Grünke et al. (2011)
[2)]Data derived from M70/2a_763 cruise

Table 3 Percentage of shared OTUs (lower triangle) and β-diversity (upper triangle), calculated as Bray-Curtis dissimilarity. β -diversity can range between 0 – 1, β=0 indicates bacterial communities with completely overlapping structure, β=1 indicates bacterial communities with distinct structure. Colors denote habitats within the same cold seep structure, and darker shades indicate the respective nearby non-seep sites.

	AMV_ seep_1	AMV_ seep_2	AMV_ seep_3	AMV_ seep_4	AMV_ non-seep_1	AmsMV_ seep_1	AmsMV_ seep_2	AmsMV_ seep_3	AmsMV_ non-seep_1	AmsMV_ non-seep_2	AmsMV_ non-seep_3	Pock_ seep_1	Pock_ seep_2	Pock_ non-seep_1
AMV_seep_1		0.4	0.3	0.4	0.4	0.3	0.4	0.3	0.6	0.5	0.4	0.4	0.3	0.5
AMV_seep_2	44		0.3	0.4	0.4	0.5	0.5	0.5	0.6	0.4	0.3	0.5	0.4	0.3
AMV_seep_3	50	48		0.2	0.4	0.4	0.4	0.5	0.5	0.4	0.3	0.5	0.4	0.4
AMV_seep_4	44	46	61		0.4	0.4	0.5	0.4	0.5	0.4	0.3	0.4	0.4	0.4
AMV_non-seep_1	41	48	45	45		0.5	0.5	0.5	0.4	0.2	0.3	0.5	0.5	0.2
AmsMV_seep_1	48	36	45	43	38		0.3	0.2	0.6	0.5	0.5	0.3	0.3	0.5
AmsMV_seep_2	42	31	40	38	30	55		0.3	0.6	0.6	0.5	0.4	0.4	0.6
AmsMV_seep_3	49	37	38	43	36	65	58		0.6	0.5	0.5	0.4	0.3	0.5
AmsMV_non-seep_1	28	28	29	29	39	26	22	26		0.4	0.5	0.7	0.6	0.4
AmsMV_non-seep_2	35	44	42	42	61	31	25	30	46		0.3	0.6	0.5	0.2
AmsMV_non-seep_3	42	56	52	52	52	37	35	37	32	50		0.5	0.4	0.3
Pock_seep_1	45	32	36	39	32	50	42	47	21	28	34		0.2	0.5
Pock_seep_2	49	41	44	48	37	52	42	49	24	34	39	65		0.5
Pock_non-seep_1	37	50	44	44	61	34	29	34	46	71	52	30	35	

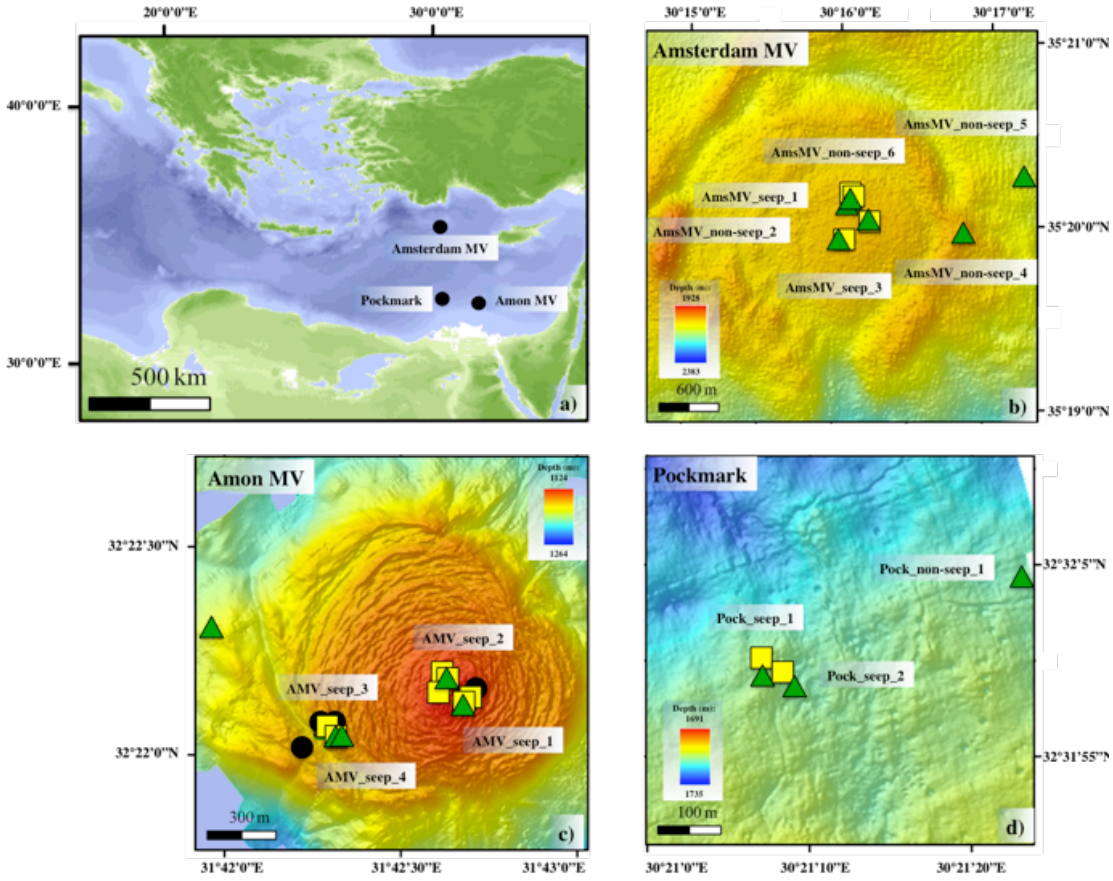

Fig. 1 Overview maps of all investigated cold seep ecosystems and the nearby non-seep sites in the Eastern Mediterranean Sea (a): the Amsterdam MV (b), Amon MV (c) and the Pockmark area (d). Map symbols depict the main sampling and measurement sites – push core and MUC sampling (green triangles) of seep and non-seep sites; benthic chamber incubations (yellow squares) and microprofiler measurements (black circles).

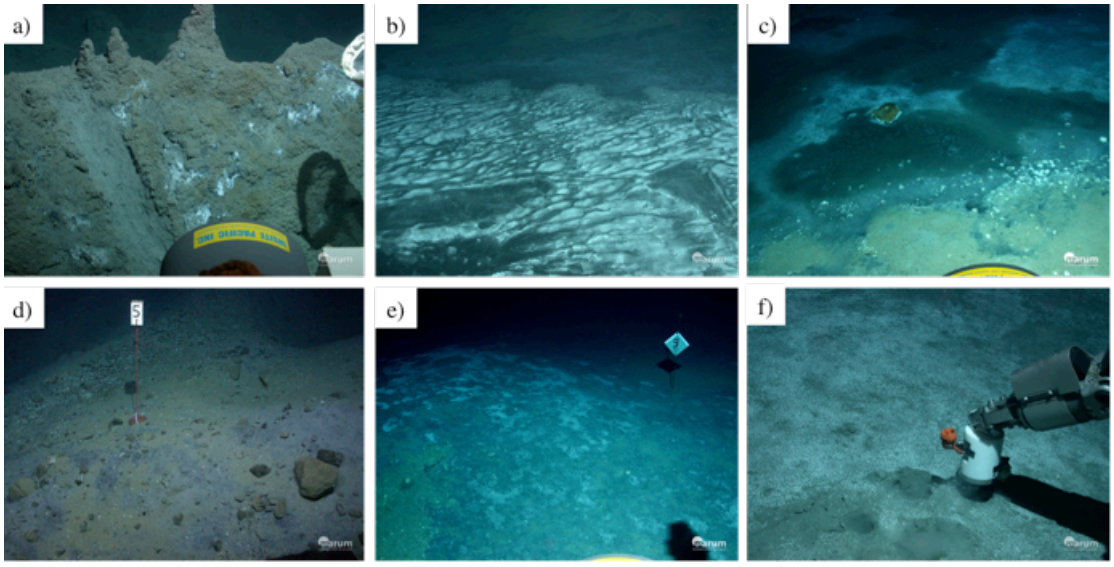

Fig. 2 ROV-photos of reduced habitats investigated in this study. Upper panel photos were taken at Amon MV: at the center of the volcano characterized by gassy sediments and patchy bacterial mats (a), as well at a lateral mud flow with extensive bacterial mats at the outer rim of the Amon MV (b,c). A typical methane-seeping habitat encountered at the Amsterdam MV is shown in photo (d). Overview (e) and zoomed in photo (f) of a bacterial mat habitat at the Pockmark cold seep.

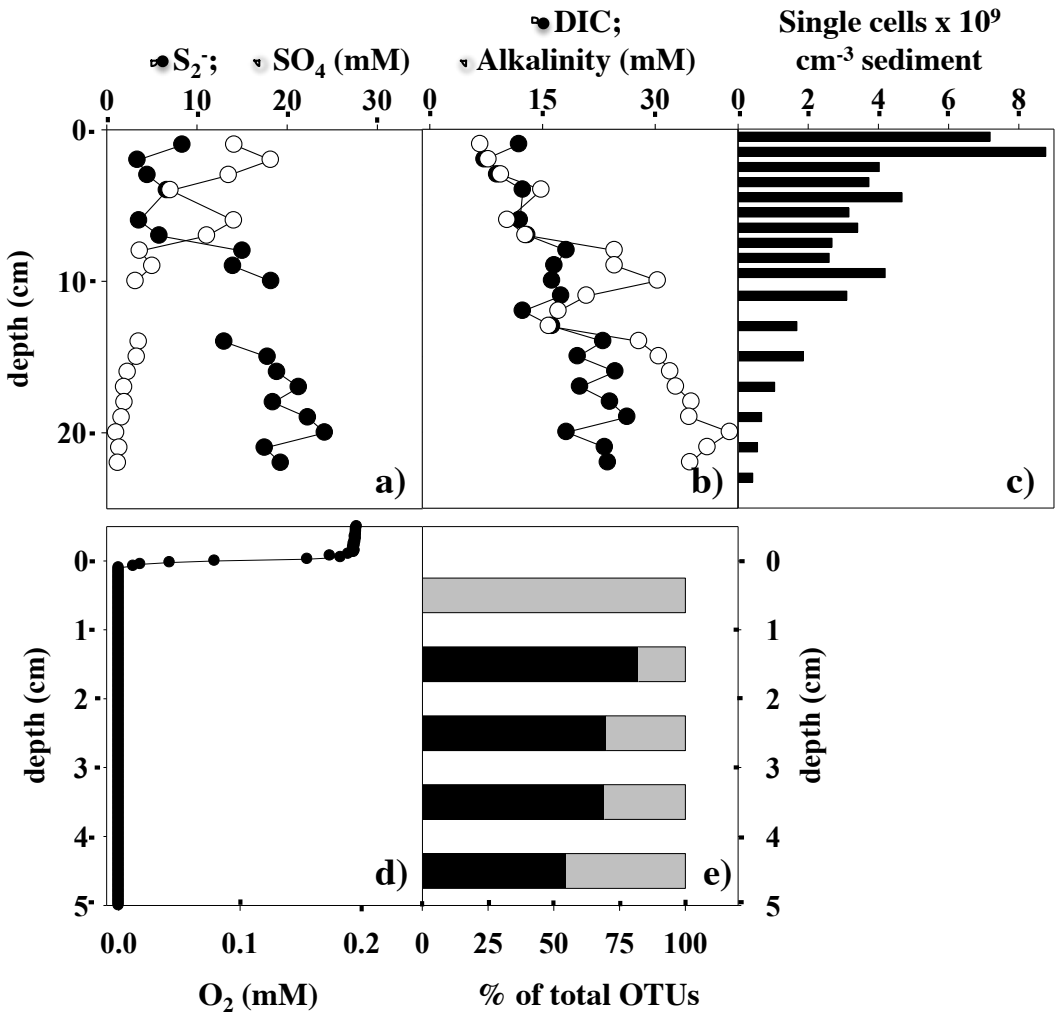

Fig. 3 Biogeochemistry and diversity of a methane-seeping reduced habitat (Pock_seep_1 site). (a) depth profiles of total sulfide (closed circles) and sulfate concentrations (open circles); (b) DIC concentration (open circles) and alkalinity (closed circles); (c) single cell numbers; (d) in situ oxygen concentration determined with microsensors; and bacterial Operational Taxonomic Unit (OTU) turnover as percentages (e). The turnover was calculated relative to the first sediment depth. Grey bars represent the percentage unique OTUs for the given depth layer and black bars represent the percentage of shared OTUs with the first depth layer).

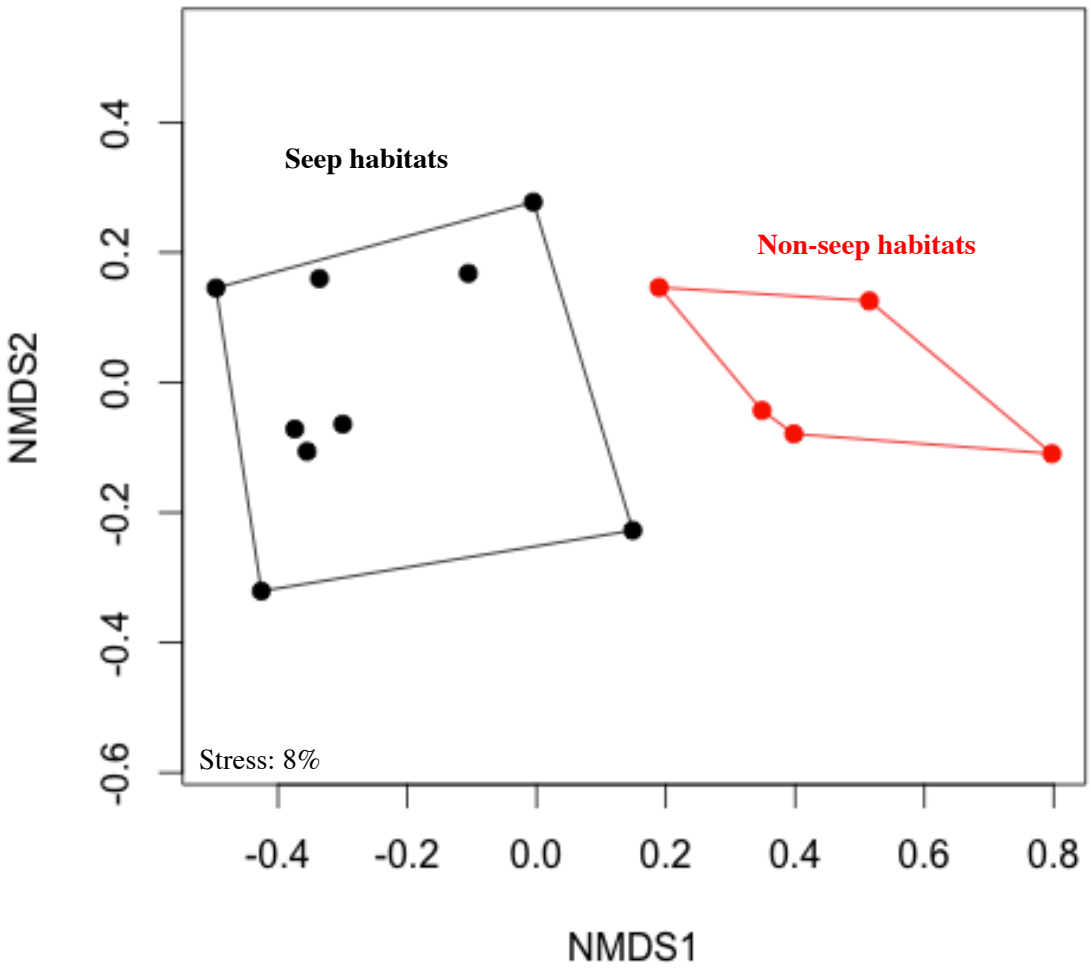

Fig. 4 Nonmetric Multidimensional Scaling Analysis (NMDS) depicting differences in the bacterial community structure between seep (black symbols) and non-seep sites (red symbols). Pooled (0 – 5 cm) samples per habitat were used to calculate Bray-Curtis dissimilarity matrix. NMDS stress of 8 %.

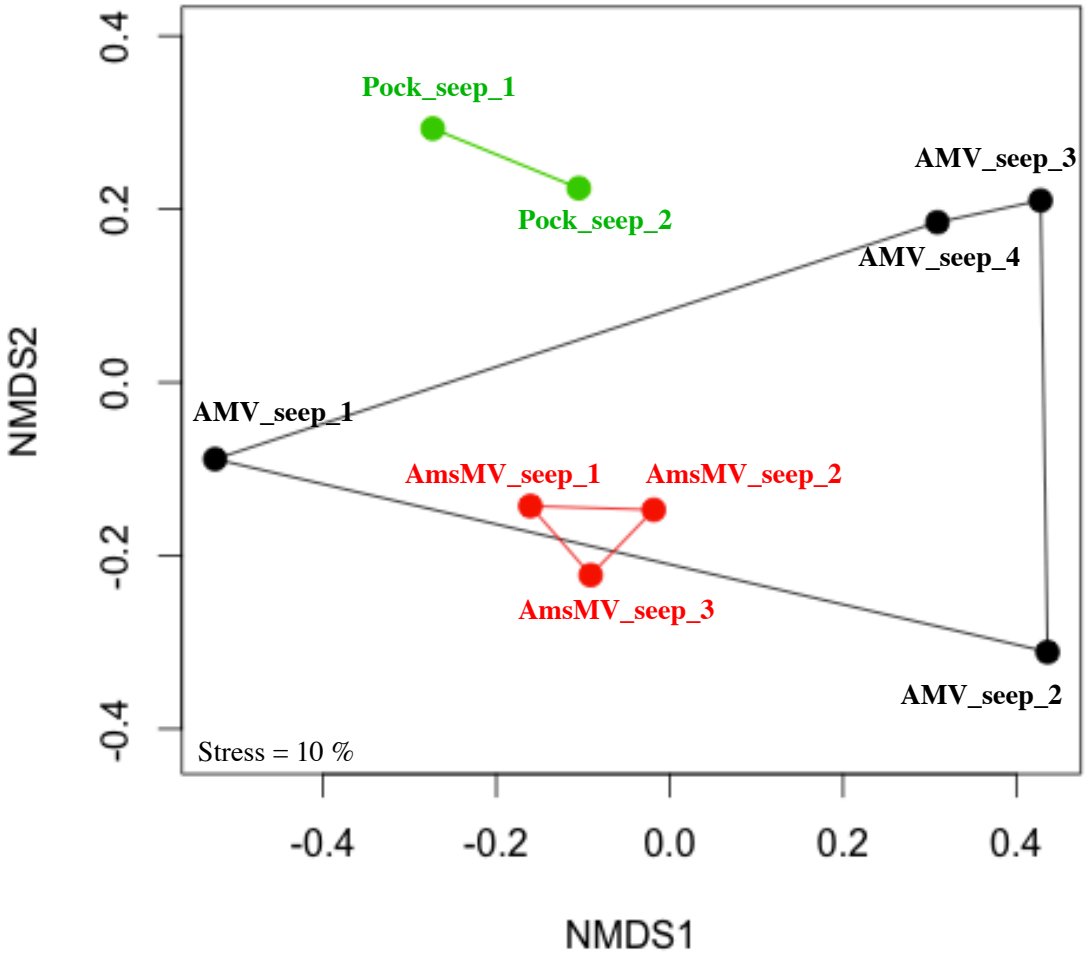

Fig. 5 Nonmetric Multidimensional Scaling Analysis plot (NMDS, based on Bray Curtis dissimilarity) depicting differences in the bacterial community structure between different seep seeps. Seep habitats are grouped and colored according to cold seep structure: Amsterdam MV seep habitats in red, Amon MV seep habitats in black and Pockmark seep habitats in green. NMDS stress of 10%.

