Effect of high CO_2 and low pH on benthic communities of the deep sea

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Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis, we ourselves are a part of the mystery that we are trying to solve. (Max Planck)

Summary

The increase of carbon dioxide (CO_2) concentration in the atmosphere will intensify climate change although the ocean moderates climate change due to its considerable capacity to store large amounts of CO_2. As a consequence the pH in seawater decreases, a process known as ocean acidification. Current state of the art of science and technology proposes mitigation strategies such as Carbon Capture and Storage (CCS) to reduce the amount of CO_2 reaching the atmosphere. Storage locations are geological formations of the deep sea, where liquefied CO_2 is sequestered. Risks to the environment, caused by CO_2 leakage are not satisfactorily studied so far, although commercial projects are already running since 1996.

The deep sea is characterized a stable high pressure, low temperature regime and organisms have adapted to this extreme environment. Changes in environmental conditions as for leakage of CO_2 will enhance mortality rates of organisms directly exposed to CO_2. This may result in a shift in community structure and potentially the loss of functional groups that maintain ecosystem functioning. In this context, "natural laboratories", such as the Yonaguni Knoll IV hydrothermal system located in the Okinawa trough characterized by high CO_2 fluxes, are appropriate sites to study responses of the deep-sea benthic community to high CO_2 concentrations and thus low pH *in situ*. In the present study, the influence of these factors on the distribution, abundance and diversity of the deep-sea benthos was investigated, including all size classes of the benthic community from a reference site to low and high CO_2 seepage sites.

Specific characteristics of the study site are presented in **Chapter I,** describing the geochemistry of the sediments and the distribution of microbial processes at the Yonaguni Knoll VI CO_2 hydrothermal system. Measured rates of, e.g. sulfate reduction (SR) and anaerobic oxidation of methane (AOM) at the reference in contrast to CO_2-impacted sites, suggest that microbial communities are adapted to the geothermal and geochemical conditions. These processes were detectable at very low pH and were generally higher at the CO_2-impacted vent site compared to the reference. However, microbial processes were limited to the upper 15 cm. This may be due to the high concentrations of dissolved CO_2 which is a highly powerful solvent and the proportionally high concentration of carbonic acid (H_2CO_3) which might be toxic for microbial cells.

In **Chapter II** responses of the bacterial communities at three distinct sites with varying relative CO_2 concentrations were explored. Distribution patterns indicated a shift in community structure from the background to the CO_2 -impacted sites with a decrease of some bacterial types and a concurrent increase in particular other types that appear to be able to cope with the low pH. However, the overall abundance of bacteria increased with high CO_2/low pH conditions, when these occurred in combination with hydrothermal fluids that may be utilized as an energy source by some bacteria.

Chapter III is concerned with metazoan meiofaunal and macrofaunal abundance, richness, and community distribution patterns in correlation with CO_2 gradients. Both size classes showed responses to low pH, that are loss and reduction of some of the taxa groups (e.g. echinoderms and polychaetes) and an increase of others (nematodes). Apparently, the complex interplay of reduced predation and/or competition due to the decrease in macrofaunal echinoderms and polychaetes led to an increase in nematodes (meiofauna) at the intermediate impacted site despite low pH.

Zusammenfassung

Der stetige Anstieg der Kohlendioxid-Konzentration (CO_2) in der Atmoshpäre wird fortlaufend den Klimawandel verstärken, obwohl der Ozean einen bedeutenden Teil der CO_2-Konzentration aufnimmt. Diese Funktion als CO_2-Senke führt zu einer Verringerung des pH-Wertes, bekannt als Ozeanversauerung. Der aktuelle wissenschaftliche und technische Stand ermöglicht es, bereits große Mengen von CO_2 in sogenannten Carbon Capture and Storage (CCS) Projekten abzuspeichern und damit die Menge an CO_2 zu verringern, die in die Atmosphäre gelangt. CO_2-Speicherstätten hierfür sind geologische Formationen in der Tiefsee, in denen das verflüssigte CO_2 sequestriert wird. Obwohl es seit 1996 kommerziell durchgeführte CCS Projekte gibt, sind mögliche Risiken durch das Austreten des gespeicherten Kohlendioxids bisher nur ansatzweise untersucht worden. Die Tiefsee ist charakterisiert durch extremen Druck und niedrige Temperatur. Aufgrund dieses hohen Anpassungsgrades von Tiefseeorganismen an ihr Habitat könnten bei CO_2-Leckagen erhöhte Mortalitätsraten auftreten. Dies hätte Veränderungen innerhalb der Gesellschaftstruktur zur Folge und würde einen Verlust von funktionellen Gruppen bedeuten, welche essentiell für die Funktionsfähigkeit des Ökosystems sind. „Natürliche Laboratorien“ wie z.B. das Yonaguni Knoll IV, ein hydrothermales CO_2-Emissionsgebiet im Okinawa Graben, stellen dabei geeignete Gebiete dar, um den Einfluss erhöhter Kohlendioxid-Konzentrationen und einen geringen pH-Wert auf benthische Tiefseegemeinschaften *in situ* zu untersuchen. In der vorliegenden Arbeit wurde der Einfluss dieser Faktoren im Hinblick auf Verteilungsmuster, Abundanzen und Diversität aller Größenklassen der benthischen Tiefseegemeinschaft an drei unterschiedlichen Gebieten untersucht: eine Referenzstation, eine Station mit einer mittleren und eine Station mit einer hohen CO_2-Konzentration.

Die Biogeochemie des Studiengebietes wird in **Kapitel I** beschrieben. Hierbei wurden die Geochemie des Sediments und die Verteilung der mikrobiellen Prozesse innerhalb des CO_2-hydrothermalen-Systems Yonaguni Knoll IV untersucht. Anhand der untersuchten Prozesse von beispielsweise Sulfatreduktion (SR) und anaerober Methanoxidation (AOM) an der Referenz sowie den vergleichenden CO_2-beeinflussten Gebieten wird vermutet, dass sich die mikrobiellen Gemeinschaften an die geothermalen und geochemischen Umweltparameter angepasst haben. Die mikrobiellen Prozesse waren, trotz des sehr niedrigen pH-Wertes, an den CO_2-beinflussten Gebieten zu messen. Allerdings waren diese auf die ersten 15 cm des Sediments beschränkt. Mögliche

Gründe hierfür wären die hohe Konzentration an gelöstem CO_2, welches als starkes Lösungsmittel wirkt, als auch die im Verhältnis sehr hohe Konzentration an Kohlensäure (H_2CO_3), die eine toxische Wirkung auf die Mikroorganismen haben könnte.

In **Kapitel II** werden die Ergebnisse der Untersuchung der bakteriellen Gemeinschaft an den drei unterschiedlichen Gebieten dargestellt. Die bakteriellen Verteilungsmuster weisen auf eine Veränderung innerhalb der Gemeinschaftszusammensetzung des Referenzgebietes, im Vergleich zu den zwei stark CO_2-beeinflussten Gebieten hin. Dies wird deutlich durch den Verlust einiger CO_2-sensitiven Bakterientypen, bei gleichzeitiger Zunahme acidotoleranter Bakterien. Interessanterweise hatte der saure pH-Wert keinen Einfluss auf die Bakterienabundanz. Anscheinend liefern die hydrothermalen Fluide Energie, die manche Bakterien für sich nutzen können.

Kapitel III befasst sich vergleichend mit Verteilungsmustern, Abundanzen und der Vielfalt der vielzelligen Meiofauna- und Makrofauna-Gemeinschaft entlang eines CO_2-Gradienten. Diese Untersuchungen ergaben, dass die Meiofaunagesellschaft weniger beeinflusst wurde als die Makrofaunagesellschaft. Dennoch wurden beide Gemeinschaften durch die hohe Konzentration an CO_2 und dem daraus resultierenden sauren pH-Wert beeinträchtigt. Möglicherweise führten geringerer Fraßdruck und/oder Konkurrenz im gemäßigt CO_2-beeinflussten Gebiet zu einer erhöhten Anzahl der Meiofauna, insbesondere der Nematoden.

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1.

Introduction

1.1 Climate Change and Ocean Acidification

The climate system is a complex, interactive system comprising the atmosphere, hydrosphere, geosphere, and biosphere (Treut et al. 2007). Modification of the composition of atmospheric gases through e.g. the increase of carbon dioxide (CO_2) emissions led to alterations and thus to climate change (Fig. 1). In recent years, climate change has been synonymously used to describe the latter factor – human-induced changes on the level of global warming - and has become a term describing post-industrial warming of the Earth's atmosphere and oceans due to the anthropogenic production and release of greenhouse gases to the atmosphere, such as carbon dioxide (CO_2) (c.f. United Nations Framework Convention on Climate Change, UNFCC).

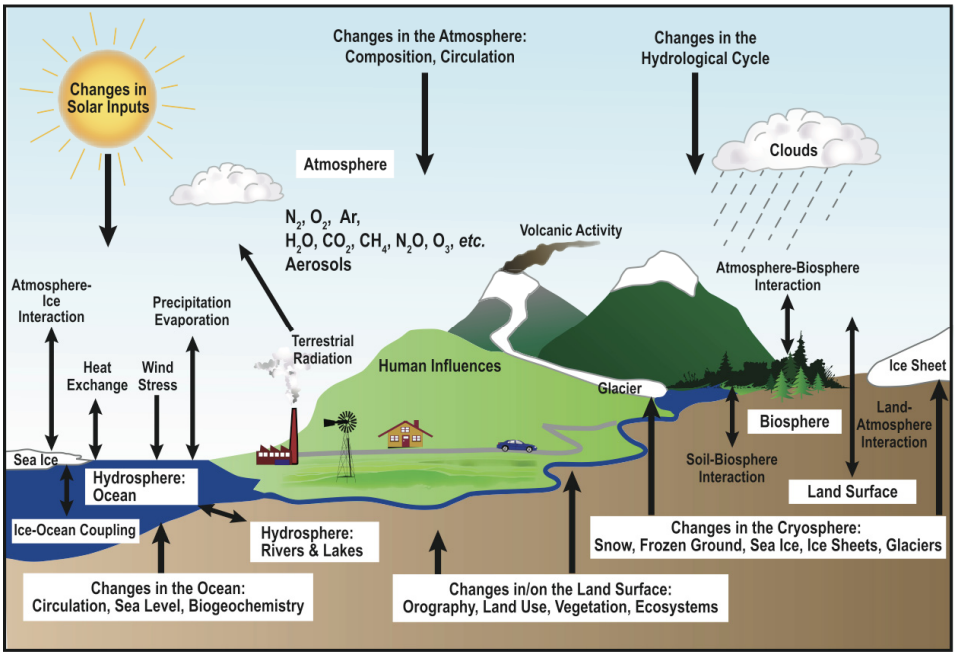

Figure 1 Schematic overview of the components of the climate system and its dynamic processes and interactions (adapted from IPCC 2007).

Carbon dioxide is, besides water vapor, methane, and nitrous oxide, a so called greenhouse gas that absorbs and emits solar radiation within the thermal infrared range, causing an elevation of the average surface temperature and thus climate change. Since the beginning of high-accuracy measurements of atmospheric CO_2 concentrations in

1958 by Charles David Keeling on Mauna Loa in Hawaii, time series of the composition of the atmosphere have been documented and, hence, constituted modern research on climate change (Treut et al. 2007). In order to evaluate atmospheric gas composition of the past and to make predictions for the future, enclosed air bubbles of ice cores dating back 10,000 years before present, which had been retrieved from Greenland and Antarctica, were analyzed. Studies on these ice cores revealed that CO_2 concentrations had been stable within a range of 280 ± 20 ppm (parts per million) up to the year 1750 (Indermühle et al. 1999). During industrialization, concentrations of the greenhouse gas rose roughly exponentially to 367 ppm in 1999 (Neftel et al. 1985; Etheridge et al. 1996; IPCC 2001) and to 397 ppm in the year 2005 (Treut et al. 2007). Other major greenhouse gases such as methane (CH_4) and nitrous oxide (N_2O) increased to a minor extent (1% and 0.25% yr^{-1}, respectively) (Graedel and McRae 1980; Fraser et al. 1981; Weiss 1981; Blake et al. 1982; Khalil and Rasmussen 1988).

While the incoming sunlight and outgoing infrared radiation determine the global climate, the increasing concentration of CO_2 as the most influencing greenhouse gas traps the incoming radiation, thereby heating up the Earth's surface (Fig. 2). An additional effect of rising concentrations of CO_2 in the atmosphere is the acidification of ocean waters, with yet largely unknown effects on marine ecosystems. Both, climate change (i.e. global warming) and ocean acidification are phenomena that have their origin in increasing concentrations of greenhouse gases, especially rising CO_2 levels.

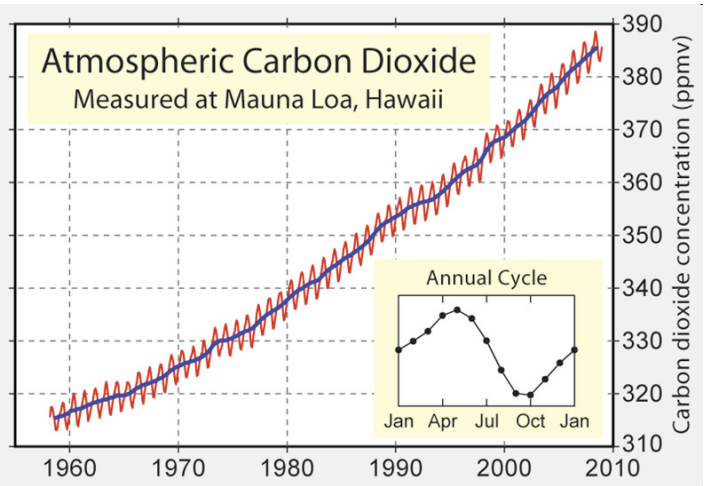

Figure 2 "Keeling Curve" showing the history of atmospheric CO_2 concentrations measured at Mauna Loa, Hawaii. The annual fluctuation of CO_2 is caused by a seasonal variation, indicating atmospheric signal of a sink, which is the massive uptake by terrestrial and oceanic plants during summer in the northern hemisphere (inset figure). Atmospheric CO_2 concentrations have reached more than 380 ppmv (parts per million per volume) in the year 2010 (www.wikipedia.org). Recently, values of 397 ppmv have been recorded.