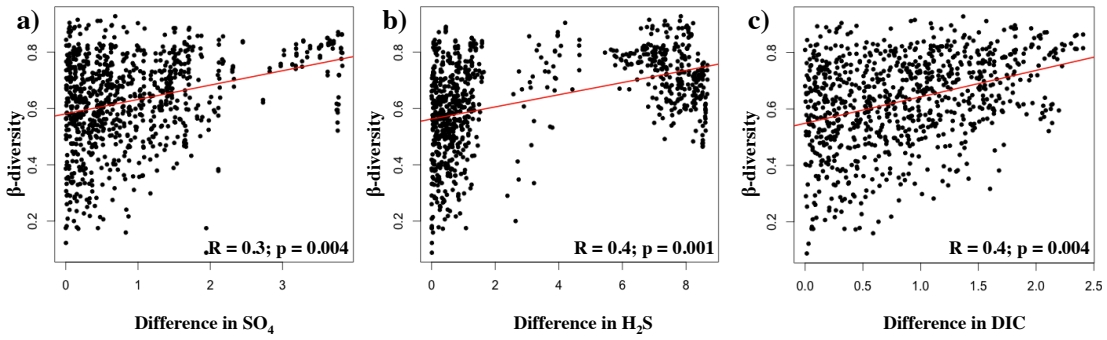

Fig. 6 Plot depicting positive correlation between β -diversity and differences in sulfate (a), sulfide (b) and DIC concentrations (c). β-diversity was calculated based on Bray-Curtis dissimilarity and differences in geochemical concentrations were calculated based on Euclidean distances. Solid line is a linear model fit. R and p values denote the Pearson correlation coefficient and the significance, respectively, based on Mantel permutation tests.

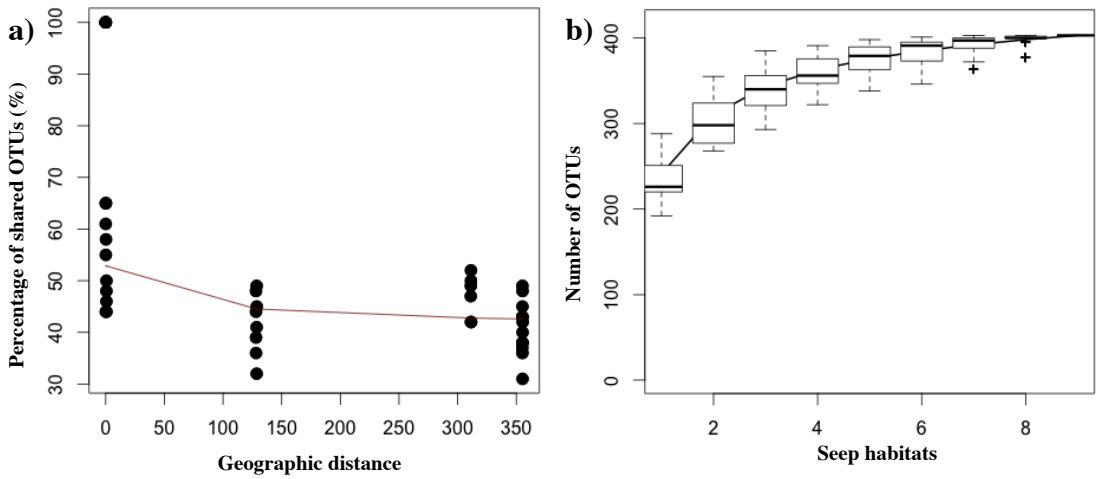

Fig. 7 Correlation of the percentage of shared OTUs and geographic distance between reduced habitats (a); OTUs accumulation across reduced habitats (b). Fig. (b) demonstrates that OTU richness increases with the number of investigated seep habitats.

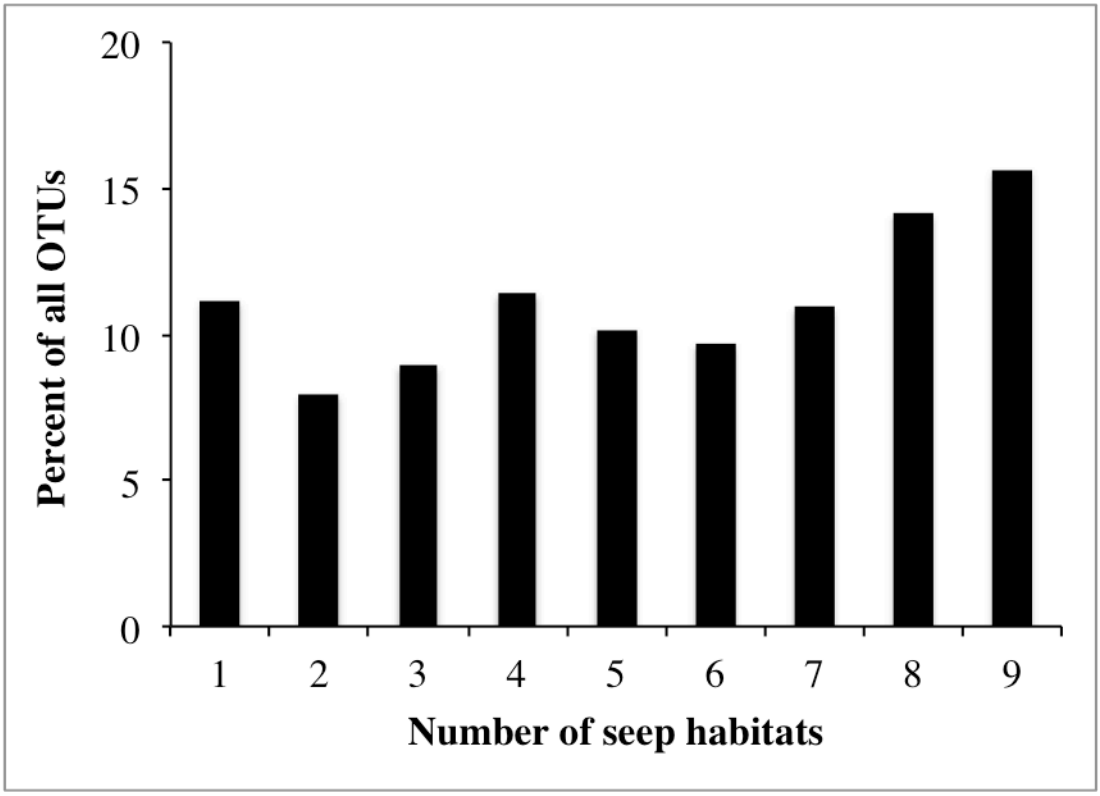

Fig. 8 Proportions of rare and common OTUs between reduced habitats.

Supplementary Material

Supplement Table 1 Overview of the main sampling sites, type of measurements and samples retrieved within this study and their corresponding Pangaea Event Label ID. Metadata of all events can be found in the PANGAEA database (www.pangaea.de).

Location	Habitat	Sampling Site ID	Measurement; Sample	Pangaea Event Label
Amon MV (Centre)	bacterial mat	AMV_seep_1	DNA	MSM13/3_929-1_PUC22
			Porewater	MSM13/3_929-1_PUC20
			SR	MSM13/3_929-1_PUC1; MSM13/3_929-1_PUC9
			MICP	MSM13/3_971-1_MICP11
			CHAM	MSM13/3_929-1_CHAM3; MSM13/3_933-1_CHAM4; MSM13/3_971-1_CHAM13
Amon MV (Centre)	hotspot	AMV_seep_2	DNA	MSM13/3_933-1_PUC13
			Porewater	MSM13/3_933-1_PUC21
			SR	MSM13/3_933-1_PUC2; MSM13/3_933-1_PUC24
			MICP	MSM13/3_971-1_MICP12
			CHAM	MSM13/3_929-1_CHAM1; MSM13/3_971-1_CHAM11; MSM13/3_971-1_CHAM12; MSM13/3_937-1_CHAM5
Amon MV (Sulphur Band)	bacterial mat at marker M14	AMV_seep_3	DNA	MSM13/3_947-1_PUC31
			Porewater	MSM13/3_968-1_PUC17
			SR	MSM13/3_947-1_PUC27; MSM13/3_947-1_PUC32
			MICP	MSM13/3_944-1_MICP4; MSM13/3_947-1_MICP7
			CHAM	MSM13/3_953-1_CHAM6
Amon MV (Sulphur Band)	black reduced sediment at marker M16	AMV_seep_4	DNA	MSM13/3_968-1_PUC15
			Porewater	MSM13/3_968-1_PUC5
			SR	MSM13/3_968-1_PUC8; MSM13/3_968-1_PUC110; MSM13/3_968-1_PUC117
			MICP	MSM13/3_968-1_MICP8
			CHAM	MSM13/3_968-1_CHAM10
Amsterdam MV	black reduced sediment at marker M5	AmsMV_seep_1	DNA	MSM13/4_984_PUC5
			Porewater	MSM13/4_984_PUC117
			SR	MSM13/4_984_PUC10; MSM13/4_984_PUC14; MSM13/4_984_PUC32
			CHAM	MSM13/4_984_CHAM1

Location	Habitat	Sampling Site ID	Measurement; Sample	Pangaea Event Label
Amsterdam MV	black reduced sediment at marker M6	AmsMV_seep_2	DNA	MSM13/4_994_PUC12
			Porewater	MSM13/4_994_PUC7
			SR	MSM13/4_994_PUC20; MSM13/4_994_PUC32
			CHAM	MSM13/4_994_CHAM3
Amsterdam MV (Centre)	black reduced sediment at marker M10	AmsMV_seep_3	DNA	MSM13/4_1022_PUC10
			Porewater	MSM13/4_1022_PUC31
			SR	MSM13/4_1022_PUC6; MSM13/4_1022_PUC20
			CHAM	MSM13/4_1022_CHAM4
Pockmark	bacterial mat at marker M10	Pock_seep_1	DNA	MSM13/4_1051-1
			Porewater	MSM13/4_1050-1
			SR	MSM13/4_1050-1; MSM13/4_1051-1
			CHAM	MSM13/4_1049_CHAM6
Pockmark	bacterial mat at marker M9	Pock_seep_2	DNA	MSM13/4_1056-1
			Porewater	MSM13/4_1056-1
			SR	MSM13/4_1056-1; MSM13/4_1057-1
			CHAM	MSM13/4_1054_CHAM7
Amon MV	beige non-seep sediment	AMV_non-seep_1	DNA	MSM13/3_959-1
Amsterdam MV (E Rim)	beige non-seep sediment	AmsMV_non-seep_1	DNA	MSM13/4_1008-1
			Porewater	MSM13/4_1008-1
			SR	MSM13/4_1008-1; MSM13/4_1007-1
Amsterdam MV (NE-E Rim)	beige non-seep sediment	AmsMV_non-seep_2	DNA	MSM13/4_1019-1
			Porewater	MSM13/4_1019-1
			SR	MSM13/4_1019-1; MSM13/4_1018-1;
Amsterdam MV (Centre)	beige seep-influenced non-seep sediment	AmsMV_non-seep_3	DNA	MSM13/4_1034-1
			Porewater	MSM13/4_1035-1
			SR	MSM13/4_1034-1; MSM13/4_1035-1
Pockmark	beige non-seep sediment	Pock_non-seep_1	DNA	MSM13/4_1058-1
			Porewater	MSM13/4_1058-1
			SR	MSM13/4_1058-1

Supplement Table 2 Mantel test for correlation between β-diversity (calculated as Bray-Curtis dissimilarities or Jaccard distance) and difference in sulphide, sulphate and DIC sediment concentrations. Mantel test was based on spearman rank coefficient; * = $p < 0.05$; Bonferroni-correction was applied to correct for multiple testing. (*) only significant without Bonferroni correction.

	Sulphide	**Sulphate**	**DIC**
Mantel statistics (Jaccard distance)	0.4*	0.3*	0.3*
Significance p-value (Jaccard distance)	0.01	0.001	0.001
Bonferroni corrected p-value (Jaccard distance)	0.04	0.042	0.04
Mantel statistics (Bray-Curtis dissimilarity)	0.4*	0.3(*)	0.4*
Significance p-value (Bray-Curtis dissimilarity)	0.001	0.004	0.001
Bonferroni corrected p-value (Bray-Curtis dissimilarity)	0.04	0.2	0.04

Chapter IV
Temporal and spatial variations of bacterial and faunal communities associated with deep-sea wood falls

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Key words: wood fall, temporal and spatial variation, fauna and bacterial communities

Abstract

Sinking of large quantities of organic matter to the food-impoverished deep-sea floor, such as sunken wood, whale carcasses and kelp, promotes the establishment of highly productive and diverse ecosystems. Such large food falls occur throughout all worlds' oceans, however their highly fragmented spatial distribution and small areal coverage poses challenges for the dispersal of their associated microbial and faunal communities. Microbial degradation of organic matter contained in the food falls causes local oxygen depletion in the seafloor and reducing conditions attractive for chemosynthetic symbioses. Our study focuses on the temporal dynamics and spatial distributions of microbial and faunal communities associated to wood falls which were deployed in the vicinity of cold seeps of the Eastern Mediterranean and the Norwegian deep seas. By combining microbial fingerprinting, *in situ* and *ex situ* biogeochemical approaches it could be shown that sunken woods have a locally confined long-term impact (> 3 y) on the sediment geochemistry. Wood experiments deployed in different oceanic realms harbored distinct faunal communities. The main wood-boring and chemosynthetic species in the Eastern Mediterranean sea were *Xylophaga sp*. and *Idas sp.* bivalves, while in the Norwegian sea *Xyloredo sp.* and siboglinid tubeworms, respectively. Similarly, bacterial communities associated to sunken woods within one geographic region (e.g. the Eastern Mediterranean sea) were more similar than communities of wood falls located in different oceans (Eastern Mediterranean versus Norwegian sea). The sunken wood attracted unique bacterial communities in comparison to those of background sediments. Moreover, temporal variation in the faunal and microbial wood-associated communities occurred within a period of 1 to 2 y, evident as species succession and variation of organism abundances. A surprisingly high turnover of the wood associated communities occurred with approximately 50 % of replacement with new bacterial types in 1-2 years. The results of this study suggest that biogeography plays an important role for the composition of both bacteria and fauna of wood-associated communities.

1 Introduction

Sunken wood, kelp and whale carcasses, commonly referred to as large organic falls, supply locally large quantities of organic matter to the otherwise food-deprived deep-sea floor (Jørgensen and Boetius, 2007; Smith and Baco, 2003; Smith et al., 1989). These local organic enrichments attract a multitude of organisms and promote the development of prolific ecosystems with one of the highest species richness known from deep-sea habitats (Baco and Smith, 2003). Although individual organic falls affect only localized areas of the deep-sea floor, they occur frequently in all parts of the world's oceans (Bernardino et al. 2010 and references therein). The carbon transported to the deep sea by large organic falls might be negligible compared to the global Particulate Organic Carbon (POC) flux, however even as one-time events such falls transport substantially higher amounts of carbon than what usually reaches the deep sea floor. For example, recent studies have estimated that a single storm event can transport up to 1.8 – 4 Tg of driftwood carbon to the ocean, that a single large whale carcass can provide an equivalent of 2000 years of background POC flux to the deep-sea floor, or a sinking swarm of swimming crabs 30-40% of the annual carbon flux (Christiansen and Boetius, 2000; Smith and Baco, 2003; Smith, 2006; West et al., 2011). These estimates clearly indicate the significance of large organic falls for the ecology of deep-sea ecosystems.

The degradation of organic matter derived from large food falls is a temporally dynamic process that involves successions of specialized communities with distinct life styles and metabolic requirements. Locally, high organic loads may deplete oxygen in the seafloor around the food fall, attracting anaerobic microbial communities to continue degradation with anoxic processes such as fermentation, sulfate reduction and methanogenesis (Goffredi et al., 2008). Such anaerobic degradation may alter biogeochemical conditions of the seafloor in the immediate vicinity of such food fall habitats, and cause sulfide production finally attracting chemosynthetic communities (Bienhold, 2011; Goffredi and Orphan, 2010; Smith and Baco, 2003; Treude et al., 2009).

Studies on the temporal succession of food fall communities in the deep sea mostly focused on whale falls as the largest type of carbon input. The degradation of whale carcasses proceeds through four successive stages that are distinguished by the fauna colonizing the whale remains and the biogeochemical conditions that evolve (Smith et

al., 1989, 2002; Smith and Baco, 2003). Specialized macro- and megafaunal organisms, e.g. sharks and hagfish initialize the degradation of organic matter from whale carcasses (Smith & Baco, 2003). Their sloppy feeding distributes pieces of meat and fat on the seafloor, and other opportunistic scavengers cause a burial of these food falls into the seafloor (Treude et al., 2009). By anaerobic respiration with sulfate, microorganisms produce sulfide, initiating the sulfophilic stage of whale carcasses (Bennett et al., 1994; Smith et al., 1989; Treude et al., 2009), which in turn attracts chemosynthetic organisms commonly found at cold seeps and hydrothermal vents, i.e. symbiotic mytilid mussels, clams, as well as chemoautotrophic bacteria. Based on the commonalities of associated fauna, it has been hypothesized that large organic falls might serve as stepping stones in the distribution and evolution of chemosynthetic fauna at seeps and vents (Distel et al., 2000; Smith et al., 1989).