Ocean acidification results from the uptake of atmospheric CO_2 by the oceans and leads to an alteration of the complex ocean carbonate system (Fig. 3). When atmospheric carbon dioxide dissolves in seawater it hydrates to form carbonic acid (H_2CO_3). This reaction is slow, compared to the dissociation (ionisation) of H_2CO_3 (Soli and Byrne 2002). In equilibrium the concentration of H_2CO_3 is only 1/1000 of the concentration of dissolved carbon dioxide [CO_2(aq)] (Riebesell et al. 2010). Both unionised species are hence forward referred to as [CO_2]. At pH<5 [CO_2] is the major form of CO_2 in solution, at higher pH it dissociates into HCO_3^- and CO_3^{2-} according to equation 1.2 and 1.3 in BOX 1. Dissolved inorganic carbon (DIC) is mostly present in three inorganic forms in seawater:

CO_2 (aq), HCO_3^- (bicarbonate), and CO_3^{2-} (carbonate). The majority of dissolved inorganic carbon in the oceans is HCO_3^- with < 85% (Riebesell et al. 2010).

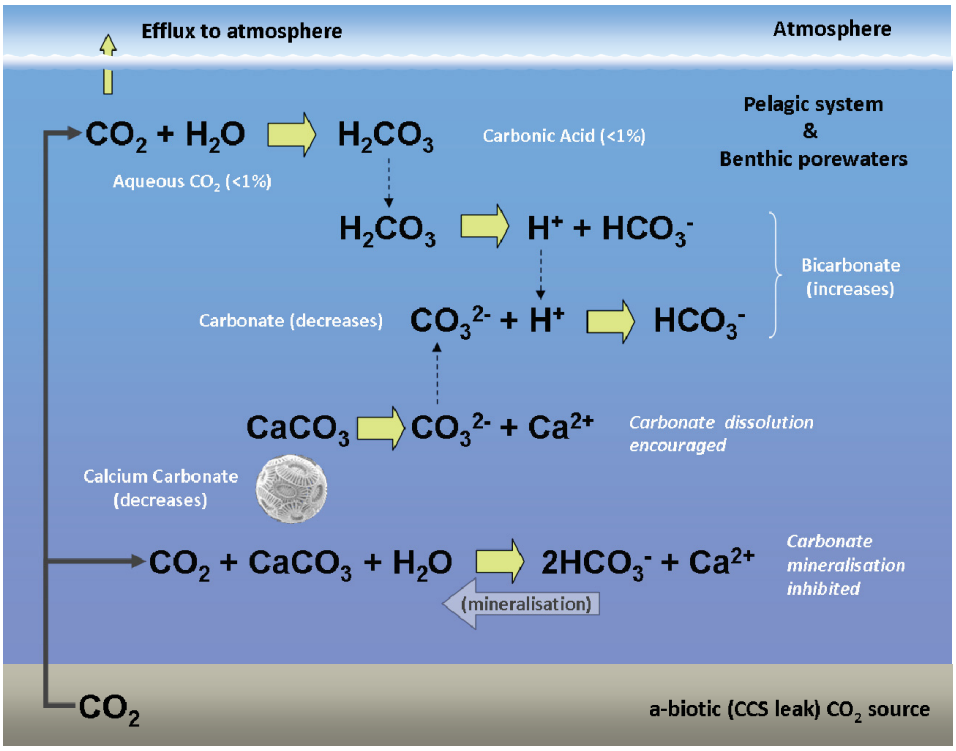

Figure 3 Ocean carbonate system under the influence of CCS leak and the escape of liquid CO_2 (www.bgs.ac.uk/qics).

Over the past 250 years, the oceanic CO_2 uptake accounted for almost a third of the CO_2 that had reached the atmosphere (Sabine et al. 2004; Sabine and Feely 2007). Without the oceans capacity to function as a carbon sink, atmospheric CO_2 would be approximately 450 ppmv (parts per million by volume), instead of 397 ppmv today (Doney et al. 2009). Although the ocean uptake alleviates the effects of CO_2 in the atmosphere, it causes pH reduction and associated alterations in chemical balances in the ocean, commonly referred to as ocean acidification (Doney et al. 2009). Not only CO_2, but also increasing inputs of dissociation products of strong acids, such as HNO_3, and H_2SO_4, as well as bases (NH_3) from fossil fuel combustion and agriculture, cause a decrease in surface seawater pH, alkalinity and DIC (Doney et al. 2007). Although these inputs only contribute a small fraction to ocean acidification, ecosystem responses especially in coastal waters could have serious effects on, e.g. fish and shellfish stocks, directly affecting ecosystem services relevant to humans (Doney et al. 2009).

BOX 1 | Acid-base equilibria in seawater and the ocean carbonate system

The gaseous carbon dioxide CO_2 (g) and $[CO_2]$ are related by Henry's law in thermodynamic equilibrium:

CO_2 (g) = $[CO_2]$; **Equation 1.0**

Solubility equilibrium of carbon dioxide between air and seawater:
CO_2 (g) ↔ CO_2 (aq) **Equation 1.1**

Dissociation reaction of the dissolved carbon dioxide:
CO_2 (aq) + H_2O (l) ↔ H^+ (aq) + HCO_3^- (aq); **Equation 1.2**
HCO_3^- (aq) ↔ H^+ (aq) + CO_3^{2-} (aq); **Equation 1.3**

At a typical seawater pH of 8.2 the speciation between CO_2, HCO_3^-, and CO_3^{2-} is 0.5%, 89% and 10.5%.

(g) = gas, (l) = liquid, (aq) = aqueous solution

(Riebesell et al.; Gattuso and Hansson 2011)

1.1.1 Effects of Ocean Acidification on marine ecosystems

Aspects of ocean acidification and associated problems to society have not yet been addressed systematically. On a scientific basis, main focus has been put on the responses of marine organisms to ocean acidification. By the end of this century it is expected that ocean pH will decrease about 0.3 - 0.4 units (Haugan and Drange 1996; Brewer 1997), accompanied by a rise in temperature and the expected warming of the ocean thereby will increase the extent of the oxygen minimum layer (Rosa and Seibel 2008). These synergistic effects however, need to be considered and included in discussions concerning ocean acidification.

Several issues on the physiological responses of marine biota to ocean acidification have been identified in previous studies. Expected primary effects include reduced calcification, which encompasses weaker skeletons, reduced extension rates, and increased susceptibility to erosion (Kleypas et al. 1999). Calcification rates will alter and for some organisms (e.g. echinoderms) decreased calcification is supposed to have substantial physiological costs. The biologically controlled mineralization for example is depending on cellular activities to direct the nucleation (initiation of the carbonate shell-forming), growth, and morphology of the mineral which is deposited (Weiner and Dove 2003). If inhibition of cellular activity appears owing to acidification, mineralization

processes will be affected. Further consequences are limited calcification rates, suppressed metabolic rates, as a consequence to decreased pH and triggered through the disruption of extracellular acid-base balance, as well as reduced ion exchange and protein synthesis (Pörtner et al. 2005; Pörtner 2008; Widdicombe and Spicer 2008). Synergistic effects of increased CO_2 concentrations, temperature extremes and hypoxia, as a consequence of climate change will enhance sensitivity to environmental extremes relative to a change in just one of theses variables (Pörtner et al. 2005; Przeslawski et al. 2005; Rosa and Seibel 2008; Russell et al. 2009; Dissanayake and Ishimatsu). Additionally, suppressed reproduction has been reported for example in sea urchins, through reduced fertilization success. Decreased developmental rates and larval size, as well as malformed or unmineralized shells of bivalve larvae were observed (Kurihara et al. 2004; Kurihara et al. 2007a). Also, many microbial processes can be affected, either directly or indirectly, as reviewed by (Weinbauer et al. 2011) and (Liu et al. 2010) via a cascade of effects by response of non-microbial groups to ocean acidification and/or through changes in seawater chemistry. They summarized different studies on, e.g. production of transparent exopolymer particles (TEP), dissolved organic matter (DOM), bacterial abundance, production and enzyme activity, organic carbon consumption and loss, nutrient cycles (nitrogen fixation and nitrification), as well as primary production. However, results were not always straight forward and for most of the studies results were inconsistent. Conflicting results on photosynthesis rates were found based on species specific responses of either little or no change in photosynthetic rates (Hein and Sand-Jensen 1997; Tortell et al. 1997; Burkhardt et al. 2001; Tortell and Morel 2002; Rost et al. 2003; Beardall and Raven 2004; Giordano et al. 2005; Martin and Tortell 2006) or enhanced biomass production (Zimmerman et al. 1997; Short and Neckles 1999; Fu et al. 2007; Riebesell et al. 2007). Increased carbon and nitrogen fixation**,** and increased C:N ratios were observed for some cyanobacteria (e.g. *Trichodesmium*) (e Ramos et al. 2007; Hutchins et al. 2007).

Just like marine biota is confronted with different consequences due to ocean acidification, humans may as well be confronted through a variety of socioeconomic connections. First impacts anticipated on humans may be through declining harvest and fishery revenues from shellfish (Cooley and Doney 2009). This would be accompanied by a loss of jobs, reduced income and indirect economic costs. Coral reefs are suggested to suffer due to ocean acidification. These highly productive ecosystems provide a broad range of valuable goods and services to humans (Brander et al. 2009): coral mining,

recreational opportunities (diving, snorkelling), coastal protection, habitat and nursery functions for both commercial and recreational fisheries, and welfare associated with the existence of diverse natural ecosystems (Brander et al. 2009).

1.1.2 Carbon Capture and Storage

The effects of current climate change and ocean acidification, caused by increased CO_2 concentrations in the atmosphere on, e.g. calcification rates, and biodiversity are severe. Currently, a variety of approaches are discussed on how to counteract both the causes as well as the effects of increased CO_2 concentrations by the use of mitigation or geoengineering strategies.

BOX 2 | Geoengineering the climate

Mitigation efforts to counteract climate change fall into two main categories:
Solar radiation management (SRM) techniques, which directly modify the Earth's radiation balance but do not treat the root cause of climate change, which is the reduction of greenhouse gases, and carbon dioxide removal (CDR) techniques, addressing the root cause by removing greenhouse gases from the atmosphere. SRM includes several strategies aiming at reducing the net incoming short-wave radiation received by deflecting the sunlight or by increasing the solar reflecting power of the atmosphere (albedo).

SRM techniques:

- **Increasing the surface reflectivity of the planet**, by brightening human structures (e.g. roof whitening, planting of crops with high reflectivity, or covering desserts with reflecting material)
- **Enhancing marine cloud reflectivity**
- **Injection of sulfate aerosols into the lower stratosphere** (mimicking the effect of volcanic eruptions)
- **Placing shields or deflectors in space** to reduce the amount of incoming solar radiation

CDR techniques:

- **Land use management to protect or enhance land carbon sinks**
- **The use of biomass for carbon sequestration and the use of carbon neutral energy sources**
- **Carbon Capture and Storage** (land- or ocean-based; direct capture of CO_2 from ambient air or from place of production and long-term storage in geological formations)
- **Enhancement of oceanic uptake of CO_2** (ocean fertilization with naturally scarce nutrients (e.g. iron), or increasing upwelling processes)

(Shepherd 2009)

Marchetti was the first to propose geoengineering in 1977, although he suggested to inject the disposed CO_2 into suitable sinking thermohaline ocean currents that spread it into the deep ocean, which has become unthinkable with current knowledge as the release of CO_2 into the water column has been banned by the London Dumping Convention (Marchetti 1977). Other geoengineering approaches do not address the source of rising CO_2 and thus do not address the problem of ocean acidification (e.g. solar radiation management (SRM)). However, mitigation strategies address both rising CO_2 and ocean acidification and are briefly discussed in the following section (BOX 2).

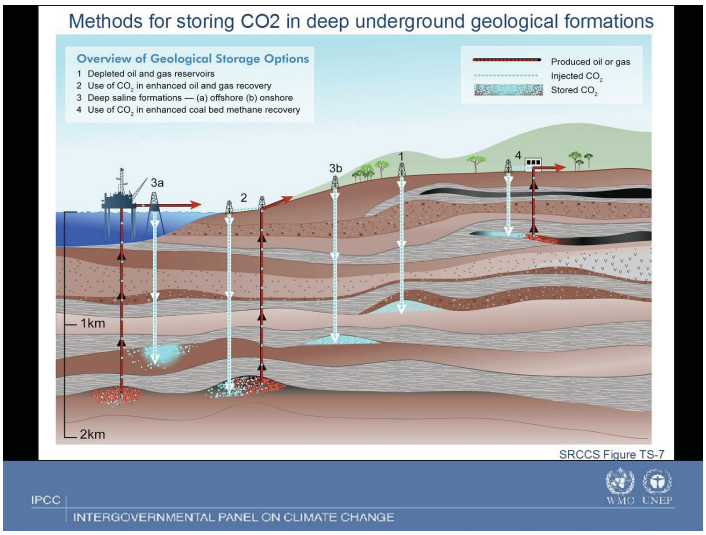

Figure 4 Overview of the methods possible for CO_2 storage in underground geological formations both on land and beneath the seafloor (source: IPCC, Carbon Dioxide Capture and Storage: Technical Summary (2005)).

Carbon Capture and Storage (CCS) is considered as one mitigation option in order to reduce atmospheric CO_2 concentrations. Sequestration in offshore geological formations has been identified the most promising storage option. The injection of CO_2 into deep-sea sediments below 3000m water depth covered with several hundred meters of sediment might present permanent geological storage option and benefit from the high pressure and low temperature predominant in deep waters (Schrag 2009). CO_2 in deep-ocean sediments will be present in liquid phase which is denser than the overlying seawater trapping the CO_2 in the sediments. Thick, low-permeability cap rocks would further prevent CO_2 from escaping (Schrag 2009). However, gravitation pronounced in these depths may further secure the storage of CO_2 and reducing leakage risks.

In general, options for CO_2 removal encompass biological, physical, and chemical methods on land or in the ocean. Since CO_2 emissions mainly result from the burning of

fossil fuels, CCS could be applied to large point sources of CO_2 such as power plants or large industrial processes (Rubin et al. 2005). This would involve technologies that collect, concentrate, and store the industrial and energy-related CO_2 in appropriate storage locations for a long period of time. The capture step involves separation of CO_2 from other gaseous substances, which can be done via post- or pre-combustion systems or the oxyfuel combustion system (for detailed description cf. IPCC special report Carbon Dioxide Capture and Storage, 2005). Storage options, either terrestrial or oceanic, comprise the usage of subsurface saline aquifers, or depleted oil and gas reservoirs (Fig. 4). The latter being first targets for sequestration due to the possible enhanced oil recovery (EOR) serving as an additional source of revenue (Schrag 2009). The insertion in open waters of the oceans has been banned by the London Protocol. This Protocol adopted by the parties in 1996, to the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (generally known as the London Convention) is meant to eventually replace the Convention of 1972. The London Protocol entered into force in 2006 and stresses the precautionary approach which requires that "appropriate preventative measures are taken when there is reason to believe that wastes or other matter introduced into the marine environment are likely to cause harm even when there is no conclusive evidence to prove a causal relation between inputs and their effects". Thus, CO_2 streams may only be considered for dumping if, e.g. disposal is into sub-seabed geological formations (London Protocol).