In contrast, still little is known about the temporal succession of communities and biogeochemical gradients at wood falls. Wood-boring bivalves are among the first organisms to colonize wood falls and are responsible for the initialization of the wood degradation by producing wood chips and fecal matter, which provide colonization surfaces and nutrients for other organisms (Turner 1973; Bienhold et al., in prep). Microorganisms along with fungi, play a crucial role in the enzymatic degradation of cellulose (Palacios et al., 2006), one of the major constituents of wood, both under oxic and anoxic conditions (Leschine, 1995). Microbially mediated anaerobic degradation of cellulose produces sulfide (Laurent et al. 2009; Bienhold, 2011), and similar to whale falls, provides the basis for the establishment of chemosynthetic communities. Despite having a crucial role in the degradation of wood falls, only few studies exist to date focusing on the ecology of microorganisms associated to wood falls (Palacios et al. 2009; Bienhold, 2011). Hence, the overall aim of this study was to contribute to the understanding of the microbial ecology of wood falls, and assess temporal dynamics and spatial variations of these ecosystems.

Therefore, identically designed wood experiments were deployed and repeatedly sampled over a time frame of 3 years. The wood experiments were deposited at three geographically distant cold seep sites, located in areas with contrasting environmental conditions, i.e. the cold Arctic deep sea (-1 °C) and the warm, oligotrophic Eastern Mediterranean sea (14 °C). Within this study we applied Automated Ribosomal Intergenic Spacer Analysis (ARISA) to describe temporal and spatial variations in

bacterial community structure, as well as *in situ* and *ex situ* biogeochemical methods to assess the influence of wood falls on the biogeochemistry of surrounding sediments. The main questions addressed are: i) is there a temporal succession of wood-associated bacterial and faunal communities ii) how does the influence of sunken wood on the surrounding sediment bacterial communities and biogeochemistry change with immersion time, iii) can we identify geographical patterns in the distribution of wood-associated bacterial and faunal communities iv) can the concept of core wood-associated bacterial community (Bienhold, 2011) be applied in a broader context, across oceans.

2 Material and Methods

2.1 Description and sampling of wood colonization experiments

Each wood colonization experiments consisted of one large wood log (200 x 30 cm) of Douglas fir, which served the purpose to attract fauna colonizers, and several small attached wood logs (30 – 50 x 10 – 15 cm) that could repeatedly be sampled (Bienhold, 2011; Fig. 1). To make sure the experiments remained at the seafloor, cement blocks were attached as weights. In total, nine wood experiments (Table 1) were deployed in the proximity of three cold seep sites i.e. the Central Province of the Nile Deep-Sea Fan characterized by carbonates and pockmarks (CP) and the Amon Mud Volcano (AMV) in the Eastern Mediterranean Sea (EMed; Dupre et al., 2007; Dupré et al., 2008; Omoregie et al., 2009; Ritt et al., 2011), and the Haakon Mosby Mud Volcano of the Norwegian Sea (HMMV; Foucher et al., 2009; Felden et al., 2010). The most prominent difference between these cold seep sites besides a different biogeography is the temperature of the bottom water, which for the Norwegian Sea can be as low as – 1º C, and for the deep Eastern Mediterranean as high as 14º C.

The Central Province area at water depths of around 1700 m encompasses numerous small pockmarks (circular depression few meters in diameter and app. one meter deep) associated with active seepage of methane, which in turn supports the development of bacterial mats and precipitation of large flat authigenic carbonate crusts that overlie reduced sediments (Dupre et al., 2007; Foucher et al., 2009; Gontharet et al., 2007). The wood experiments in the Central Province were deployed in different habitats: on

bare inactive sediments (EMed-CP-wood#5, EMed-CP-wood#7), carbonate crust (EMed-CP-wood#2) and in the proximity of carbonate crust (EMed-CP-wood#1, EMed-CP-wood#6) (Table 1; Bienhold, 2011). Two sets of samples, after one year (2007) and three years of immersion (2009) were acquired from the EMed-CP-wood#1, EMed-CP-wood#2 and EMed-CP-wood#5 experiments deployed in 2006 (Table 1; Fig. 1a - i). EMed-CP-wood#6 was deployed in 2007 and sampled after 1 day of deployment, as well as two years later in 2009. Detailed description of the sampling procedure and biogeochemical measurements performed on these wood experiments in 2007 is given in (Bienhold, 2011). In 2009 an additional wood experiment (EMed-CP-wood#7) was deployed in the vicinity of the EMed-CP-wood#5 to serve as a reference for the investigation of the impact of woods on the surrounding sediments (Table 1). At the Amon MV (Ø 2 km, water depth 1120 m), two wood experiments were deployed in 2006 at the rim of the so-called "sulfur band" habitat that is located at the foot of the MV and is characterized by a lateral outflow of briny mud (Dupre et al., 2007; Girnth et al., 2010). The immediate environment of the experiments was characterized by bare sediments with no indication of any recent seepage of methane or development of reduced conditions (Table 1). Both experiments (EMed-AMV-wood#3 and EMed-AMV-wood#4) were sampled after three years of submersion.

At the Haakon Mosby Mud Volcano (Ø 1.4 km, water depth 1250 m) two wood experiments (HMMV-wood#1 and HMMV-wood#2) were deposited in 2007 on a so-called "siboglinid tubeworm" habitat (Table 1; Niemann et al. 2006; Jerosch et al. 2007; Lichtschlag et al. 2010a). This habitat was characterized by sediment densely populated with two symbiotic species of siboglinid Annelids i.e. *Sclerolinum contortum and Oligobrachia haakonmosbiensis* (Gebruk et al., 2003; Lösekann et al., 2008). Samples from HMMV-wood#1 were obtained after two (2009) and three years (2010) of submergence (Fig. 1j – k). Due to limited ROV diving time, samples from HMMV-wood#2 were only obtained in 2009. Summarized description of the location, type of habitat, deployment and sampling events of the different wood experiments is given in Table 1. All metadata are stored in the PANGAEA database (http://www.pangaea.de/), and PANGAEA references for the samples are cited accordingly (Supplement Table 1, Bienhold, 2011).

2.2 Visual observations and sampling of wood experiments

ROV-based high quality video and photo surveys were performed on the wood experiments prior to sampling, in order to assess the condition of the sunken woods, to qualitatively deduce potential changes in the degradation rates with increasing time and to observe associated megafauna. Wood log samples were recovered from the HMMV and Eastern Mediterranean experiments, and subsampled for bacterial community analysis, as well as the assessment of macro-, and mega-faunal colonizers. The *in situ* and on board subsampling procedures of the wood logs were conducted as described in Bienhold (2011). After 3 years, the degradation of some of the woods had advanced substantially, thus in some case it was not possible to recover three complete wood logs per experiment. Moreover, some of the wood logs were so heavily degraded that it was not possible to distinguish the surface from the subsurface. However, when possible, 2x3 subsamples of the surface (0-2 cm) and the subsurface (2-4 cm), as well as an additional sample from the bark, were obtained from each wood log, resulting in maximum 21 samples per wood experiment. Part of the wood samples was stored at -20°C for DNA analyses and part for bacterial cell counts (4% Formalin/Seawater). Mega- and macrofauna were collected and fixed for taxonomical analysis.

For most of the Eastern Mediterranean experiments additional sampling of the adjacent wood-influenced (0.5 m – "at wood") and reference (10 m – "away from wood") sediments was performed in order to determine the degradation effects of the wood on the benthic bacterial communities and sediment biogeochemistry. Biogeochemical measurements and sediment retrieval was not possible at the EMed-CP-wood#2, as this experiment was deposited on top of hard carbonate substrate. Due to limitation in ROV-bottom time, no biogeochemical analyses of wood-influenced sediments were performed for the HMMV woods. Sediments were sampled with the help of ROVs using push core liners (Ø 8 cm, 20 - 30 cm sediment depth), or ship-based Multiple Corer (MUC; Ø 9.5 cm). On board the cores were sliced in 1 cm resolution and subsamples for DNA analyses and bacterial cell counts were fixed as described above.

2.3 Microbial community analysis

2.3.1 DNA extractions

Total DNA was extracted from finely cut wood material (0.3 g) or 1g of sediment using UltraClean Soil DNA Isolation Kits (MoBio Laboratories Inc., Carlsbad, CA). DNA was finally eluted in 50 µl 1 x Tris-EDTA buffer (Promega, Madison, WI, USA), and its concentration was determined with a microplate spectrometer (Infinite® 200 PRO NanoQuant, TECAN Ltd, Switzerland).

2.3.2 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA amplification (Fisher and Triplett, 1999) of bacterial DNA, in triplicates, and subsequent capillary electrophoresis analysis were performed following the procedure described in Bienhold (2011). Due to technical adjustments, it was not possible to use the same GoTaq DNA Polymerase (Promega Corporation, Madison, USA) as applied in the study by Bienhold (2011), hence for the purpose of this study peqGOLD Taq Polymearase (PEQLAB Biotechnologie GMBH, Erlangen, Germany) was used. The dataset generated during this study was binned together with the dataset provided by Bienhold (2011) in order to ensure comparativeness between the two datasets (using a bin size of 2 bp; Interactive Binner function, http://www.ecology-research.com). The three ARISA PCR replicates were merged to form a consensus profile, under more stringent conditions where a peak was considered present if it appeared in at least two out of the three PCR replicates.

2.3.3 Statistical analyses

Overall patterns in bacterial community structure and composition were visualized with Non-metric MultiDimensional Scaling (NMDS) analysis based on ARISA OTU tables with Bray-Curtis and Jaccard distances, as implemented in the R package *vegan* (Oksanen et al., 2011). Analysis of similarity (ANOSIM) was used to test for significant differences between *a posteriori* groupings of samples. To assess spatial patterns in the bacterial community structure, samples submerged for the same period of time were compared. Samples originating from the same wood experiments, but sampled at different time points were used to assess the temporal succession of bacterial communities. All statistical analyses were performed in R (v. 2.9.1) (R

Development Core Team 2009, http://www.R-project.org) using *vegan* (Oksanen et al., 2011) and custom R scripts.

2.4 Biogeochemical analysis

2.4.1 Sediment porewater analyses

Detailed description of porewater extraction and fixation for nutrient (NO_3, NO_2, NH_4), DIC, alkalinity, sulfide and sulfate samples, as well as list of all biogeochemical measurements performed during this study are available in supplementary information (SupplementTable 1).

2.4.2 *In situ* quantification of Total Oxygen Uptake (TOU)

The total oxygen uptake of sediments located at (0.5 m) and away (10 m) from EMed-AMV-wood#4 and EMed-CP-wood#6 experiments (Supplement Table 1), was determined *in situ* with a benthic chamber module (Wenzhöfer and Glud, 2002; Felden et al., 2010). The centrally stirred benthic chamber (BIC) module enclosed 284 cm^2 (Ø 19 cm) of sediment surface and 10 – 25 cm of overlying bottom water (equivalent to 4 – 6 L). Once at the seafloor, the benthic chamber was handled and operated by a ROV, which ensured precise positioning of the chamber module to the sites of interest. Upon deployment of the chamber to the seafloor, a one-way valve released the excess water and thus provided smooth placement on the sediment. The exact height of the enclosed water body was determined with the help of the ROV-camera system. The decline in bottom water oxygen concentration during the incubation was continuously measured with an oxygen optode (AADI, Norway). In general, the initial decrease in oxygen concentration with time was used to calculate the TOU (mmol m^{-2} d^{-1}), as given in Felden et al. (2010):

$$\boldsymbol{TOU = \frac{dC}{dt}\, x\, \frac{Vchamber}{A\,chamber}}$$

where, dC/dt (µmol L^{-1} h-1) is the change of oxygen concentration over incubation time, $V_{chamber}$ (cm^3) is the volume of the overlying water in the enclosed chamber, and the $A_{chamber}$ (cm^2) is the area of the sediment enclosed by the chamber.

2.4.3 *In situ* and *ex situ* quantification of Diffusive Oxygen Uptake (DOU)

Diffusive oxygen uptake and Oxygen Penetration Depth (OPD) of sediments at (0.5 m) and away (10 m) from the wood experiments were determined via microsensor measurements, performed both *in situ* using a modified deep-sea microprofiler (MICP) module (Glud et al., 2009) and *ex situ* on retrieved push cores. List of all microsensor measurements performed in 2009 is given in SupplementTable 1. *Ex situ* microsensor measurements (including O_2, H_2S and pH profiles) performed at the wood experiments in 2007 are available in (Bienhold, 2011). Calibration of the O_2-microsensors and measurement of *in situ* high-resolution (200 µm) O_2 concentration profiles was performed as described elsewhere (Revsbech 1989; Beer et al. 2006; Treude et al. 2009; Lichtschlag et al. 2010b). The laboratory set up used to conduct *ex situ* oxygen microsensor measurements was done according to Bienhold et al. (in prep). Based on the Fick's first law of diffusion, the *in situ* and *ex situ* DOU was calculated from the linear oxygen concentration gradient in the DBL, according the formula (Jørgensen and Des Marais, 1990)

$$J = D * \frac{\delta C}{\delta z}$$

where J is the diffusive flux in mmol m^{-2} d^{-1}, D is the diffusion coefficient of oxygen in water (D = 1.6 x 10^{-9} m^2 s^{-1}), corrected for the temperature and salinity (Li and Gregory, 1974) and $\delta C/\delta z$ is the concentration gradient (mmol m^{-2}) through the DBL.

2.4.4 *Ex situ* measurements of sulfate (SR) and methane consumption (MOx)

At each of the investigated sites (SupplementTable 1), three sediment replicate subcores (Ø 2.8 cm) were injected at 1 cm resolution with either 25 µl $^{14}CH_4$ (dissolved in water, 2,5 kBq) or 5 – 10 µl $^{35}SO_4^{-2}$ (dissolved in water, 50 kBq) for the quantification of sulfate reduction (SR) and Methane Oxidation (MOx) rates, according to the whole-core injection method (Jørgensen, 1978). The injected subcores were incubated for app. 12 h at *in situ* temperature of 14° C in the dark. Afterwards, the cores were sliced at 1 cm resolution and fixed with NaOH (2.5%, w/v, in glass gas tight bottles) and 20 ml Zincacetate (20%, w/v) for SR and MOx analyses, respectively. Substrate (methane and sulphate) concentrations were measured by gas chromatography (5890A Hewlett Packard) and anion exchange chromatography

(Waters IC-Pak anion exchange column, waters 430 conductivity detector), respectively. Turnover rates were measured in the home laboratory and calculated according to Treude et al. (2003) and Kallmeyer et al. (2004).

3 Results

3.1 In situ visual observations

The degradation of all sunken woods deposited in the Central Province area had progressed with time, and EMed-CP-wood#1, -#2, -#5 were substantially more degraded in 2009, after 3y of submergence, as compared to 2007 (Fig. 1 a-i). The small wood logs were largely degraded, and only parts of the bark of the large wood logs were still present. In contrast, the Amon MV sunken woods still contained most of the originally deployed material, with most of the small wood logs still attached to a more or less complete large wood log. The degree of degradation of EMed-CP-wood#6, located at the Pockmark and submerged for 2y in total, was similar to the condition of the Amon MV sunken woods after 3 years. Big piles of wood chips of approximately 3 cm height, produced by the activity of the wood-boring bivalves, were spread over the sediments surrounding the log (0.5 m, areal coverage of approximately 4.5 m^2) at all experiments in the Eastern Mediterranean. Blackened, reduced spots, indicating precipitation of iron sulfide, existed within the woodchip accumulations and at the transition to the sediments. The extent of these reduced spots was most pronounced at EMed-CP-wood#6, encompassing the complete surrounding sediment. White debris, most probably derived from broken shells of woodborers, laid spread near the wood experiments. White sea urchins, amphipods, large crustaceans and occasionally snails were the most prominent fauna dwelling on the wood chips and the sunken woods in the Eastern Mediterranean.

The degree of degradation of the HMMV wood experiments appeared more variable among wood experiments and among wood logs within single experiments (Fig. 1 j, k). Both sunken woods were deployed on soft sediment densely populated by chemosynthetic siboglinid tubeworms. In the case of HMMV-wood#1 the sediments were covered with white bacterial mats found as close as < 0.5 m away from the wood parcels. The presence of these organisms is indicative of sulfidic conditions. Striking difference to the EMed sunken woods was that no wood chips could be observed

around the HMMV experiments. The only larger fauna residing on the sunken woods were few crustacean and pycnogonids.

3.2 Wood-associated faunal communities

3.2.1 Temporal variations of wood-associated fauna

The faunal communities related to wood falls in the Eastern Mediterranean have undergone temporal succession within the 3y since the deployment of the experiments. Mainly, wood-associated fauna in 2009 was different from 2007 in terms of abundances of certain taxa, e.g. substantially increased numbers of sipunculids were observed in 2009, but substantially lower abundances of wood-boring bivalves (Table 2). However, in contrast to 2007, two different species of wood-boring bivalves were found in association with the sunken woods in 2009. In addition to *Xylophaga dorsalis* already recovered in 2007, a second species from the subfamily *Xylophagainea* (C Borowski & A. Nunes-Jorge, Max Planck Institute for Marine Microbiology, pers. communication), termed "*Xylo2*" for the purpose of this study, was recovered from the sunken wood experiments in 2009. The taxonomic identification of "*Xylo2"* is still ambiguous, as the molecular analysis revealed 99% identity of this species to both *Xyloredo* and *Xylophaga* species (C. Borowski and A. Nunes Jorge, Max Planck Institute for Marine Microbiology, pers. communication). Mostly empty shells of *Xylophaga dorsalis* were recovered. The chemosynthetic bivalves present on the wood experiments in 2009 were *Idas modiolaeformis* (Lorion et al., 2012; S. Dupperon, Université Pierre et Marie Curie, pers. communication), which had also been recovered in 2007 (Table 2; Bienhold, 2011). Sea urchins and occasional crabs were the largest fauna colonizing the experiments and the sediments covered with wood chips. Up to 4 – 5 sea urchins were observed per experiment, amounting to only one fifth of the number of specimens encountered 2y before.