The first CCS project was launched in 1996 (Sleipner Vest) by Statoil and others were added for commercial use in 2005: the Weyburn Enhanced Oil Recovery (EOR) project in Canada, and the In Salah natural gas project in Algeria, each of them capturing and storing 1-2 $MtCO_2$ per year (Rubin et al. 2005). By own account, Statoil has by now injected 12 million tonnes of CO_2 into the Sleipner storage site, an aquifer composed of porous sandstone filled with saline waters. No leakage has been reported since the beginning of the project. Based on seismic images, Eiken et al (2011) report only small pressure build-up in the reservoir and stating that about 5% of the pore space in the reservoir has been occupied by CO_2. The plume injected reached an area of 3.1 km^2 by 2008. However, although the injected CO_2 plume is reported to be stable and no leakage has been reported so far, leakage risks can not be ruled out. Thus, besides "normal" ocean acidification, leakage from CCS sites needs to be considered, to better estimate potential risks.

1.2 Life at the extremes

In order to evaluate potential effects of ocean acidification or the leakage of CO_2 from CCS sites, we need to look at the natural variability of pH in the oceans (that organisms are exposed to) and how different organisms cope with it and have adapted to it. Seawater is usually slightly alkaline with pH values naturally varying in a range of 7.5 and 8.4. Locally, the seasonal amplitude might be up to 0.1 pH units (Haugan and Drange 1996). When it comes to long-term exposure of CO_2 caused by leakage, and thus a reduction in pH, it is expected that the first response will be an increasing mortality rate of organisms directly in contact with CO_2 (Barry et al. 2004). Evolution of organisms might in some cases be positively correlated to alterations in environmental conditions but this is restricted to impacts that occur on long-term. However, short-term impacts probably impair adaptability. Nevertheless, adaptive evolution might occur at extreme changes in natural parameters, e.g. when exposed to increased acidification as recently reported by (Collins 2012; Lohbeck et al. 2012) on the world's single most important calcifying organism *Emiliania huxleyi*. In order to understand consequences and adaptation based on long-term exposure to high CO_2 concentrations, evaluation of evolutionary adaptation of organisms to extreme environments can be helpful. The following section provides insight into some extreme habitats organisms have adapted to. The first question that needs to be answered is: what is extreme? The term extreme is anthropocentric as its definition is based on a human perspective taking a human perception of hostile conditions as a basis. Extreme environments exhibit extreme conditions that are inhospitable for most of the organism and include thereby places where humans generally cannot live. These may include extremes in temperature, pressure, acidity or alkalinity, radiation and others.

Studies of extremophile microorganisms are interesting, because one can investigate how life functions under environmentally extreme conditions, e.g. high temperature or salinity. Extremophiles may also provide answers on how early Earth was colonized. Further it is questionable whether extremophiles have only recently adapted to harsh conditions they live in or if they have evolved from ancient types of organisms living under extreme conditions and having adapted to them (Rainey and Oren 2006). Answers to these questions may elucidate our view on the origin of life and on what the limits of life are, also in the context of climate change, including ocean acidification and global warming.

All organisms tolerate a certain range of environmental conditions, a certain temperature, pressure, solar radiation, salinity, or pH range, due to their optimized metabolic functions (Pörtner 2002). A negative correlation of, e.g. thermal tolerance and increasing complexity of organisms is based on structural and kinetic coordination of molecular, cellular, and systemic processes (Pörtner and Farrell 2008). For example, the functional constraints of an organism, defines and limits the temperature range they can live in (Pörtner and Farrell 2008). Other abiotic parameters (pH, salinity, pressure) as well restrain organismal performance. Functions of an organism appear more affected than single cells or molecules; animals and plants may be more affected than unicellular organisms (Pörtner 2002).

The term extremophile (see BOX 3) is mainly used for the domains *Bacteria* and *Archaea*, or unicellular eukaryotic organisms that thrive in extreme environments. The majority of all extremophiles are found for bacteria and archaea. However, up to date several eukaryotic extremophiles are known, although the study of extremotrophic and extremophilic eukaryotic organisms has been largely neglected compared to studies on bacteria and archaea. Extremophiles are found in all domains of life: *Archaea*, *Bacteria*, *and Eukarya*. But also viruses and phages are extremophiles, as they attack archaea and bacteria that live in extreme conditions. Some organisms may be exposed to merely one extreme, while others may live in habitats where they are exposed to a number of extremes. For example, bacteria that live at hydrothermal vents in the deep sea are exposed to high temperatures and potential toxic chemical compounds emitted from the vents. These kinds of extremophiles are termed polyextremophiles (Rothschild and Mancinelli 2001). However, a much larger fraction and diversity of organisms exists that tolerate harsh conditions, but do not have their growth optima at the extreme condition. These organisms are defined as extremotrophs (see BOX 3) (Mueller et al. 2005). Despite of living in extreme habitats, many extremophiles have a low level of adaptability to changing environmental conditions (Rainey and Oren 2006) and limiting factors for growth are probably nutrients and other energy resources (Kristjansson and Hreggvidsson 1995). Besides extremophiles and extremotrophs, many other organisms rely on dormant stages to survive extreme conditions but these organisms are not capable of growing and reproducing under extreme conditions.

BOX 3 | Selected categories of Extremophiles

Extremophiles are organisms that are adapted to and thrive in physically or geochemically extreme conditions and have their growth optimum at or near extreme ranges of environmental variables. Extremophiles have been firstly termed in a paper of R. D. MacElroy, 1974 and henceforward the term has been widely used.
Extremotrophs are organisms that can tolerate and grow under extreme conditions, but do not necessarily have their optimum growth at theses extremes (Mueller 2005).
Acidophile: an organism that has a pH optimum for growth at or below 3-4.
Piezophile (formerly termed barophiles): an organism that lives optimally at hydrostatic pressures of 40 MPa or higher.
Oligotroph; an organism capable of growth in nutritionally depleted habitats.
Thermophile: an organism that can thrive at temperatures between 60°C and 85°C (Horikoshi 2011).
Neutrophile: an organism that lives under normal conditions.

(Horikoshi 2011)

1.2.1 Organisms in acidic environments

As ocean acidification has become a hot topic, it is important to understand the principles that underlie physiology and adaptation strategies of acidophiles in order to predict survival rates of non-acidpophiles in the future ocean. But why are organisms in general not capable of thriving at very low pH? Reasons are found on the cellular level, because the exposed microbial cell components can be hydrolyzed or important proteins denatured. Furthermore, dissociation and solubility of many molecules that directly or indirectly affect microbes are also influenced by pH. For instance, metal ions (e.g. Cd^{2+}, Cu^{2+}) are more soluble at a low pH and thus can reach toxic concentrations (Atlas and Bartha 1997).

Microorganisms that tolerate acidic conditions, i.e., environments with very low pH values, occur in acidic lakes and rivers, acidic main drainages, geothermal sites and commercial bioreactor cultures (Edwards et al. 2000; Norris et al. 2000; Lopez-Archilla et al. 2001; Simmons and Norris 2002; Gonzalez-Toril et al. 2003; Hallberg and Johnson 2003; Johnson and Hallberg 2003; Dopson et al. 2004; Prokofeva et al. 2005; Reysenbach et al. 2006). Within the group of acidophilic mircroorganisms (BOX 3) a number of prokaryotes and eukaryotes are obligate acidophilic. They became increasingly interesting for academic and biotechnological studies, due to their fascinating ecology and physiology. While one would assume that diversity among extremophiles is rather low due to the extraordinary high adaptation levels required, particularly eukaryotic extremophiles have been reported to be diverse (Amaral-Zettler et al. 2002; Rainey and

Oren 2006). Communities of extremophiles may be functionally diverse, as for instance in the acid main drainages microbial communities where unicellular eukaryotes such as protists and fungi were reported to affect bacterial and archaeal communities via grazing and other ecological processes (Baker et al. 2004).

Studies on the Rio Tinto ecosystem confirmed a high diversity on microbial level. This ecosystem is characterized by pH values of 2.2 and high concentrations of heavy metals (Lopez-Archilla et al. 2001). These extreme conditions are the result of the metabolic activities of chemolithotrophic microorganisms, e.g. sulfur-oxidizing bacteria. The food chain reported for the Rio Tinto ecosystem was exclusively microbial and short, where primary production was the sum of photosynthesis and chemosynthesis. Three different highly diverse functional groups could be identified according to their ecological role: (1) Primary producers (photosynthetic algae and chemolithotrophic bacteria) (2) decomposers (heterotrophic bacteria and fungi) and (3) consumers (heterotrophic protists). The relatively high biodiversity of this ecosystem discloses an interesting level of adaptation both for prokaryotic and eukaryotic microorganisms (most of them photosynthetic) to low pH and high concentrations of heavy metals (Lopez-Archilla et al. 2001; Amaral-Zettler et al. 2002; Amaral-Zettler et al. 2003). The phylogenetic diversity based on sequences obtained from the study on the small subunit rRNA genes included fungi, animals, green algae, land plants, stramenopiles, and alveolates (Amaral-Zettler et al. 2002).

Acidophiles have also been reported from deep-sea hydrothermal vents where they thrive on the vent fluids being released from subsurface reservoirs through volcanic activity. Chemosynthesis, i.e., the utilization of chemical compounds (e.g. H_2S, CH_4) to fix inorganic carbon and produce biomass, presents the basis of life at these chemosynthetic deep-sea habitats. The majority of acidophiles is known to be mesophilic (20-40°C), some are thermotolerant (40-60°C) while others are thermophilic (exclusively archaea), with a growth optimum of >60°C (Hallberg and Barrie Johnson 2001; Johnson 2003). All organisms that are capable to grow at temperatures above 100°C are known to belong to the domain Archaea. Archaea are more successful under extreme conditions, because archaeal lipids consist of ether bonds that are much more robust compared to bacterial and eukaryal lipids with labile ester bonds (Rainey and Oren 2006). Therefore, the upper limit of thermotolerance for eukaryotic life is at about 60°C. First studies on thermophilic bacteria were conducted by Tom Brock on hot springs of the Yellowstone National Park in the 1960s (Rainey and Oren 2006). They revealed a large variety of

organisms adapted to this extremely hot environment, including phototrophs which can survive and grow to about 72-73°C and heterotrophs which even existed at temperatures of 91°C. Further studies on deep-sea volcanoes showed that archaea could even grow at temperatures higher than those of the boiling point of water under normal atmospheric pressure (Stetter 1996). Extremely variable and changing chemical conditions at hydrothermal vents generate a wide range of geochemical niches and potential energy sources, that can be inhabited by a variety of microorganisms. Although vent fluids are released with temperatures that can reach up to 350°C, this does not exclude life. Chemolithoautotrophs can gain energy by exploiting the chemical disequilibria that result from relatively slow inorganic reactions in the interface of oxidized seawater and reduced hydrothermal vent fluids (Fisher et al. 2007). Also, many chemolithotrophs have been isolated and cultured from deep-sea vents (Nakagawa and Takai 2006). Both bacteria and archaea thrive as polyextremophiles at the hot, toxic environments of deep-sea hydrothermal vents.

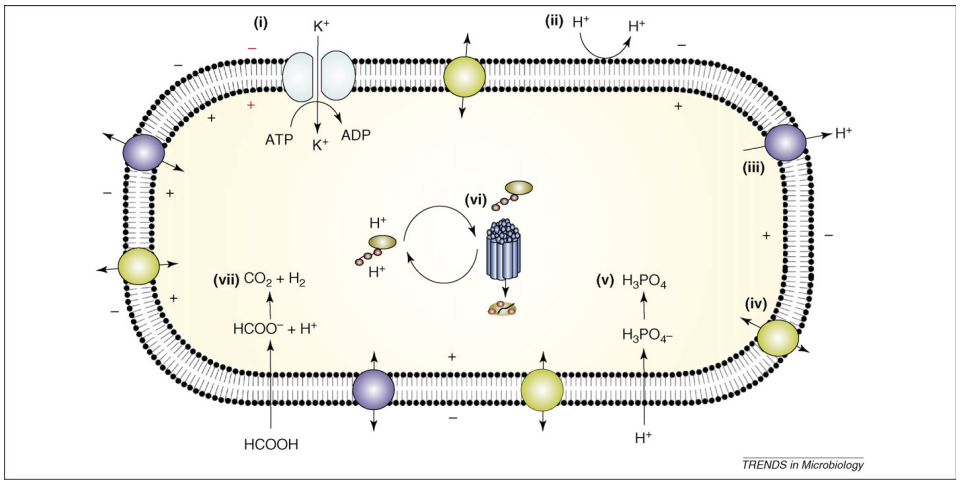

Figure 5 Processes associated with pH homeostasis in acidophiles showing (i) reversed membrane potential (Δψ), (ii) impermeable cell membrane, (iii) maintance of ΔpH through active proton transport, (iv) secondary transporter, (v) presence and availability of enzymes and/or chemicals to bind and sequester protons, (vi) DNA and protein repair system, (vii) degradation of organic acids (adapted from Baker-Austin and Dopson, 2007). Note: in (v) $H_3PO_4^-$ needs to be $H_2PO_4^-$.

1.2.2 Adaptation strategies to acidic environments

In order to grow at low pH, acidophiles require several physiological adaptations to maintain a pH gradient of several pH units across the cellular membrane while using the influx of protons through the F_0F_1 ATPase to produce ATP (Madshus 1988). However, knowledge on adaptation strategies for eukaryotic organisms remains scarce. Amaral-Zettler et al. (2003) conducted *ex situ* physiological experiments on monocultures of *Chlamydomonas sp.*, to evaluate if cytosolic pH would deviate from neutral. They reported that the acidophilic chlamydomonad isolates maintained an internal pH of 6.6 at an external pH of 2. They proposed the existence of active transport mechanisms regulating the internal pH and hypothesized a novel diversity in H^+ ATPase, besides the two major families known, the V/F/A ATPase and the P-type ATPase (Amaral-Zettler et al. 2003).