In contrast to EMed wood experiments, much lower numbers of faunal organisms were found in association with HMMV wood experiments, and it was therefore difficult to establish whether the HMMV wood-associated fauna communities had changed with time. Siboglinids specimens found at the top outer side of the sunken woods were sampled in 2009 and 2010, however only one individual of wood boring bivalve and no pycnogonids were recovered in 2010 (Table 2; for detailed description

of the fauna colonizing the HMMV experiments, see following chapter on spatial variations).

3.2.2 Spatial variations of wood-associated fauna

No prominent difference in the structure of faunal communities colonizing the Amon MV and Central Province sunken woods were observed (Table 2). Similar to the CP wood experiments, very few specimens of living woodborers, predominantly "*Xylo2"*, were observed at either of the wood experiments at the AMV. The most abundant colonizers at the Amon MV were *Idas modiolaeformis* (S. Duperron, Université Pierre et Marie Curie, pers. communication), as well as sipunculids that were smaller than the ones detected at the CP woods. Other colonizers associated to the sunken woods at the AMV were amphipods, found in great numbers, as well as amphinomids and sea urchins (most probably *Asterechinus elegans*) represented by only few specimens.

The HMMV wood-associated fauna differed substantially from the fauna found to colonize the Eastern Mediterranean wood experiments (Table 2). Namely, none of the two wood-boring bivalve species encountered in the EMed were found at the HMMV wood experiments, and instead a different bivalve species from the subfamily *Xylophagainae* (termed "*Xylo3*" for the purpose of this study), most probably belonging to the genus *Xyloredo* (C. Borowski, Max Planck Institute for Marine Microbiology, pers. communication), represented the main woodborer responsible for the extensive wood degradation of the HMMV experiments. The Arctic *Xylo3* woodborer was characterized by having burrows with white calcareous linings, which stretched longitudinally throughout the complete wood logs. These bivalve structures occupied extensive parts of the logs, and in some cases completely substituted the typical structure of the wood logs (Fig. 2).

In contrast to the EMed sunken woods, no chemosynthetic bivalves were found associated to the HMMV experiments. However, few chemosynthetic siboglinid tubeworms *Sclerolium contortum* (Gaudron S., Université Pierre et Marie Curie, pers. communication), known to depend on sulfide for their survival, were found on the upper side of the log, on the wood bark. In contrast to the EMed, pycnogonids were the dominant predatory animals associated to HMMV woods and no sea urchins were

observed on the wood, but also never in the background HMMV area (Gebruk et al., 2003).

3.3 Biogeochemistry and bacterial communities of wood fall ecosystems

3.3.1 Influence of wood falls on the sediment biogeochemistry

In contrast to measurements performed after 1 y of deployment, no free sulfide was detected anymore at any of the wood-influenced sediments ("at wood") in the Eastern Mediterranean in 2009, after 3y of immersion (Table 3). However, a blackened reduced layer, most probably originating from sulfide precipitation with iron, was observed on the boundary between the wood chips and the sediment at all experiments in the EMed. It is possible that the inability to detect sulfide in 2009 was an artefact of the ex situ rhizon-extraction of the porewater, during which the sulfide in the fluffy wood chip layer may have been oxidized. Correspondingly, the sediment sulfate reduction rates were around 1 mmol m^{-2} d^{-1} (Table 3), similar to the rates detected two years before. No (an)aerobic oxidation of methane was detected at any of the investigated sites, hence SR was most likely related to wood degradation. Higher sulfide porewater values (max. 1.2 mM) were detected only in the deeper sediment layers (> 8 cm) at EMed-CP-wood#6 (data not shown), most probably due to proximity of this experiment to an active seep site.

Similar geochemical conditions prevailed at the wood-influenced sediments after 1y and 3y of deployment at both EMed-CP-wood#1 and EMed-CP-wood#5 experiments, with diffusive oxygen uptake (DOU) of 3.7 – 6.2 mmol m^{-2} d^{-1} and oxygen penetration depth (OPD) as shallow as 4.6 mm (Table 3 & Fig. 3). Moreover, even in the second year of deployment, the diffusive uptake (2.7 mmol m^{-2} d^{-1}) and penetration of oxygen (5.4 mm) remained unchanged, as revealed from EMed-CP-wood#6. As detected previously, oxygen was completely consumed within the wood chip layer at all investigated experiments (Fig. 3). Comparable diffusive oxygen fluxes (3.4 – 3.6 mmol m^{-2} d^{-1}) were detected also in the sediments surrounding the sunken woods at the Amon MV (EMed-AMV-wood#3, EMed-AMV-wood#4; Table 3).

Total oxygen flux measured after 2y (EMed'09-CP-wood#6) and 3y (EMed'09-AMV-wood#4) of submergence was more than two times higher (60 – 63 mmol m^{-2} d^{-1}) as compared to the first year of immergence of EMed'09-CP-wood#1.

Even after 3y, the influence of the wood at all investigated experiments in the EMed was confined only to the sediments located in the vicinity of the experiments (0.5 m). The impact of the sunken woods on these surrounding sediments was evident from the elevated fluxes (diffusive and total) and shallower penetration of oxygen detected next to the experiments, as compared to the reference sediments located app. 10 m from the wood experiments (Table 3). At the reference sediments EMed'09-Away_wood#1 and EMed'09-Away_wood#4 oxygen penetrated deeper than 16 and 63 mm, respectively, while the total benthic oxygen uptake was 3 – 4 times lower compared to the wood-influenced sediments (Table 3).

Although, the oxygen consumption measurements indicated intensified degradation of organic matter in the vicinity of the wood experiments, the nutrient and DIC concentrations (data not shown) remained low and more or less constant throughout the complete core depth, and were similar to the values detected at sediments not influenced (10 m away) by the wood.

No biogeochemical samples were available from the sediments associated with HMMV wood experiments, however the occurrence of thiotrophic bacterial mats on the sediments in contact with the wood experiments also indicate development of sulfidic conditions. Normally, bacterial mats of the HMMV are confined to the central mud flows, and some patches of bare sediments within the siboglinid-covered outer rim (Lichtschlag et al. 2010a; Felden et al. 2010).

3.3.2 Influence of the wood falls on the sediment bacterial communities

All depth layers of the wood-influenced sediments had higher OTU number (85±53 SD, n = 16) compared to the reference sediments (49±43, n = 16) at both investigated wood experiments (EMed-CP-wood#1, EMed-CP-wood#5) over different years. This difference was the most pronounced at the EMed'07-CP-wood#5 experiment, where 135 OTUs were detected within the wood chip layer at the wood-influenced site, and only 17 OTUs in the topmost surface layer at the reference site. The relative proportion of OTUs common to wood-influenced and reference sediments at EMed-CP-wood#5 increased with immersion time (9 to 35%; Table 4). However, no such

trend was observed at the EMed-CP-wood#1 and similar percentages of shared OTUs (39 and 45%) between the wood-influenced and reference sediments were detected after 1y and 3y of immersion (Table 4). Moreover, no statistically significant differences in the bacterial community structure (including information on the relative abundances of OTUs) between wood-influenced and reference sediments were detected at either of the experiments (EMed-CP-wood#1 and EMed-CP-wood#5) over different years (Fig. 4, Supplement Table 2). In contrast, the structure and composition of wood-associated bacterial communities remained significantly distinct from the surrounding sediment-associated communities through the course of different years (Fig. 4, Supplement Table 2). Based on the NMDS analyses (Fig. 4), the similarity between the sediment (including reference sites) and the wood-associated bacterial communities was higher for the topmost surface layers after 3y of immersion compared to the deeper sediment layers. While the NMDS analyses revealed temporal shift in the wood-associated bacterial community structures at both EMed-CP-wood#1 and EMed-CP-wood#5 experiments (Fig. 4), the structure of sediment-associated bacterial communities did not change significantly with time (Supplement Table 2).

3.3.3 Comparison of wood-associated bacterial communities in relation to immersion time

An increase in the total number of OTUs with increasing length of immersion was detected for all wood experiments in the Eastern Mediterranean, as well as at the HMMV, with most pronounced differences detected for the EMed-CP-wood#6 experiment, between the time of the deployment and after 2 years of immersion (Fig. 5). At the same time, a substantial proportion of the initial OTU types were lost from the woods: on average only 54±5 SD % (n = 3) of the OTUs found in 2007 were also retrieved in 2009. In the case of EMed-CP-wood#6, up to 75% of the original fresh-wood OTUs were lost, and only 25% were recovered after 2y of deployment (Fig. 5).

Temporal succession in the bacterial community structure associated to experiments deposited at the CP and HMMV was confirmed using NMDS analyses. Significant shifts in the structure and composition of wood-related bacterial communities has occurred with time at all investigated CP experiments, as revealed from the comparison of woods deployed for 1y and 3y (EMed-CP-wood#1, EMed-CP-wood#2,

EMed-CP-wood#5; Fig. 6a and Supplement Table 3). The temporal variations were much stronger compared to the differences in the communities between different wood experiments deployed for the same duration (Fig. 6a, Supplement Table 3). Less pronounced, but still significant temporal shifts were revealed for the wood-related bacterial community of HMMV wood experiments during a period of 1 year (73% of shared OTUs for HMMV-wood#1l Fig. 6b; Supplement Table 3).

3.3.4 Spatial variations of wood-associated bacterial communities

To investigate spatial variations in wood-associated bacterial communities, experiments submerged for the same period of time, but deployed at different sites were compared. Pairwise OTU sharing analysis revealed that any two wood experiments (including EMed and HMMV woods) had 48 – 78% OTUs in common (Fig. 7b). In contrast, reference sediments in the EMed (EMed'09-CP-Away_wood#5 and EMed'09-CP-Away-wood#1) shared only 34 % of the total OTUs.

When the abundance of OTUs, and not only presence-absence, was considered (calculated as Bray-Curtis distances), it was observed that woods deposited at different geographic regions, i.e. HMMV versus EMed woods, had significantly different wood-associated bacterial communities (Fig. 7a, Supplement Table 4). Less pronounced, but still significant differences in the bacterial community structure were observed between wood experiments located at proximate seep sites within single geographic region (CP versus AMV woods) (Fig. 7a, Supplement Table 4). Lastly, no significant differences in the structure of bacterial communities existed between replicate wood experiments located within the same cold seep structure (e.g. within the CP, AMN and HMMV) (Fig. 7a, Supplement Table 4).

4 Discussion

4.1 Temporal variations of the wood-associated communities

4.1.1 Temporal variation of wood-related fauna communities

This study provides evidence that sunken woods are highly dynamic ecosystems with prominent temporal succession in the wood-associated faunal and bacterial communities already within the first 3 years of immersion. Although no quantitative

assessment of the faunal community associated to the sunken woods was conducted during this study, and the sampling procedure was biased towards larger animals, temporal shift of the most abundant faunal representatives was observed. For wood falls in the Eastern Mediterranean, succession of wood-boring bivalve species and substantial increase in the abundances of sipunculids were observed. Furthermore, particular successional stages can be defined, similar to what has been previously documented for other large organic falls (Bennett et al., 1994; Fujiwara et al., 2007; Lundsten et al., 2010; Smith and Baco, 2003; Smith et al., 2002). Based on the faunal and biogeochemical investigations of this and other studies (Lorion et al., 2012; Turner, 1973, 2002; Voight, 2008; Zbinden et al., 2010), we propose the presence of four overlapping successional stages in the degradation of wood falls: 1) a *specialists stage*, occurring within the first couple of months of the wood arrival at the sea floor and characterized by invasion of woodborers that initialize the degradation of wood; 2) an *opportunist stage*, initiated already before the sulphophilic stage and lasting for 1 – 2 years, with a peak during the main growth of wood-borer populations, when detritus-feeders and predator organisms, i.e. sipunculids, pycnogonids, sea urchins, get attracted by the accumulation of biomass; 3) a *sulphophilic stage* (duration > 1 - 2 years), during which enhanced cellulose degradation leads to sulfidic conditions and a colonization by chemosynthetic organisms, i.e. *Idas sp.*, siboglinids, takes place; 4) a *senescence stage,* initiated after the third year of degradation, characterized by long term cellulose degradation in the surrounding sediments and possible dispersal of the woods chips, as well as decline of numbers of large faunal organisms including reduction of woodborer biomass (Bernardino et al., 2010).

Symbiotic wood-boring bivalves such as the deep-sea genus *Xylophaga sp.* (Distel and Roberts, 1997) play a key role in the degradation of wood in deep-sea marine environments, and hence, are very important for the ecology and biogeochemistry of wood falls ecosystems (Bienhold, 2011and references therein). Interestingly, we were able to observe shifts in the occurrence of wood-boring species within a period of three year of submergence. In the Eastern Mediterranean Central Province, *Xylophaga dorsalis* dominating the wood-experiments, as well as other small colonization devices (Gaudron et al., 2010), declined in abundance after the first year of deposition, and was almost substituted by a different and less abundant *Xylophagainae* species (*"Xylo2";* C. Borowski, Max Planck Institute for Marine Microbiology, pers.

communication). For a long time only one wood boring species (*Xylophaga dorsalis*) was known from the Mediterranean Sea. However, just recently a study on wood falls in a submarine canyons in Blanes discovered three unidentified species of *Xylophaga spp*. that co-colonized sunken woods and had variable abundances with time (Romano et al., 2011).

These findings suggest that different species of wood boring bivalves have adopted different ecological strategies with, e.g. certain species initializing the degradation of wood, forming openings through the bark, borrows inside the wood, wood chips and fecal matter as well as biomass, and others being important in the later stages of the degradation. Moreover, potentially more than one species of wood-boring bivalve might be relevant for the complete degradation of wood falls in the deep Eastern Mediterranean sea. Another possibility that can explain the patterns observed in this study could be the observation that different *Xylophaga* species have variable modes of spawning i.e. periodic versus continuous (Tyler et al., 2007), which could have an impact on the structure of the pioneering colonizer communities. To better understand the commonality and causality of such events continuous long-term monitoring should be employed that would investigate the ecology of the host species as well as the metabolic potentials of their symbionts.

4.1.2 Temporal succession of wood-related bacterial communities

Cellulose is the major component of plant and woody material that reaches the deep sea floor. Degradation of cellulose is a complex process that proceeds via a cascade of different enzymatic catalytic steps mediated by complex bacterial and fungal communities (Leschine 1995). Despite being the second most important carbohydrate in marine environments (Wilson 2011), the consortia of microorganisms responsible for cellulose hydrolysis in marine environments are largely unknown. A recent sequence-based study identified potentially diverse cellulose-degrading bacteria e.g. *Alphaproteobacteria, Flavobacteria, Actinobacteria* and *Clostridia* comprising a core wood-related community on sunken woods in the EMed (Bienhold, 2011). Also, the study confirmed the loss of terrestrial signature in the bacterial community with increasing immersion time (Bienhold et al. in prep), and moreover revealed a rapid temporal succession in the wood-associated bacterial communities. This study supports the high turnover of the original community, and shows that relative OTU

richness increased with immersion time, i.e. only half of the OTUs from the first year were found after three year of deposition. The results of our study are in agreement with the findings by Palacios et al. (2009) who showed that variations in deep sea wood-related microbial communities could come from differences in the immersion time of sunken woods. Moreover, an earlier study revealed that not only deep-sea wood falls, but also shallow water sunken woods represent highly dynamic ecosystems with prominent temporal bacterial species successions (Austin et al. 1979).

Temporal succession of bacterial communities might occur as a response to changes in faunal activity and succession, as well as in changes in geochemical conditions during the degradation of wood. Especially when oxygen becomes depleted, cellulose is predominantly degraded anaerobically with sulfate in marine ecosystems, producing sulfide (Leschine 1995). Even for the aerobic heterotrophic wood degradation, diverse microorganisms are involved in the anaerobic degradation of cellulose. Together with anaerobic processes and the availability of sulfide an even higher diversity of microbial communities could be promoted, explaining the increase in OTU richness with time. We speculate that the surrounding sediments contribute little to the increased number of bacterial types colonizing the sunken woods after prolonged immersion time, as deduced from the relatively stable number of shared OTUs between wood experiments and the surrounding sediments during different years. However, further studies, based on coupled geochemical – microbiological approaches are needed in order to discern where wood colonizing bacteria come from and to reveal the factors inducing temporal succession of bacterial communities at sunken wood ecosystems.

4.2 Influence of sunken wood on surrounding sediments

4.2.1 Influence of sunken wood on sediment geochemistry

Whale falls have a long-term (several decades) impact on the geochemistry of sediments surrounding the falls, because of the massive input of organic carbon in the form of lipids, causing the production of sulfide and methane (Fujiwara et al., 2007; Goffredi and Orphan, 2010; Goffredi et al., 2008; Treude et al., 2009). In contrast to lipid and protein-based organic matter, wood cellulose is much more difficult to degrade due to the high degree of polymerization, rigid microfibril organization,

insolubility in water and association to lignin. Hence, very few highly specialized microorganisms can metabolize cellulose via production of cellulases. Only recently, a study showed for the first time that also sunken woods immersed for 1y alter the geochemistry of the surrounding sediments and promoted sulfide production within the wood chips layer accumulated on top of the sediments (Bienhold, 2011). Our study confirmed these findings and furthermore revealed that, similar to whale falls, sunken woods can have a long-term impact (>3y) on the sediment geochemistry. Moreover the impact of the sunken woods on the surrounding sediments remained relatively constant over time, as revealed by similar geochemical fluxes and rates measured after one and three years of immersion. However, in contrast to whale falls (Treude et al., 2009), the influence of the sunken woods on the sediment geochemistry did not expand laterally even after 3y of immersion, and instead remained spatially restricted to the nearby sediments (< 0.5 m from the wood experiments). However, it remains unknown if cellulose degradation in sediments can lead to methane production. A prerequisite would be the complete consumption of sulfate in the porewaters, to suppress substrate competition between sulfate reducing bacteria and methanogens (Oremland and Polcin, 1982). In the experiments conducted here, we could not observe a complete consumption of sulfate in the sediment porewaters around the wood falls, hence, excluding the development of methanogenic zones.

Interestingly, the feeding behavior and succession of wood-boring bivalves may be important for the areal impact of sunken wood, as well as for the associated biogeochemical processes. For example, in contrast to the EMed wood boring bivalves (Bienhold, 2011), the ones responsible for the degradation of HMMV sunken woods did not produce massive amounts of wood chips, but formed massive amounts of carbonaceous tubes gluing the wood together despite of its progressing degradation.