Similar to neutrophiles, acidophiles require a circumneutral intracellular pH of 6.5 – 7.5, but tolerate several orders of magnitudes higher pH gradients than neutrophiles.

$$\text{pH gradient } (\Delta \text{ pH}) = \text{pH}_{in} - \text{pH}_{out}$$

However, the Δ pH is linked to cellular bioenergetics since it is the major contributor to the proton motive force (PMF). During the process of ATP-production based on the proton influx, cellular protonation intensifies and will rapidly dissipate ΔpH if not counteracted (Baker-Austin and Dopson 2007). This would quickly lead to adverse effects on the functions of proteins and nucleic acids, as well as on other processes such as DNA transcription, protein synthesis and enzyme activity (Madshus 1988). In their review, Baker-Austin and Dopson (2007) collated several adaptation strategies that have been identified mainly based on pH homeostatic mechanisms (Fig. 5), including:

1. A highly impermeable membrane for H^+
2. Membrane channels with reduced pore size and selectivity for protons
3. Chemiosmotic gradient created by a Donnan potential
4. Active proton pumping
5. Cytoplasmatic buffering to maintain intracellular pH
6. Active mechanisms of organic acid degradation
7. Repair mechanisms
8. Stabilizing mechanisms
9. Small genome size

In the following, I will briefly explain the mechanism behind some of these adaptation features. Due to the highly impermeable membrane of acidophiles, the influx of protons into the cytoplasm is restricted which maintains ΔpH (Konings et al. 2002). The membrane structure determines whether the cell can sustain an appropriate PMF (BOX 4) while balancing between proton permeability, proton influx through energetic and transport systems, and outward proton pumping (Baker-Austin and Dopson 2007).

BOX 4 | Cellular properties to maintain pH homeostasis

Proton motive force (PMF): Generated as a result of a charge separation across the cell membrane between external milieu and the cytoplasm created by pH gradient (ΔpH) and the membrane potential (ΔΨ). It gives a measure for the energetic state of the cell membrane.
Reversed membrane potential (ΔΨ): The influx of potassium ions (K^+) generates a positive charge in the cytoplasm of acidophiles, which counteracts the proton entry into the cell and thereby subtracting the large PMF created by the ΔpH.
Donnan potential: The electric potential between two ionic solutions separated by an ion-exchange membrane which restrains any fluid exchange.
Secondary transporter: Membrane proteins that use the trans-membrane electrochemical gradient to drive transport processes.
Antiporter: Membrane protein that transports two species of ions or other solutes in opposite directions across the membrane. One species is transported from high to low concentration thereby gaining the energy to transport the other species from low to high concentration (secondary transporter).
Symporter: Membrane protein that transports two species of ions or other solutes in the same direction across the membrane. The energy gained from the transport of one species from low to high concentration is used to transport the other species against the electrochemical gradient (secondary transporter).
Uncoupler (protonophore): Uncharged compounds with dissociable protons (mainly protonated acids or a conjugate base) that can easily pass the membrane into the cell with an almost neutral milieu where the proton then dissociates. During this process they "uncouple" proton transport from cellular processes.

(Adapted from Baker-Austin and Dopson, 2007)

When exposed to high proton concentrations it is beneficial to reduce the passage into the cell. This includes both the size and the permeability of membrane channels, which could then also represent an important mechanism for pH homeostasis. *Acidithiobacillus ferroxidans* for instance has the ability to control size and permeability of the pore entrance while causing a positive charge (+2 at pH 2.5), which is remarkable as *E. coli* has a charge of -4 at neutral pH (Guiliani and Jerez 2000). A chemiosmotic gradient created by a Donnan potential inhibits the proton influx by means of an inside positive reversed membrane potential (ΔΨ). This positive potential is caused by an enhanced influx of potassium ions, which is greater than the outward flux of protons

(Baker-Austin and Dopson 2007). It is assumed that cation transporters are involved in the generation of the Donnan potential and some acidophiles have been reported to contain a disproportionally high number of them (She et al. 2001; Fütterer et al. 2004; Tyson et al. 2004). The active proton pumping is based on primary and secondary active transport accomplished by different integral membrane proteins such as the proton ATPase, antiporters and symporters (Michels and Bakker 1985; Fütterer et al. 2004; Tyson et al. 2004; Golyshina and Timmis 2005). Another mechanism to sustain pH homeostasis is the buffering capacity of the cytoplasm based on cytoplasmic buffer molecules that have alkaline amino acids such as lysine, histidine and arginine. Organic acids are potentially harmful for acidophiles as they may be transported into the cells by diffusion in a protonated form, followed by a dissociation of the proton. Thus, they function as uncouplers of the respiratory chain at low pH (Alexander et al. 1987; Kishimoto et al. 1990; Ciaramella et al. 2005). However, the active degradation of organic acids might therefore be a pH homeostasic mechanism solely used by heterothropic acidophiles (Baker-Austin and Dopson 2007). It is specifically interesting that all acidophiles that are able to grow at extreme acidic pH values, which may be < pH 0, are heterotrophs capable of degrading organic acids (Angelov and Liebl 2006).

Another important feature to be reported are repair mechanisms to counteract damages on DNA and protein level caused by acidification are operated by chaperones, which are proteins involved in the folding and refolding of proteins after synthesis. Crossman et al. (2004) found a large number of genes determining DNA repair in *Picrophilus torridus*. Ferrer et al. (2007) found a uniquely high proportion of iron proteins in the proteome of *Ferroplasma acidiphilum*, an archaeal microorganism. These high proportions may contribute to the pH stability of enzymes at acidic pH values as the iron possibly maintains the 3D structure and thus the function of the proteins. So far, there seems to be no generally valid DNA-based adaptation for microorganisms growing at low pH, although Fütterer et al. (2004) noticed a minor increase of isoleucine in the acidophile *P. torridus* and Schäfer et al. (2004) suggested this could contribute to acid stability. Many acidophiles are found in hot environments, and heat may additionally aggravate the problem of adaptation. Besides acidotolerance, thermotolerance is another key challenge to these organisms.

Usually, seawater pH is in the range of 7.5 – 8.4, slightly alkaline, while the higher value is found in the water column and lower values in the benthos. Natural variation in the ocean pH has varied in the geological past and estimates indicate that deep-waters of

the Atlantic and Pacific had been 0.3 ± 0.1 units higher and thus more basic during the last glacial (Sanyal et al. 1995). Some marine ecosystems are characterized by naturally low pH values, such as some deep-sea hydrothermal vents that leak CO_2. Organisms inhabiting these sites have adapted to extreme pH values as low as 4.5; however adaptation has evolved over a long period of time.

1.2.3 Previous acidification experiments and mesocosm studies

Even though some marine organisms have adapted to low pH and high concentrations of CO_2, ocean acidification through atmospheric CO_2 increase or local acidification through potential leakage from CCS sites may challenge and endanger a variety of marine organisms, ecosystem, and ecosystem functioning. Some recent studies have addressed the resulting problems on various approaches, nevertheless still little is known on the responses of individual species or whole communities to ocean acidification, and especially not on the responses to leakage from CCS.

Ocean acidification as a serious effect of increasing CO_2 concentrations in the atmosphere has been approached by numerous studies, which address possible threats to marine organisms. Threats include reduced calcification rates and decalcification, hypercapnia, hypoxia, reduced reproduction and growth rates or even death. Numerous studies have investigated all size classes from microbes to megafauna, but were in general biased toward studying the response of calcifying organisms, probably because effects begin to show quickly and are obvious (Takeuchi et al. 1997; Orr et al. 2005; Hoegh-Guldberg et al. 2007; Fabry 2008; Fabry et al. 2008; Hofmann et al. 2008; Jokiel et al. 2008; Kuffner et al. 2008; Wood et al. 2008; Ries et al. 2009; Silverman et al. 2009; Hofmann et al. 2010). Some calcifiers can exist for a short time without their calcareous shell (e.g. some forms of corals and phytoplankton (Fine and Tchernov 2007), while others (e.g. echinoderms) depend on their skeleton to maintain organismal function (Pörtner 2008).