4.3 Spatial variations of wood-related communities

4.3.1 Spatial variations of wood-related faunal communities

Similar to cold seeps and hydrothermal vents, all types of large organic falls have a highly fragmented distribution and represent island-type habitats in the vast oligotrophic deep sea (Smith and Baco 2003). Given the fragmented and isolated nature of these habitats, it is of crucial importance to better understand the dispersal

patterns, and hence the biogeography and interconnectivity of biota communities associated to these habitats (Tyler et al. 2003; Vanreusel et al. 2009; Weaver et al. 2009). Community effort focusing on the biogeography primarily of vent, and to certain extent of seep communities has identified six biogeographic provinces for vent fauna, determined by the geographic location of the ocean basins and their isolation along the mid-ocean ridge system (Van Dover et al. 2002; Bachraty et al. 2009), and broader geographic ranges for seep fauna where depth, rather than geographic distance, was the major factor responsible for structuring seep fauna (Olu et al. 2010). On the other hand, the research on the biogeography of organic fall communities is still in its infancy (Smith and Baco 2003). Similar to cold seeps, water depth has been suggested to influence the distribution of whale fall communities (Goffredi et al. 2004; Braby et al. 2007; Fujiwara et al. 2007; Lundsten et al. 2010). Despite the non-quantitative sampling approach, the results of this study revealed strong qualitative differences in the wood-related fauna community according to ocean realms i.e. Norwegian versus Eastern Mediterranean, where wood experiments were located. Wood experiments located at two different cold seeps in the EMed (CP and AMV; 130 km distance) had more or less identical abundant taxa i.e., wood-boring bivalves "*Xylo2*", chemosynthetic mussels *Idas modiolaeformis* (seep-type fauna) and sipunculids, and many types of the background megafauna (sea urchins and crabs). In contrast, the HMMV sunken woods hosted distinct fauna as compared to EMed experiments, also representative of the background seep and deep-sea fauna. These included a different woodborer species "*Xylo3*" (most probably belonging to the genus *Xyloredo*), the chemosynthetic siboglinid tubeworm *Sclerolinum contortum*, few polychaetes, as well as pycnogonids represented the majority of the HMMV wood-related fauna community. Gaudron et al. (2010) identified a similar faunal community in a colonization experiment filled with wood substrate at the HMMV. The EMed woodborer *Xylophaga dorsalis* has been reported also from cold northern waters i.e. offshore of Iceland (Turner 2002) and Norway (Schander et al. 2010), however no specimens were found to colonize the HMMV wood experiments. Interestingly, no chemosynthetic bivalves were recovered from the HMMV wood experiments, but these were also not represented at the Norwegian margin cold seeps. Instead chemosynthetic siboglinid tubeworms were attached to the bark of the sunken woods, potentially indicating sulfide production within the wood logs. Few other studies have reported on siboglinids associated to sunken wood, indicating that wood

falls might represent a natural habitat for these chemosynthetic organisms (Gebruk et al., 2003; Gaudron et al., 2010; Schander et al., 2010). Although up to twelve species of sipunculids have been found to live in the Arctic Ocean (http://www.arcodiv.org/seabottom/worms/Sipunculids.html), none were previously reported from wood experiments in Northern waters. The results of this study suggest that the wood-associated fauna investigated in this study belong to two different biogeographic provinces, i.e. the Eastern Mediterranean and the Norwegian Sea, with frequent fauna exchange occurring within single provinces (e.g. seep and wood falls), and limited dispersal among provinces.

Temperature-dependent oxygen concentrations have been proposed to structure benthic communities (Lundsten et al., 2010 and references therein), which could also limit the dispersal of wood-associated fauna between the cold Norwegian and warm Mediterranean Sea. Moreover, isolation of the Arctic waters (Van Dover et al., 2002) has been assumed to act as a barrier and hence, result in highly specific and divergent vent and seep communities located in the Arctic Ocean. The same mechanisms could potentially govern the distribution of wood-related fauna, and hinder dispersion of faunal communities between the cold Norwegian and warm Eastern Mediterranean deep sea.

4.3.2 Spatial variations of wood-related microbial communities

Wood-related bacterial communities had a similar spatial distribution pattern to fauna colonizing deep-sea sunken woods, in that their composition and structure was different among sunken woods located in different oceanic regions. Bacterial communities of sunken woods located at the same cold seep site, and to certain extent at proximate seep sites, i.e. AMV and CP were overlapping and had highly similar structure. Conversely, the bacterial community structure of wood experiments at the HMMV was more distinct from the EMed, potentially indicating the existence of provinces for wood-related bacterial communities and dispersal limitation among the Eastern Mediterranean and the Norwegian Sea. In line with the results from this study, Zinger et al. (2011) proposed a certain degree of provincialism of benthic bacterial communities. Other authors have found no evidence that the geographic location played a role in structuring wood-associated communities (Palacios et al., 2009). Our findings challenge this view, at least for wood falls in two geographically distant sites

as different as the Eastern Mediterranean and the Norwegian Sea. To reveal patterns in the structure of bacterial communities Palacios et al. (2009) examined sunken woods that differed in many variables e.g. the type of wood, length of immersion, water depth of immersion, as well as used sediment along with wood samples to perform analyses. In such a set up the influence of other major factors, such as time of immersion, could have potentially blurred the impact of geographic distances on the bacterial community structures.

4.4 Sunken wood hosting distinct bacterial communities at the deep sea floor

Three-year submerged woods, investigated within this study, were characterized by distinct bacterial communities when compared to the wood-influenced and reference sediments, indicating highly specialized assemblages of microorganisms colonizing the wood falls. These results corroborate and expand on results of an earlier study that revealed highly similar communities of wood-associated bacteria after 1 year of submersion of different woods (Bienhold, 2011). Our study extends the time scale for which the presence of a core wood-related bacterial community was previously suggested (Palacios et al. 2009; Bienhold, 2011), and shows that sunken woods support highly distinct communities of bacteria, increasing in richness of bacterial types with longer periods (> 3y) of submergence.

Organic falls cause perturbations in the natural environmental conditions (Goffredi and Orphan 2010; this study), and in the case of wood falls, most prominently alter the geochemical conditions of the topmost surface layers of the sediments. In concordance, the influence of the sunken wood on the sediment bacterial communities was most pronounced in the surface sediment layers covered by wood chips, resulting in higher similarities of these communities to the wood, instead of to the deeper sediment communities. Over time, the sediment area influenced by whale falls expands laterally, with evident temporal changes in the bacterial diversity and the local sediment geochemistry (Treude et al. 2009; Goffredi and Orphan 2010). However, this was not the case for sunken woods, where even after 3y of immersion, imprint of the wood was evident only on the sediments surrounding the wood (< 0.5 m). Based on the comparison of temporal variations of wood- and sediment-hosted

bacterial communities we conclude that the sunken woods are more temporally dynamic habitats compared to typical deep-sea sediments.

5 Conclusions

Our study reveals that large falls of sunken wood can have long-term (>3y) impact on the surrounding sediment biogeochemistry, providing an energy source for unique bacterial and faunal organisms, including chemosynthetic organisms. The degradation of the wood falls by wood boring bivalves was accompanied by a community succession of both microbes and fauna, including the replacement of the dominant woodborer species and decline in their abundances. Based on the findings of this study we propose four overlapping stages in the degradation of wood falls, including 1) a *specialist stage*, characterized by woodborer colonization and initiation of wood degradation; 2) an *opportunist stage*, characterized by arrival of detritus-feeders and predator organisms; 3) a *sulphophilic stage*, during which cellulose is degraded anaerobically and the production of sulfide attracts chemosynthetic organisms and, 4) a *senescence stage*, characterized by long-term cellulose degradation and decline in woodborer biomass. Diversity of wood-associated bacteria increased with time and surprisingly high bacterial turnover with approximately 50% of replacement with new bacterial types occurred within a period of only 2y. Sunken wood of different oceanic realms i.e. the Eastern Mediterranean and the Norwegian seas harbored distinct faunal communities. Moreover, the bacterial communities associated to the woods located in these different geographic regions differed substantially from communities colonizing woods within the same geographic region, indicating dispersal limitation and an influence of biogeography on diversity patterns.

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Table 1 Location of wood experiments in the Norwegian and the Eastern Mediterranean deep sea, description of the benthic habitats and date of deployment and samplings events.

Wood experiment	Location	Habitat	Position	Deployment; Cruise	Sampling; Cruise
EMed-CP-wood#1	Eastern Mediterranean; Central Pockmark	Close to carbonate crusts	N 32°32.0496 E 30°21.1248	Nov 2006; Bionil[1)]	Nov 2007; Medeco[2)] Oct-Nov 2009; MSM13[3)]
EMed-CP-wood#2	Eastern Mediterranean; Central Pockmark	On carbonate crust	N 32°31.9626 E 30°21.1752	Nov 2006; Bionil[1)]	Nov 2007; Medeco[2)] Oct 2009; MSM13[3)]
EMed-AMV-wood#3	Eastern Mediterranean; Amon Mud Volcano	Edge of sulfur band	N 32°21.9920 E 31°42.1788	Nov 2006; Bionil[1)]	Nov 2009; MSM13[3)]
EMed-AM-wood#4	Eastern Mediterranean; Amon Mud Volcano	Edge of sulfur band	N 32°22.0159 E 31°42.2205	Nov 2006; Bionil[1)]	Nov 2009; MSM13[3)]
EMed-CP-wood#5	Eastern Mediterranean; Central Pockmark	On sediments	N 32°32.0790 E 30°21.3840	Nov 2006; Bionil[1)]	Nov 2007; Medeco[2)] Nov 2009; MSM13[3)]
EMed-CP-wood#6	Eastern Mediterranean; Central Pockmark	Edge of carbonate crust	N 32°32.0124 E 30°21.1920	Nov 2007; Medeco[2)]	Nov 2007; Medeco[2)] Nov 2009; MSM13[3)]
EMed-CP-wood#7	Eastern Mediterranean; Central Pockmark	On sediments; Reference	N 32°32.0775 E 30°21.3862	Nov 2009; MSM13[3)]	Nov 2009; MSM13[3)]
HMMV-wood#1	Norwegian Sea; Hakon Mosby Mud Volcano	Rim of gray mats and siboglind tubeworms	N 72° 00.3900 E 14° 43.6398	Jun 2007; ARKXXII/1b[4)]	Jul 2009; ARKXXIV/2[4)] Oct 2010; MSM16[5)]
HMMV-wood#2	Norwegian Sea; Hakon Mosby Mud Volcano	Rim of gray mats and siboglind tubeworms	N 72° 00.3780 E 14° 43.0957	Jun 2007; ARKXXII/1b[4)]	Jul 2009; ARKXXIV/2[4)]

[1)] *Expedition Bionil: RV Meteor; ROV Quest 4000*
[2)] *Expedition Medeco: RV Pourquoi Pas?; ROV Victor 6000*
[3)] *Expedition MSM13/3 & 4: RV Maria S Merian; ROV Quest 4000*
[4)] *Expedition ARKXXIV/2: RV Polarstern; ROV Quest 4000*
[5)] *Expedition MSM16: RV Maria S Merian; ROV Genesis*

Table 2 List of most prominent and abundant fauna colonizing the wood experiments in the Norwegian and Eastern Mediterranean sea.

	EMed'07-CP-wood#1	EMed'09-CP-wood#1	EMed'07-CP-wood#2	EMed'09-CP-wood#2	EMed'07-CP-wood#5	EMed'09-CP-wood#5	EMed'09-AMV-wood#3	EMed'09-AMV-wood#4	EMed'09-CP-wood#6	HMMV'09-wood#1	HMMV'10-wood#1	HMMV'09-wood#2
Sipunculids*	+	+	+	+	+	+	+	+	+	-	-	-
Amphinomid*	+	+	+	+	+	+	+	-	+	-	-	-
Xylophaga dorsalis	+	+	+	+	+	-	-	-	-	-	-	-
"Xylo2"	-	+	-	+	-	+	+	+	+	-	-	-
"Xylo3"	-	-	-	-	-	-	-	-	-	+	+	+
Idas modiolaeformis	+	+	+	+	+	+	(+)	(+)	+	-	-	-
Galatheidae	-	+	-	+	-	+	-	-	-	-	-	-
Amphipod	+	-	+	-	+	-	+	+	-	-	-	+
Gastropod	-	+	-	+	-	-	-	-	-	-	-	-
Sea urchin*	+	+	+	+	+	+	-	+	+	-	-	-
Glycera noelae sp. nov.	+	-	+	-	+	+	-	-	-	-	-	-
Pogonophora	-	-	-	-	-	-	-	-	+	+	+	+
Pycnogonids	-	-	-	-	-	-	-	-	+	+	-	-

* Fauna sampled in 2007 has been taxonomically identified to species level.
For detailed description refer to Bienhold et al. (in prep)

Table 3 Summary of biogeochemical measurements performed at wood-influenced (0.5 m – "At wood") and reference sediments (10 m – "Away wood") in the Eastern Mediterranean after one year submergance (2007; Bienhold et al. in prep.) and 3 years (2009, this study). The table combines information on the *in situ* Total Oxygen Uptake (TOU), *in situ* and *ex situ* Dissolved Oxygen Uptake (DOU), Oxygen Penetration Depth (OPD), total sulphide flux as determined *ex situ* with microsensors and *ex situ* average integrated (0 – 10 cm) Sulphate Reduction (SR) rates. n.d. = not determined. * = *in situ* measurement. Where both *in situ* and *ex situ* data are available, the latter is placed in parenthesis.

	TOU mmol m^{-2} d^{-1}	**DOU** mmol m^{-2} d^{-1}	**OPD** mm	**H_2S** mmol m^{-2} d^{-1}	**SR** mmol m^{-2} d^{-1}
EMed'07-CP-At wood#1	25*	4.3 ± 0.9	6.7	31.6 ± 6.7	1.3
EMed'09-CP-At_wood#1	n.d.	3.7 (4.5)	5 (5)	n.d.	1.3
EMed'07-CP-Away_wood#1	1*	2.3 ± 0.4	6.7	15.5 ± 6.1	2.5
EMed'09-CP-Away_wood#1	n.d.	1.5*	15.9*	n.d.	0.2
EMed'09-AMV-At_wood#3	n.d.	4.8	7.1	n.d.	2.4
EMed'09-AMV-At_wood#4	60*	3.6* (5.8)	6.3* (4.2)	n.d.	1.7
EMed'09-AMV-Away_wood#4	20*	1.5* (2.6)	>63* (>25)	n.d.	0.1
EMed'07-CP-At_wood#5	n.d.	4.4 ± 0.5	5	19.3 ± 7.6	2
EMed'09-CP-At_wood#5	n.d.	6.2	4.6	n.d.	0.1
EMed'07-CP-Away_wood#5	n.d.	1.0 ± 0.4	> 32	0	0.1
EMed'09-CP-At_wood#6	64*	3.8	5.4	n.d.	0.8
EMed'09-CP-Away_wood#6	16*	n.d.	n.d.	n.d.	n.d.
EMed'09-CP-At_wood#7	n.d.	0.3	>55	n.d.	0

Table 4 Percentage of shared OTUs between wood samples, wood-influenced sediment and reference sediment samples at EMed-CP-wood#1 (a) and EMed-CP-wood#5 (b) experiments, after 1y and 3y of immersion.

a)	**EMed'07-CP-wood#1**	**EMed'09-CP-wood#1**	**EMed'07-CP-At_wood#1**	**EMed'09-CP-At_wood#1**	**EMed'07-CP-Away_wood#1**	**EMed'09-CP-Away_wood#1**
EMed'07-CP-wood#1						
EMed'09-CP-wood#1	59					
EMed'07-CP-At_wood#1	45	44				
EMed'09-CP-At_wood#1	42	51	43			
EMed'07-CP-Away_wood#1	38	40	39	35		
EMed'09-CP-Away_wood#1	42	44	40	45	33	

b)	**EMed'07-CP-wood#5**	**EMed'09-CP-wood#5**	**EMed'07-CP-At_wood#5**	**EMed'09-CP-At-wood#5**	**EMed'07-CP-Away-wood#5**	**EMed'09-CP-Away-wood#5**
EMed'07-CP-wood#5						
EMed'09-CP-wood#5	47					
EMed'07-CP-At_wood#5	46	33				
EMed'09-CP-At-wood#5	47	46	38			
EMed'07-CP-Away_wood#5	8	9	11	9		
EMed'09-CP-Away-wood#5	36	33	28	35	11	

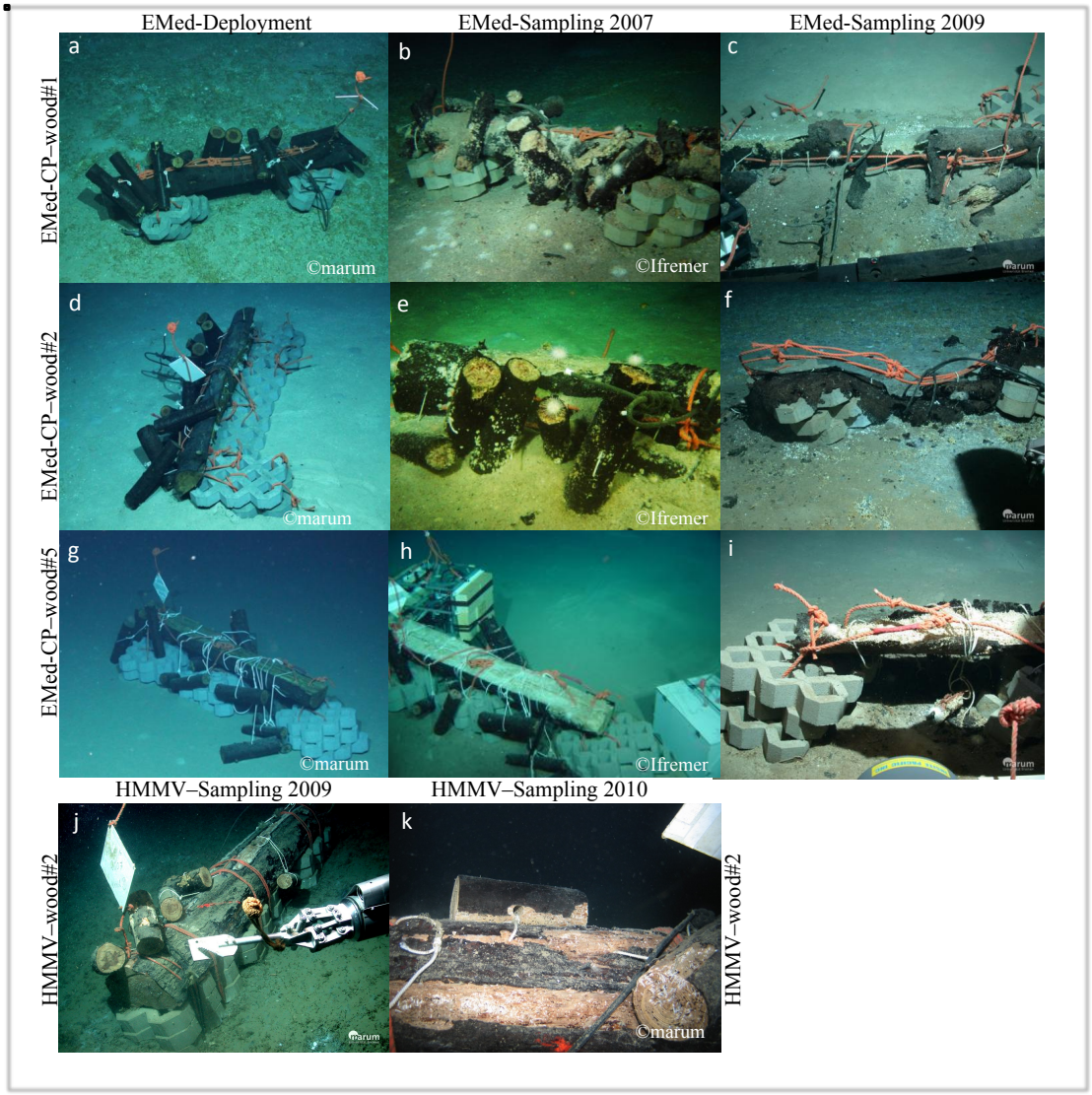


Fig. 1 ROV-based images portraying the condition of the wood experiments during the deployment and sample recovery in the (a – i) Eastern Mediterranean sea (Central Province cold seep) and the (j – k) Norwegian sea (HMMV cold seep).

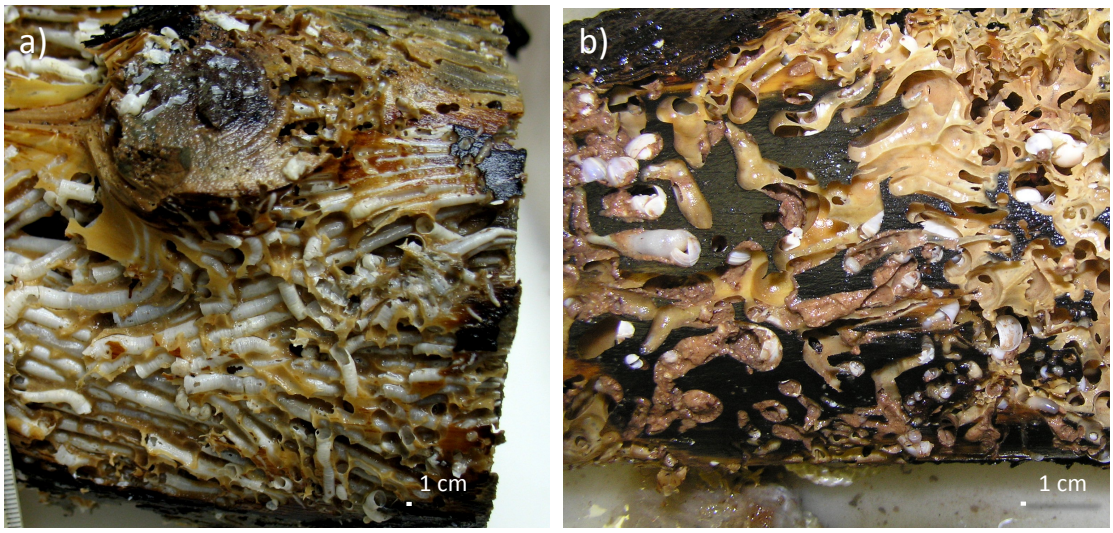


Fig. 2 A close-up of wood logs prior to sampling, depicting prominent differences in the degradation of wood experiments by wood-boring bivalves in the Norwegian (a) and Eastern Mediterranean Sea (b). Burrows with calcareous linings build by "Xylo3" (most probably Xyloredo sp.) at the HMMV wood experiment are visible on the left panel (a).

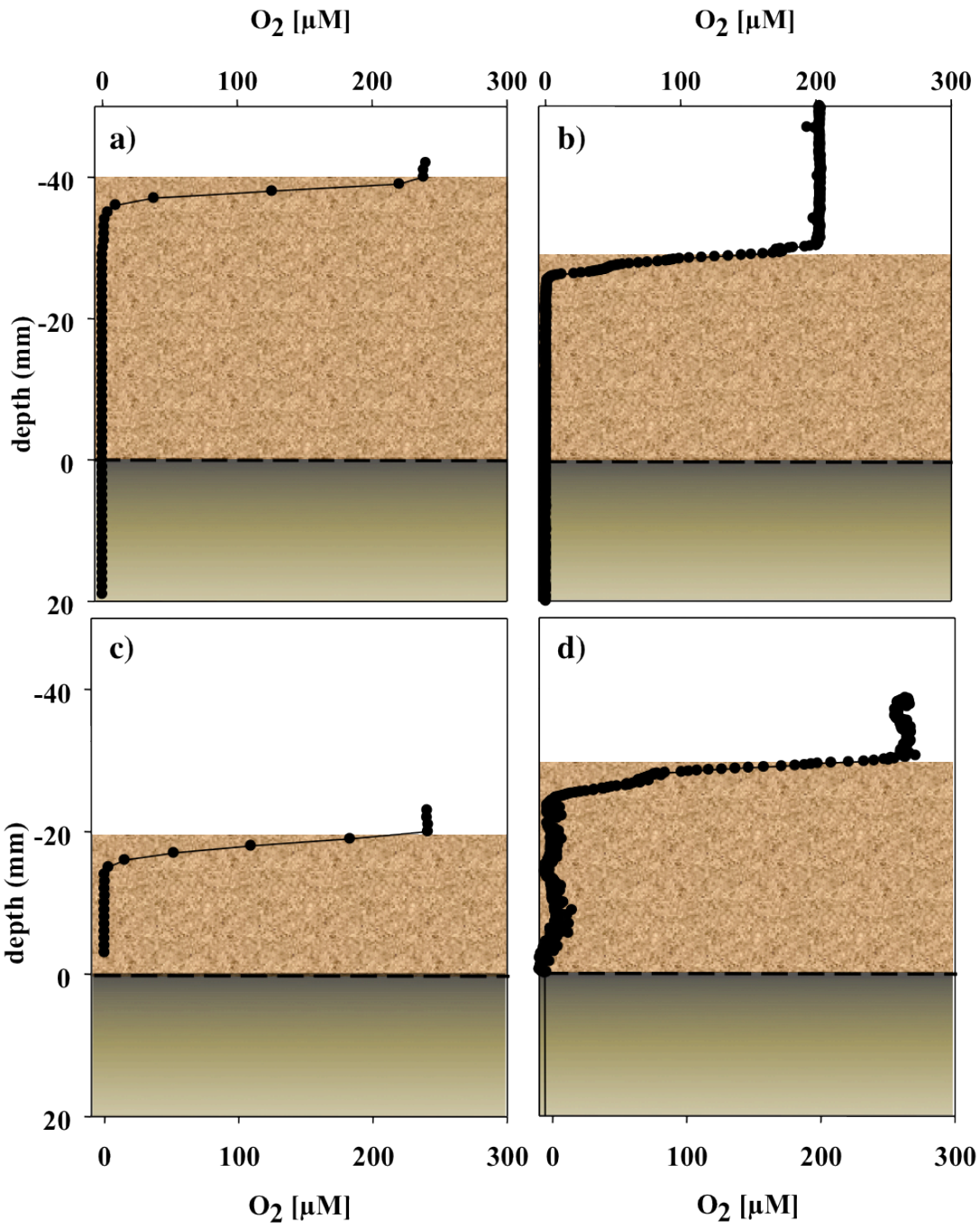


Fig. 3 Microsensor measurements of oxygen concentration at wood-influenced sediments after 1y (a, c) and 3y (b, d) of immersion of EMed-CP-wood#1 (a, b) and EMed-CP-wood#5 (c, d). Oxygen measurements in (b) was performed *in situ* and in (a, c, d) *ex situ*. The sediment surface is indicated with dashed black line, denoting the boarder between the wood chip (brown colored) and sediment layer (olive green colored).

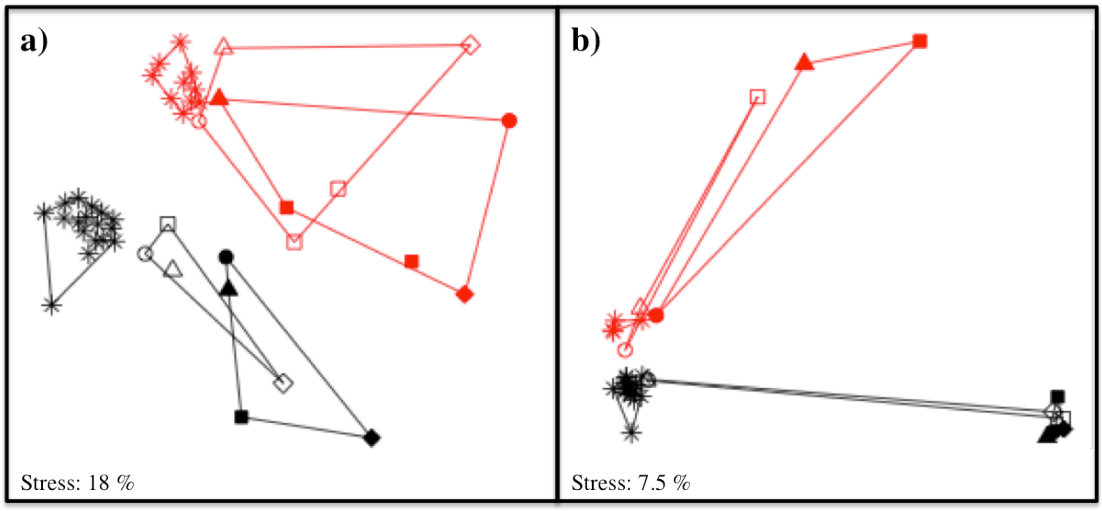


Fig. 4 NMDS analysis, based on Bray-Curtis dissimilarity, depicting difference in the structure of bacterial communities from (a) EMed-CP-wood#1 and (b) EMed-CP-wood#5 experiments. The different symbols indicate bacterial communities in wood (*), of wood-influenced sediment (open labels) and of reference sediment (closed labels). Black colored are communities sampled after 1yr of immersion and red colored after 3yr. Different numbers of the sediment communities refer to the depth horizon sampled (circle = 0-1 cm, triangle = 1-2 cm, square = 4-5 cm and rhomb = 9-10cm). NMDS plot (a) stress = 18% and NMDS plot (b) stress = 7.5%.

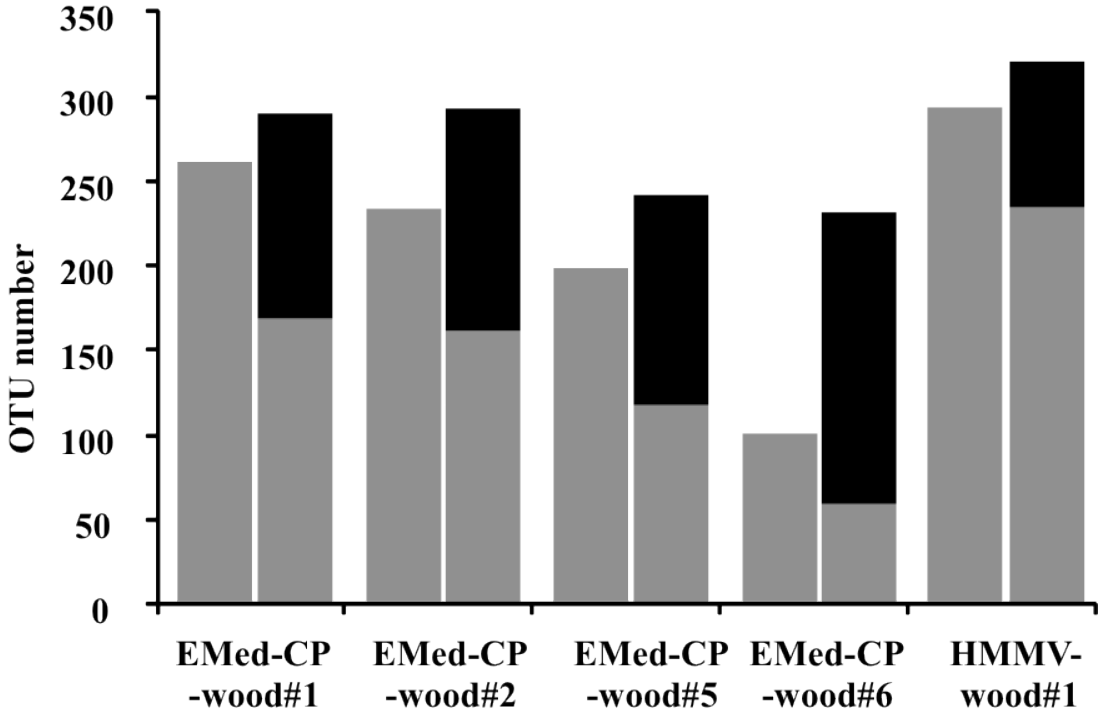


Fig. 5 Temporal turnover of ARISA OTUs between wood experiments in the Eastern Mediterranean, sampled in 2007 and 2009, and Arctic experiments sampled in 2009 and 2010. Single-colored gray bars represent the total number of OTUs found at wood experiments during the first sampling period in 2007 and 2009, in the Eastern Mediterranean and Arctic Ocean, respectively. Two-colored bars represent the total OTU number of wood experiments found during the second sampling period, in 2009 and 2010, in the Eastern Mediterranean and Norwegian Sea, respectively. The black parts of the bars represent the unique OTUs for the second sampling period, while the gray part of the same bars denotes the number of OTUs found during both sampling periods.

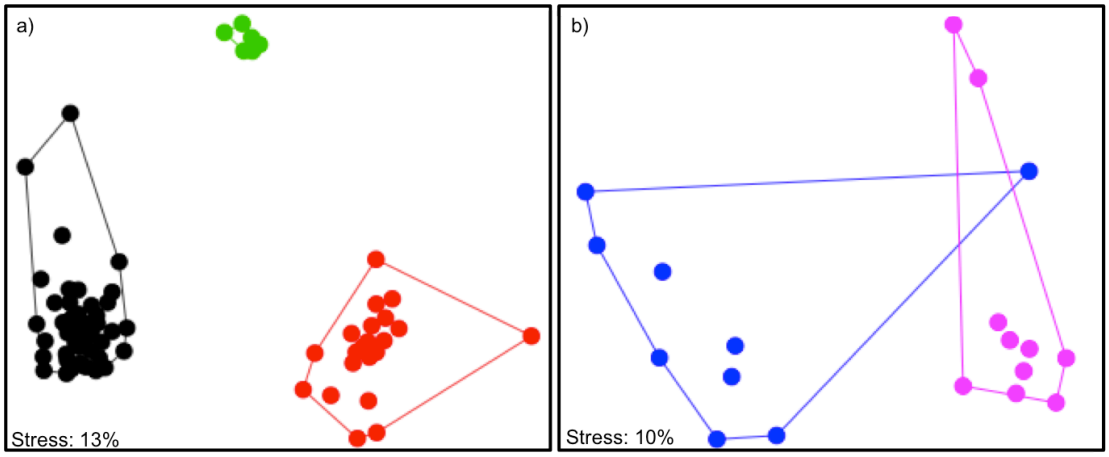
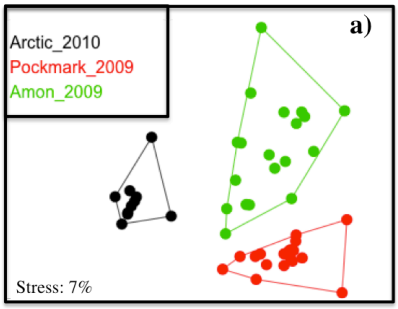


Fig. 6 NMDS analysis, based on Bray Curtis dissimilarity, depicting temporal variations in the wood-associated bacterial communities structure at Central Province in the Eastern Mediterranean (a), and the Norwegian sea (b). Samples are color coded according to immersion time. The CP NMDS analysis include samples derived from EMed-CP-wood#1, EMed-CP-wood#2, EMed-CP-wood#5, immersed for 1y and 3y, as well as EMed-CP-wood#6 deployed for 1d (a). The HMMV NMDS analysis includes samples derived from HMMV-wood#1 immersed for 2y and 3y (b). NMDS plot (a) stress = 13% and NMDS plot (b) = 10%.



b)

	Emed'09-CP-wood#1	Emed'09-CP-wood#2	Emed'09-CP-wood#5	Emed'09-CP-wood#3	Emed'09-CP-wood#4
Emed'09-CP-wood#2	69				
Emed'09-CP-wood#5	58	56			
Emed'09-CP-wood#3	73	59	55		
Emed'09-CP-wood#4	72	62	54	78	
HMMV'10-wood#1	61	55	48	63	62

Fig. 7 3D-NMDS analysis based Bray-Curtis dissimilarity, depicting spatial variations in the wood-related bacterial community structure between CP, AMV and HMMV experiments (a). Data are plotted on the first two axes. Complete 3D configuration of the plot is available in Supplement Fig. 1. Samples are color-codded according to cold seep site. The NMDS analysis includes wood samples immersed for 3y at the different cold seep sites (EMed'09-CP-wood#1, EMed'09-CP-wood#2, EMed'09-AMV-wood#3, EMed'09-AMV-wood#4, EMed'09-CP-wood#5 and HMMV'10-wood#1). NMDS stress = 7%. Percentage of shared OTUs between wood experiments deployed for the 3y at spatially distant and close seeps (b).

Supplementary Material

Supplement text 1:

Porewater analysis

Porewater from every centimetre sediment depth, from the 0.5 cm to 20 cm, was extracted using Rhizon moisture samplers (Seeberg-Elverfeldt et al., 2005; pore size 0.1 µm) inserted into holes of predrilled push cores liners at all investigated sites. Subsamples (1 ml) for sulphate and sulphide analyses were fixed with 0.5 ml of zinc acetate and stored at 4 °C till further processing. The total sulfide concentrations (H_2S + HS^- + S_2^-) were determined with the diamine complexation method (Cline, 1969). Sulfate concentrations were measured by non-suppressed anion exchange chromatography (Waters IC-Pak anion exchange column, waters 430 conductivity detector) after filtration and dilution. DIC concentrations were measured on subsamples stored headspace-free in gas tight vials (2 ml), with the flow injection method (conductivity detector: VWR scientific model 1054) according to Hall and Aller (1992). On board, approximately 10 to 15 ml of porewater were stored frozen at - 20 °C for nutrient analyses. In the home laboratory nutrient concentrations (nitrate, nitrite and ammonium) were determined with a Skalar Continous-Flow Analyzer according to the method of Grasshoff et al.

Supplement Table 1 Location of wood experiments in the Arctic Ocean and the Eastern Mediterranean sea, description of the benthic habitats and date of deployment and samplings events. Deoxyribonucleic acid (DNA), Anaerobic Oxidation of Methane (AOM), Sulfate Reduction (SR), benthic chamber (CHAM) and microprofiler (MICP).

Location	Wood experiment	Sampling habitat	Sample/ Measurement	Pangaea Label ID
Eastern Mediterranean; Central Pockmark	**EMed-CP-wood#1**	wood	DNA	MSM13/3_918-1_WOOD1; -2; -3; -4
			AOM;SR	MSM13/3_899-1_PUC16; MSM13/3_899-1_PUC21; MSM13/3_899-1_PUC10
		wood-influenced sediment	Porewater	MSM13/3_899-1_PUC28
			DNA	MSM13/3_899-1_PUC21
			CHAM	MSM13/3_899-1_CHAM1
			MICP	MSM13/3_918-1_MICP1
			AOM;SR	MSM13/3_899-1_PUC13; MSM13/3_899-1_PUC14; MSM13/3_899-1_PUC21
		reference sediment	Porewater	MSM13/3_899-1_PUC25
			DNA	MSM13/3_899-1_PUC13
			CHAM	MSM13/3_899-1_CHAM2
			MICP	MSM13/3_918-1_MICP2
	EMed-CP-wood#2	wood	DNA	MSM13/3_899-1_WOOD1; -2
Eastern Mediterranean; Amon Mud Volcano	**EMed-AMV-wood#3**	wood	DNA	MSM13/3_962-1_WOOD1; -2; -3; -4; -9
		wood-influenced sediment	AOM;SR	MSM13/3_962-1_PUC4; MSM13/3_962-1_PUC27; MSM13/3_962-1_PUC31;
			Porewater	MSM13/3_962-1_PUC24
			DNA	MSM13/3_962-1_PUC7
	EMed-AM-wood#4	wood	DNA	MSM13/3_944-1_WOOD1; -2; -3
			AOM;SR	MSM13/3_944-1_PUC2; MSM13/3_944-1_PUC20; MSM13/3_944-1_PUC21
		wood-influenced sediment	Porewater	MSM13/3_944-1_PUC9
			DNA	MSM13/3_944-1_PUC22
			CHAM	MSM13/3_962-1_CHAM7
			MICP	MSM13/3_947-1_MICP6
			AOM;SR	MSM13/3_962-1_PUC; MSM13/3_962-1_PUC14; MSM13/3_962-1_PUC16;
		reference sediment	Porewater	MSM13/3_962-1_PUC20
			DNA	MSM13/3_962-1_PUC22
			CHAM	MSM13/3_962-1_CHAM8
			MICP	MSM13/3_947-1_MICP6

Location	Wood experiment	Sampling habitat	Sample/ Measurement	Pangaea Label ID
Eastern Mediterranean; Central Pockmark	EMed-CP-wood#5	wood	DNA	MSM13/3_976-1_WOOD4
		wood-influenced sediment	AOM;SR	MSM13/3_976-1_PUC11; MSM13/3_976-1_PUC19; MSM13/3_976-1_PUC27
			Porewater	MSM13/3_976-1_PUC30
			DNA	MSM13/3_976-1_PUC115
		reference sediment	AOM;SR	MSM13/3_976-1_PUC10; MSM13/3_976-1_PUC22; MSM13/3_976-1_PUC23
			Porewater	MSM13/3_976-1_PUC117
			DNA	MSM13/3_976-1_PUC24
	EMed-CP-wood#6	wood	DNA	MSM13/3_976-1_WOOD2
		wood-influenced sediment	AOM;SR	MSM13/3_918-1_PUC30; MSM13/3_918-1_PUC16; MSM13/3_918-1_PUC13
			Porewater	MSM13/3_918-1_PUC24
			DNA	MSM13/3_918-1_PUC16
		reference sediment	CHAM	MSM13/3_976-1_CHAM14
			CHAM	MSM13/3_976-1_CHAM15
	EMed-CP-wood#7	wood	DNA	MSM13/3_976-1_WOOD6
		wood-influenced sediment	DNA	MSM13/3_976-1_PUC24
Norweigian Sea; Håkon Mosby Mud Volcano	HMMV-wood#1	wood	DNA	PS74/183-1_WOOD-2; -5; -6
		wood-influenced sediment		MSM16/2_844_WOOD1; -2; -3
	HMMV-wood#2	wood	DNA	PS74/183-1_WOOD-4; -8; -9;

Supplement Table 2 Analysis of similarity (ANOSIM), based on Bray-Curtis dissimilarities. Testing for significant differences in the bacterial community structures between wood samples, wood-related and reference sediments at (a) EMed-CP-wood#1 and (b) EMed-CP-wood#5 experiments immersed for 1y and 3y. ***p < 0.001, **p < 0.01, p < 0.05 after Bonferroni correction; (*) only significant without Bonferroni correction.

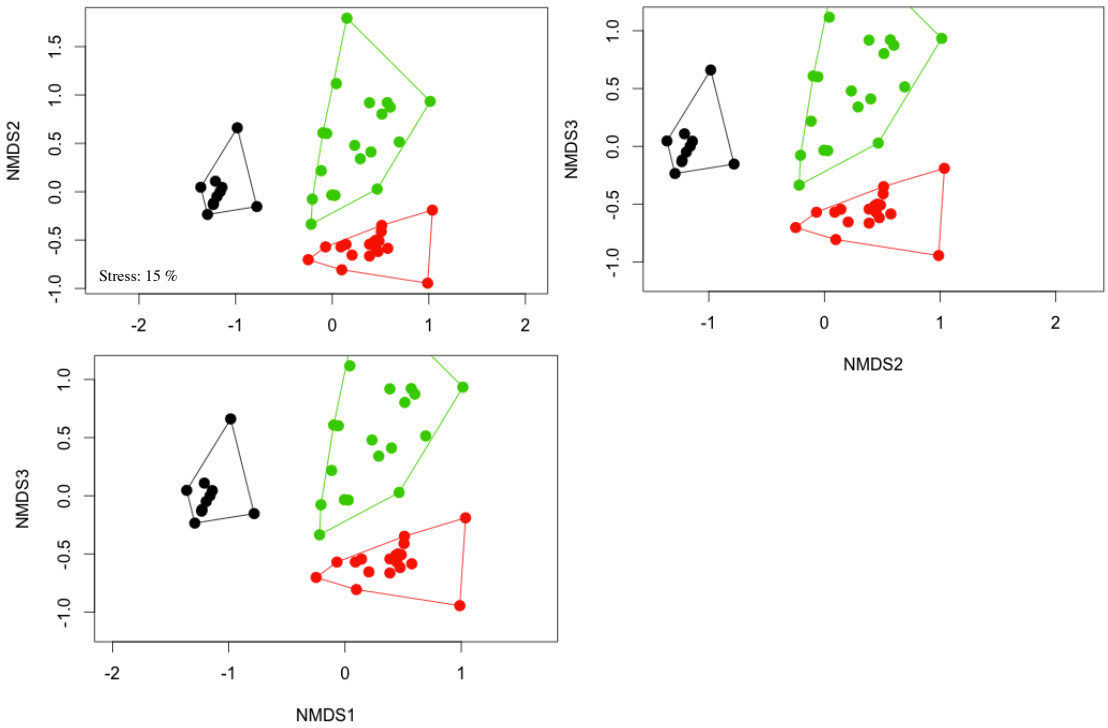
a)	**EMed'07-wood#1**	**EMed'07-Away_wood#1**	**EMed'07-At_wood#1**	**EMed'09-wood#1**	**EMed'09-At_wood#1**
EMed'07-Away_wood#1	1**				
EMed'07-At_wood#1	0.91**	0.48			
EMed'09-wood#1	1**	0.98**	0.93**		
EMed'09-At_wood#1	0.96**	0.59	0.41	0.72**	
EMed'09-Away_wood#1	0.98**	0.72	0.78	0.74**	0.09
b)	**Emed'07-wood#5**	**Emed'07-Away_wood#5**	**Emed'07-At_wood#5**	**Emed'09-wood#5**	**Emed'09-At_wood#5**
Emed'07-Away_wood#5	1**				
Emed'07-At_wood#5	0.82**	0.33			
Emed'09-wood#5	0.99**	1(**)	0.65(**)		
Emed'09-At_wood#5	0.93**	1.00	0.63	0.80(**)	
Emed'09-Away_wood#5	0.99**	1.00	0.78	1(**)	0.48

Supplement Table 3 Temporal variations of wood-associated bacterial communities. Analysis of Similarity (ANOSIM), testing for significant difference among wood experiments in the EMed immersed for variable periods of time. ***$p < 0.001$, **$p < 0.01$, $p < 0.05$ after Bonferroni correction.

	EMed'07-CP-wood#1	EMed'07-CP-wood#2	EMed'07-CP-wood#5	EMed'07-CP-wood#6	EMed'09-CP-wood#1	EMed'09-CP-wood#2	EMed'09-CP-wood#5
EMed'07-CP-wood#2	0.5**						
EMed'07-CP-wood#5	0.3**	0.45**					
EMed'07-CP-wood#6	1**	1**	1**				
EMed'09-CP-wood#1	1**	1**	1**	1**			
EMed'09-CP-wood#2	1**	1**	1**	1	0.4		
EMed'09-CP-wood#5	1**	1**	1**	1	0.3	0.2	
EMed'09-CP-wood#6	1**	1**	1**	1	0.2	0.1	0.4

Supplement Table 4 Spatial variations of wood-associated bacterial communities. Analysis of Similarity (ANOSIM), testing for significant difference among wood experiments immersed for 3y at different cold seep sites. ***p < 0.001, **p < 0.01, p < 0.05 after Bonferroni correction.

	Ark'10-wood#1	**EMed'09-CP-wood#1**	**EMed09-CP-wood#2**	**EMed'09-CP-wood#5**	**EMed09-AMV-wood#3**
EMed'09-CP-wood#1	1.0**				
EMed'09-CP-wood#2	1.0**	0.4			
EMed'09-CP-wood#5	1.0**	0.3	0.2		
EMed'09-AMV-wood#3	0.8**	0.6**	0.4*	0.4	
EMed'09-AMV-wood#4	1.0**	0.7**	0.4**	0.5	0.0



Supplement Fig. 1 3D-NMDS analysis based Bray-Curtis dissimilarity, depicting spatial variations in the wood-related bacterial community structure between CP, AMV and HMMV experiments (a). NMDS stress = 15%.

3 Discussion and perspectives

Cold seeps represent oasis of life at the deep-sea floor, with microbial and faunal biomasses a few orders of magnitude higher than in surrounding environments. Their productivity is surprisingly high and is comparable to that of the most prolific ecosystems in the entire oceans. Such high production of biomass at the deep-sea floor is only possible because seep microbes are capable of harnessing the energy from the ocean's geosphere, delivered at seep sites at the seafloor in the form of methane. Seeps have a highly fragmented worldwide distribution, but a small areal coverage (app. 1 km) of usually extreme local heterogeneity, which along with their remoteness makes them a challenge for exploration. Within this PhD the usage of advanced deep-sea technology for targeted sampling and *in situ* measurements, as well as expensive research vessels, made it for the first time possible to ask questions as to how seep microbial diversity changes along spatial scales ranging from a few meters to hundreds of kilometers. Here, we combined different geochemical and molecular fingerprinting techniques, as well as multivariate statistical analyses to quantify microbial consumption rates, evaluate seep microbial communities in context of their environment, and specifically assess the link between diversity and *in situ* methane fluxes. Moreover, within this study we investigated the temporal and spatial dynamics of microbial communities of yet another, though less famous, reduced ecosystem, the wood falls. The results presented in the chapters of this thesis might eventually help to the better develop urgently-needed conservation strategies which aim to protect seep ecosystems and their associated biodiversity which are under increased threat by enhanced anthropogenic activities in the deep-sea.

3.1 Energy as driving factor of reduced deep-sea hotspot ecosystems

Cold seeps and sunken wood are peculiar ecosystems of the deep-sea, which are fueled by completely different types of energy sources than the majority of the deep sea floor. Mainly, while most of the deep-sea floor communities rely on the production of organic matter in the euphotic zone, seep and wood fall communities gain energy from local sources such as methane and sulfide in the case of seeps, and cellulose and sulfide at wood falls. Hence, specialized fauna and microbes, capable of utilizing methane, sulfide and cellulose as their primary energy source, dominate cold seep and wood fall ecosystems (Sibuet and Olu, 1998;

Knittel and Boetius, 2009; Bienhold et al., 2011). Our studies confirmed these findings, and furthermore revealed that in response to the unique type of energy sources both wood falls and cold seeps harbor distinct bacterial communities with surprisingly little overlap to the background deep-sea sediments (Chapter III, IV). In contrast to deep-sea sediments populated by *Planctomycetacia*, both wood falls and cold seeps in the deep Eastern Mediterranean sea harbored substantially higher relative abundances of *Deltaproteobacteria*, a class known to comprise members capable of perform sulfate reduction (Figure 9). Furthermore, associated to cellulose-rich energy sources, higher relative abundances of *Alphaproteobacteria* and *Clostridia* were observed, typical for deep-water masses and anoxic conditions, respectively. At cold seep sediments the anaerobic *Anaerolineae* and *Epsilonbacteria* contributed higher percentage to the overall seep community. Overall, the results of this study indicate that cold seeps and wood falls hotspot ecosystems, driven by unique energy sources, contribute substantially to the overall biodiversity of the deep-sea floor.

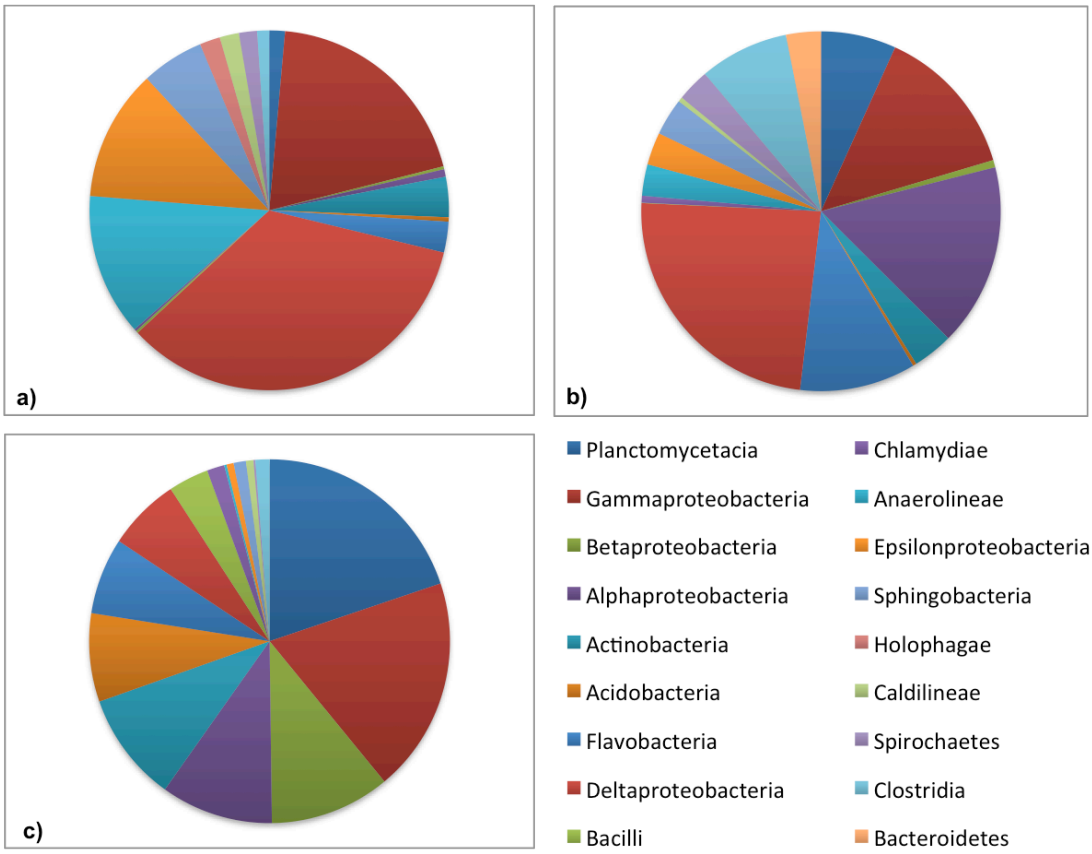


Figure 9 Average bacterial taxonomic composition in: a) cold seep sediments (0 – 10 cm), n = 6 (Pop Ristova P., unpublished), b) wood experiments (Pop Ristova P., unpublished), n = 5, c) deep-sea surface sediments (Bienhold C., unpublished), n = 4 in the deep Eastern Mediterranean sea, as derived with 454 molecular analysis.

In addition to the type of energy source, this PhD study for the first time revealed that the magnitude, or availability of energy plays an important role in structuring bacterial communities at cold seep sites. Sulfide and methane fluxes, indicative of the potential energy available to seep communities, differed by one to three orders of magnitude between seep habitats investigated in this study (Chapter I, III), confirming the high heterogeneity of seep sediments (Cordes et al., 2010 and references therein). In accordance, the two individual studies presented in this thesis revealed the same patterns of significant relationships between seep bacterial β-diversity (change in species composition and/or structure between habitats) and difference in energy availability, represented as methane or sulphide content. Hence, a general conclusion can be drawn that seep habitats with similar biogeochemistry/energy availability have more similar bacterial community structure as opposed to habitats with distinct geochemistry (Chapter I, III). Our study corroborates the findings of previous studies, which showed significant relationships of β-diversity patterns with energy availability in different marine benthic ecosystems ranging from oligotrophic continental margins to coastal sands (Böer et al., 2009; Bienhold et al., 2012). Together, these results suggest that energy availability might be an important factor generally structuring benthic bacterial communities. However, it remains unclear whether relationship between energy availability and species richness, shown for many organisms ranging from bacteria, plants to animals (Bienhold et al., 2012), exist in the case of cold seep bacteria. A way to explore this would be with application of new generation sequencing technologies, which offer high-throughput and high-resolution detection of bacterial taxa (Loman et al., 2012), and hence presumably include the so-called "rare biosphere" – the low abundant entities of life (Pedrós-Alió, 2006, 2007, 2012; Sogin et al., 2006; Huber et al., 2007). Shifts in the bacterial community structure were weakly correlated to variations in the magnitude of microbially-mediated processes, such as anaerobic oxidation of methane and sulphate reduction (Chapter I). Future studies should focus more on this relationship, perhaps by applying metagenomic approaches (Martín-Cuadrado et al., 2007), in order to reveal whether links between the functional diversity, community structure and the energy availability exist for cold seep microbial communities.

Distributions and densities of larger seep organisms, including symbiont-bearing megafauna and non-symbiont-bearing macro- and meiofauna, are directly or indirectly linked to bottom water methane concentrations, as was recently shown (Olu-Le Roy et al., 2007; Olu et al., 2009; Van Gaever et al., 2009; Menot et al., 2010). Here, we could extend this link, and furthermore show that methane fluxes determine the sediment geochemistry at cold seeps,

which selects for different types of chemosynthetic megafauna (Chapter I). Further relationships could be inferred between the relative abundance of methanotrophic symbionts in the mytilid mussel *Bathymodiolus aff. boomerang* and their potential energy source, detected as methane effluxes and bottom water concentrations (Chapter II). Tight coupling of geochemical, microbiological and faunal processes and activities at cold seeps would indicate that fauna and microbes might influence each other's distributions. As already started in this study (Chapter I), it would be worth that future studies closely examine this relationship, in order to gain better understanding what other contemporary processes might influence the structure of microbial communities at cold seeps.

Overall, the different studies of this PhD thesis suggest a general relationship between seep bacterial communities and the energy availability at cold seep ecosystems, indicating strong effects of the contemporary environmental conditions, on the structuring of seep bacterial communities at local and regional scales. Based on the findings in this and other studies, we propose that energy availability at cold seeps is one of the dominant factors that shapes the structure of seep communities across all organismal size classes, ranging from microorganisms to meio-, macro- and megafauna.

3.2 Interconnectivity of reduced deep-sea hotspot ecosystems

Cold seeps and wood falls are widespread ecosystems, but occur as isolated, fragmented habitats at the deep-sea floor, with relatively small local areal coverage (Sibuet and Olu, 1998; Wolff, 1979). In this thesis, for the first time we addressed questions regarding the interconnectivity of microbial populations associated to these ecosystems (Chapter III, IV). For both types of ecosystems it could be shown that the associated bacterial communities potentially freely disperse on a scales of meters to few hundreds of kilometers between structures within the same geographic province (i.e. the Eastern Mediterranean) (Chapter III, IV and see Appendix). In contrast, very little overlap in the bacterial and faunal communities associated to wood experiments in the Eastern Mediterranean and the Norwegian Sea was revealed, indicating a strong selection by local contemporary factors and possibly dispersal limitation between these two oceanic regions. It has yet to be confirmed whether dispersal limitation for seep bacterial communities also exists between sites that are geographically separated and environmentally different such as the warm Eastern Mediterranean and the cold Arctic deep-sea waters. However, given the analog nature of these ecosystems, as isolated and fragmented habitats with communities highly specialized to their unique type of

energy source, we believe that future studies will uncover similar patterns for seep bacterial communities as for wood fall ecosystems. Dissimilarities between wood-associated communities according to geographic region detected in our study verify what was proposed for global benthic bacterial communities (Zinger et al., 2011) and confirmed for vent fauna - another type of isolated ecosystems (Van Dover et al., 2002), and suggest a certain degree of provincialism for both bacterial and faunal communities associated to wood falls. The findings of this thesis mark the beginning of the effort to understand how bacterial communities of isolated habitats are structured along spatial scales. However, to fully comprehend the interconnectivity of these ecosystems we need to reveal from where bacteria colonize these ecosystems and understand their dispersal mechanisms. At the moment, we have little evidence that surrounding sediments represent colonizing grounds for either seep or wood fall ecosystems. Furthermore, even if terrestrial bacteria brought down by the wood could potentially survive high hydrostatic and osmotic pressures, as well as cold temperatures, on the long run the terrestrial wood-associated community does not contribute substantially to the wood falls ecosystems in the deep-sea. This was suggested by the findings of the investigation of temporal dynamics of wood fall ecosystems (Chapter IV). Widespread seeding by geofluids from hydrocarbon seeps and ocean crust ecosystems and consequent transport by oceanic currents has been proposed to transport spores of microbes over great geographic distances (Hubert et al., 2009). While this can likely represent an important source for seep bacteria (Inagaki et al., 2006), seabed fluids can potentially only contribute a fraction of the wood-associated bacterial community, in particular the fraction that is associated to chemosynthetic processes e.g. sulfide production at wood fall ecosystems. Nevertheless, to unravel the dispersal mechanisms in microbial communities at both ecosystems, it might prove helpful to systematically investigate the water column at given spatial distances from the habitats or along bottom water currents.

On spatial scales of few meters to hundreds of kilometers (> 300 km), in this study no correlation between the bacterial community structure and geographic distances could be detected for seep bacteria. However, dispersal seemed not to be limited, as proximate sites (separated by few meters) shared as much bacteria types as distant sites. Hence, these results indicate that on local and regional scales the distribution of seep microbial communities resemble the fragmented and patchy character of their habitats and energy sources (Chapter III). Similarly, the geographic distance between seep sites had little effect on the distributions of cold seep megafauna (Cordes et al., 2007; Olu et al., 2010). However, this is in contrast to

studies that explored this relationship for bacterial communities in many different marine and terrestrial ecosystems, over a much broader range of spatial scales (Martiny et al., 2006, 2011 and references therein; Schauer et al., 2010; Bienhold et al., 2012). It will be interesting to see if the lack of effect of geographic distance on seep community structure will still hold true if cold seep sites from different oceanic regions are compared. The spatial scaling approach applied in this study helped for the first time to identify microbial diversity hotspots at cold seep sites (Chapter III). Namely, with more than 50 % of bacterial turnover, the small reduced seep habitats, usually not bigger than few meters in diameter, contribute substantially not only to the cold seeps', but also to the overall microbial diversity of the deep-sea floor.

Cold seep ecosystems face a threat of being irreversibly affected by increased anthropogenic activities such as deep-sea mining, oil and gas exploration and trawling for deep-sea fish (Continental Shelf Associates, 2006; Baco et al., 2010). Recent, unfortunate example is the major accident in the Gulf of Mexico when large amounts of oil and gas leaked in the deep-sea due to the explosion of safety valves of the Deepwater Horizon, and which had as a consequence detrimental effect on the nearby seep communities ((Ramirez-Llodra et al., 2011 and references therein). However if mass extraction of gas hydrates becomes a reality, many methane seeps might become subject to disturbance more significant than that of oil and gas extraction, unless protection is put in place, as in the Gulf of Mexico (Ramirez-Llodra et al., 2011). Hence, studies such as ours that aim at identifying areas, which are important sources of biodiversity at the deep-sea floor, can help to better devise conservations strategies and establish Marine Protected Areas (MPAs).

3.3 Perspectives

3.3.1 *In situ* technologies: towards more accurate assessment of cold seep processes

Studies included in this thesis highlight the importance of the usage of *in situ* technologies for the exploration of cold seeps and the understanding of seep microbial communities in the context of their environment. At cold seeps, which are characterized by high fluid advection, gassy sediments laden with methane hydrates, and extremely steep and often temporally and spatial variable biogeochemical gradients, sample recovery generally causes severe biases on the consequent shipboard or home laboratory analyses (Boetius and Wenzhöfer, 2009). In contrast, usage of *in situ* technologies i.e. benthic chambers and microsensors, to quantify

biogeochemical fluxes and rates, and detect gradients, overcome this problem and provide unbiased picture of the environmental conditions at seep sites, and furthermore allow assessment of the high heterogeneity of cold seep sediments (Boetius and Wenzhöfer, 2009). Therefore, in addition to the standard porewater techniques, within this PhD thesis we used an *in situ* approach to quantify habitat-specific methane effluxes and investigate their effect on the overall microbial community structure at cold seep sites. However, biological studies including *in situ* geochemical approaches are still rare, hence our ability to infer a general relationship between the diversity of seep communities and the environmental factors at cold seep ecosystems is hindered. More frequent application of *in situ* geochemical methodologies is needed if we are to understand the complex interplay of geochemical, biological and geological processes that shape cold seep ecosystems. In addition, increased number of *in situ* measurements will in future improve the current estimates of methane emissions, and allow to better assess how much methane seeps contribute to the ocean waters and the atmosphere (Felden et al., 2010). However, although quite few numbers of *in situ* sensors are available today for high-resolution characterization of cold seeps, still *in situ* quantification of e.g. dissolved inorganic carbon or sulfate is not possible (Boetius and Wenzhöfer, 2010). Given the importance of *in situ* technologies it is evident that improving the current *in situ* instruments would lead to higher accuracy and better comparability of measurements (Tengberg et al., 1995). Moreover, devising new instruments or applying existing ones in the cold seep research allows conducting new kinds of measurements. For example, the introduction of eddy-correlation systems allows for the first-time a non-invasive assessment of the benthic oxygen fluxes across hard-bottom substrates (Berg and Huet, 2008; Glud et al., 2010), which are typical for cold seep sites; development of an *in situ* Raman-based probe allows improved *in situ* determination of dissolved methane concentrations in sediments, and the already the few available results indicate a much larger near-surface reservoir of dissolved methane than previously thought (Zhang et al., 2011). Hence, *in situ* technologies continue having impact on the quality of deep-sea research.

3.3.2 From snapshots to long term observations

Most studies at cold seeps provide only snapshots of the biological or geochemical processes of the investigated locations at a given time. This is the case, because the cold seep research in the deep-sea is limited by the bottom time of Remotely Operated Vehicles (ROVs) or submersibles (usually not more than 12 h), as well as the battery lifetime of *in situ*

instruments. This is a serious constraint, because the few results obtained from longer monitoring or repetitive measurements, indicate that cold seeps are temporally highly dynamic ecosystems with prominent variations in the methane effluxes (Tryon et al., 1999; Tryon and Brown, 2004; Feseker et al., 2008;). Hence, establishment of long-term autonomous observatories at cold seep sites is needed, which will continuously monitor and record a variety of geochemical, physical and biological parameters. This will help to understand the causes of temporal variability and the mechanisms that trigger eruptions at mud volcanoes, as well as it will allow better studying of the consequences of such events on the local geochemistry and seep communities. A recent deployment of an autonomous long-term observatory (LOOME; http://www.esonet-noe.org/Demonstration-missions/LOOME) at the Håkon Mosby Mud Volcano has been proved successful in providing valuable data that contribute towards better understanding of the temporal dynamics of cold seeps (A. Boetius, personal communication). A next step towards the exploration of temporal variability would be to establish cabled observatories at cold seeps that will provide continuous supply of power to various instruments and sensors and allow remote controlling and real-time data transfer. In the long run, autonomous long-term observatories at cold seeps could even be equipped with ecogenomic sensors - a new class of autonomous sensors that enable the use of molecular biological techniques in ocean observing frameworks (Scholin, 2009; Preston et al., 2011). Eventually this would provide information on the temporal dynamics of microbial communities at cold seep sites, and perhaps even allow predictions to be made about future changes.

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To write down in words what I feel for you and how much your support means to me, seems far more complex then writing this thesis. You make me strong and happy, and I love you for that.

4 Appendix

4.1 Additional contributions to publications

Biogeochemistry and bacterial diversity of deep-sea wood falls

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In preparation for submission to PLoS Biology

Keywords: wood fall, reduced habitats, organic substrate, colonization, deep sea, bacterial diversity

Abstract

Large organic food falls to the deep sea such as whale carcasses and wood logs support the development of reduced, sulfidic habitats in an otherwise oxygenated, oligotrophic deep-sea environment. These transient hot spot ecosystems may serve the dispersal of highly adapted chemosynthetic organisms such as thiotrophic bivalves and siboglinid worms forming conspicuously dense communities at hot vents and cold seeps. Here we investigated the biogeochemical and microbiological processes leading to the development of sulfidic niches that may be colonized by chemosynthetic organisms, on wood falls deployed at a depth of

1690 m at the Nile deep sea fan (Eastern Mediterranean). Wood-boring bivalves of the genus *Xylophaga* played a key role in the degradation of the wood logs, facilitating the development of anoxic zones and anaerobic microbial processes such as sulfate reduction. Macrofaunal organisms and bacterial communities associated with the wood included types reported from other chemosynthetic deep-sea habitats, confirming the potential role of large organic food falls as stepping stones for vent and seep communities. In addition, numerous opportunistic organisms such as polychaetes, crabs and heterotrophic bacteria were attracted to the wood falls, underlining the importance of large food falls as biological hotspots in the deep sea. Bacterial communities that had developed on and around the wood falls after one year were distinct from the reference wood (<1 day submerged) and background sediments. Although overall bacterial community structure also differed between wood experiments, our results suggest the presence of a core bacterial community associated with the wood, and we identify candidate taxa that may play an important role in the utilization of wood in the deep sea, including sulfate-reducing *Deltaproteobacteria*.

Biogeochemical processes associated with mud volcanism on the Nile Deep Sea Fan - The Amon Mud Volcano

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In preparation for submission to Biogeosciences journal

Keywords: Chemosynthetic communities, oxygen uptake, methane efflux, sulfate reduction, cold seep

Abstract

The highly active Amon mud volcano (MV), located at 1250 m water depth between the Central and Eastern province of the Nile Deep Sea Fan, was investigated during the BIONIL expedition with RV METEOR (M70/2) and MSM13-3 in 2006 and 2009, respectively. The Amon MV can be subdivided in four habitats: a central dome (I); the surrounding hummocky area with patches of bacterial mats (II); a wide slope area covered by biogenic mounds (III); and (IV) a lateral mud flow (sulfur band) at the foot of the Amon MV. Here we investigated the spatial and temporal variation in biogeochemical processes at these habitats and their relation to fluid flow regimes. Total and diffusive oxygen uptake was quantified in situ with a benthic chamber and a microsensor-profiler, respectively. Microbial sulfate and methane consumption were measured by radiotracer incubations. Pore water chemistry was

investigated to gain an understanding about the flow patterns and composition of subsurface fluids.

Our results show that the concentric structure, morphology and biogeochemisty of these habitats are mainly determined by spatial variations in gas and fluid flow on scales of meters to hundreds of meters. Similar habitat distribution patterns in 2006 and 2009 illustrate the persistent of these zoning at the Amon MV. The biogeochemical hot spot of Amon MV was the large patchy bacterial mat habitat surrounding the central dome, with high rates of hydrocarbon oxidation, sulfide production and oxygen consumption (maximum 52 mmol m^{-2} d^{-1}). Another benthic hot spot habitat was found at the southeastern foot of the Amon MV; a lateral outflow of sulfidic, briny mud covered by thiotrophic bacterial mats and siboglinid tubeworms. Here, the high oxygen uptake was fueled by sulfide that was transport into the habitat and not only by local microbial production. Beside the established habitats a succession of the chemosynthetic communities was also observed. Our data give clear evidence that the Amon MV changed from an eruption/high fluid and gas flow state (2006) towards a period of lower fluid and mud flow (2009), associated with more stable conditions permitting a higher benthic activity. Changes were not only visible during ROV surveys but also by replicate biogeochemical measurements at the four habitats.

4.2 Poster and Oral Presentations

Pop Ristova P, Bienhold C, Wenzhöfer F, Boetius A (2012). Wood falls as biological and biogeochemical hotspots in the deep sea. DIWOOD workshop, Bremen, Germany, 23-24 January 2012.

Pop Ristova P, Bienhold C, Wenzhöfer F, Boetius A (2011) Wood falls as biological and biogeochemical hotspots in the deep sea. HERMIONE workshop (WP5, Chemosynthetic ecosystems), Ghent, Belgium, 16-18 November 2011.

Pop Ristova P, Ramette A, Wenzhöfer F, Felden J& Boetius A (2011) Spatial variations of bacterial communities and related biogeochemical activity of cold seep sites in the Eastern Mediterranean deep sea. *SAME - Symposium on Aquatic Microbial Ecology, Rostock/Warnemünde*. Oral Presentation.

Pop Ristova P, Wenzhöfer F, Ramette A, Zabel M, Fischer D, Kasten S & Boetius A (2011) Biogeochemical activities and associated biodiversity at the gigantic pockmark REGAB - a multidisciplinary study. *EGU conference, Vienna*. Poster Presentation.

Pop Ristova P, Wenzhöfer F, Bienhold C & Boetius A (2009) Microbial diversity and in situ biogeochemistry in different chemosynthetic habitats. *ASLO Aquatic Science Meeting conference, Nice.* Poster Presentation.

Pop Ristova P, Wenzhöfer F, Ramette R, Zabel M, Fischer D, Kasten S& Boetius A (2010) Biogeochemistry of gas-driven, chemosynthetic habitats at the giant pockmark REGAB. *International conference on gas in marine sediments, Listvyanka.* Oral Presentation.

Pop Ristova P, Wenzhöfer F, Felden J & Boetius A (2010) Spatial variations of bacterial communities and related biogeochemical activity of cold seep sites in the Eastern Mediterranean deep sea. *HERMIONE first annual meeting, Malta.* Oral Presentation.

Pop Ristova P, Wenzhöfer F, Felden J & Boetius A (2010) Spatial variation of bacterial communities and related biogeochemical activity of cold seep sites. *Marum PhD days*, Bremen. Oral Presentation.

4.3 Cruise Participations

RV Meteor, M76/3b cruise, Southeast Atlantic Ocean, REGAB pockmark (cold seeps, wood experiments). 17 July – 24 August 2008.

RV Polarstern, ARKXXIV/2 cruise, Barents Sea, Haakon Mosby Mud Volcano, Hausgarten Observatory (cold seeps, wood experiments). 10 July – 03 August 2009.

RV Maria S Merian, MSM13/3, Eastern Mediterranean Sea, Nile Deep Sea Fan (cold seeps, wood experiments). 25 October – 18 November 2009.

RV Maria S Merian, MSM13/4, Eastern Mediterranean Sea, Anaximander Mountains, Nile Deep Sea Fan (cold seeps, wood experiments). 21 November – 14 December 2009.

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Erklärung

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