Laboratory and pelagic mesocosm studies on phytoplankton, in particular coccholithophorids, have been conducted and showed opposing feedback of e.g. *Emiliana huxleyi* to enhanced pCO_2 levels. While Igelsias-Rodriguez (2008) confirmed that calcification rates doubled at pCO_2 levels almost twice the average of todays, others reported a decrease in calcification rates and malformed organisms (Riebesell et al. 2000; Riebesell et al. 2001; Riebesell 2004; Rost and Riebesell 2004; Engel et al. 2005; Iglesias-Rodriguez et al. 2008; Riebesell et al. 2008; Rost et al. 2008). Benthic mesocosm studies

indicated diverging responses, either disclosing significant influence on the abundance of unicellular (Bernhard et al. 2009; Ricketts et al. 2009) and metazoan meiofauna (Carman et al. 2004), or resulting in high rates of mortality for example of nematodes (Barry 2003; Baker et al. 2004; Barry et al. 2004; Thistle et al. 2006; Barry and Drazen 2007; Kurihara et al. 2007b; Thistle et al. 2007; Fleeger et al. 2010). Physiological experiments on different model organisms exhibited effects on organism performance at the level of reproduction, behavior, and growth, especially on lower marine invertebrates, characterized by a low capacity to compensating disturbances in acid-base balance (Pörtner et al. 2004; Pörtner 2008; Pörtner and Farrell 2008). Most of the studies mentioned are very specific regarding the organism and were implemented in artificially changed environments in a set time frame. Some studies, however, have taken advantage of studying acidification effects *in situ* at natural laboratories, in order to evaluate the composition, level and mode of disturbance and dysfunction of organisms, in response to increased acidification (Inagaki et al. 2006; Hall-Spencer et al. 2008; Tunnicliffe et al. 2009; Fabricius et al. 2011). Yet, studies on whole communities, including microorganisms, meiofauna, macrofauna and megafauna, under naturally occurring high acidification regimes, comparable to CCS CO_2 leakages, are missing so far. The present PhD study conducted at a natural high CO_2 site will contribute to the understanding of responses on ecosystem level to high CO_2 concentrations and resulting low pH as in potential scenarios of CO_2 leakage from CCS sites.

1.3 Natural laboratories to study the risks of CCS and consequences of ocean acidification

Natural CO_2 seeps in the ocean are considered "natural laboratories" where effects of increased CO_2 on marine organisms can be studied. Due to the long-term existing gradients they provide, organisms and communities have the chance to adapt through migration, succession, or by means of physiological adaptation and eventually evolution at the gene level.

Thus, they provide insights into long-term effects on deep-sea organisms subsequent to the aftermath of leakages from CCS. Enforced by pressure increase through the introduction of CO_2, other gases will be extruded and in combination with CO_2, will lower pH significantly. The responses of marine organisms to elevated CO_2 concentrations and concomitant chemical species can be directly evaluated at CO_2 seeps, where conditions may in many ways resemble conditions that would be generated during

leakage events at CCS sites. Long-term effects on species composition, richness, abundance, as well as appearance (e.g. malformed shells) can be investigated along natural CO_2 and/or pH gradients. An evaluation of responses may be performed from individual species to community level.

So far, our understanding of ocean acidification is mainly based on short-term laboratory perturbation experiments of individual organisms or derive from deterministic models (Fabricius et al. 2011). Although perturbation experiments are essential and provide insights into instantaneous responses of organisms to increased CO_2 levels, they mostly lack information of co-limiting factors for example nutrients, currents, or surface irradiance (Tunnicliffe et al. 2009; Fabricius et al. 2011). Hence, *in situ* studies provide information on populations that have acclimatized to such conditions and may provide better data for models of future CO_2 responses (Hall-Spencer et al. 2008; Kuffner et al. 2008) However, confounding effects will make interpretation of the data complicated. As natural CO_2 vents are promising study sites to evaluate long-term consequences of high CO_2 concentrations and low pH, observations at the Yonaguni Knoll IV hydrothermal system at a natural CO_2 vent were conducted.

1.3.1 Ecosystem responses and community ecology

In addition to studying a natural site this PhD work also investigated the response of possible leakage at CCS sites on an entire ecosystem, i.e., comprising bacteria, meio- and macrofauna as well as megafauna. Acidification induced by local leakages could potentially reduce marine biodiversity in a number of ways, including the loss of species sensitive to high CO_2 and low pH, and impacts on taxonomy if sensitivity to acidification is a function of an organisms taxonomic group (Widdicombe and Spicer 2008). In addition, the loss of keystone species or a reduction in their activity, as for example predation, bioturbation, or grazing, would alter and reduce habitat heterogeneity and complexity, whilst also reducing biological regulation and competition (Widdicombe and Spicer 2008). From the information currently available, it is emerging that the deep sea is a fragile ecosystem where any changes in parameters may have severe effects on deep-sea communities, owing to their high adaptation to deep-sea parameters. Information still lacks on important deep-sea parameters such as temperature, salinity, bottom currents, and organic and inorganic nutrients (Danovaro et al. 2001). Bottom- up and top-down controls should be taken into account by including all different size classes of the benthic community. Seafloor sediments contain 10 – 10,000-fold more microbial

cells per unit volume than in the productive ocean-surface-waters (Jorgensen and Boetius 2007) and cell numbers can reach the order of 10^9 cells per g sediment in the upper 10 cm of the deep-sea surface sediments, comparable to sediments in coastal waters (Deming and Colwell 1982; Boetius et al. 1996; Guezennec and Fiala-Medioni 1996). Opposing results of a decrease in standing stock of the benthic meio-, macro-, and megafauna caused by an exponential decrease in the rate of nutrient input from sinking phytodetritus with increasing depth and distance from coastal waters have been reported (Rex et al. 2006). Bacterial biomass, however only marginally decreases and bacterial biomass in abyssal sediments can be as high as 95% (Rowe et al. 1991; Pfannkuche 1992). Bacteria not only dominate the sediments of the deep-sea surface in terms of abundance and biomass, but also provide important features for ecosystem functioning, such as the recycling and turnover of organic matter and thereby play an essential role in the global carbon cycle, as well as in the nitrogen cycle by nitrification and nitrogen fixation (Liu et al. 2010; Joint et al. 2011; Weinbauer et al. 2011). At deep-sea hydrothermal vents chemolithoautotrophic microorganisms are the primary producers, as they can use a huge range of chemical compounds deriving from hydrothermal fluids and thus support life in theses environment (Jorgensen and Boetius 2007). Many organisms depend on the energy flow of these chemoautotrophs, including symbiotic animals as the giant tubeworms, numerous molluscs and shrimps (Boetius 2005; Jorgensen and Boetius 2007).

Meiofauna can play an important role in the energetics of benthic communities but determining their diet remains problematic due to their small size (Leduc et al. 2009). Despite their low biomass, meiofauna are important for the carbon flux of benthic communities due to their high turnover rate (Kuipers et al. 1981). Nematodes for example are able to digest twice their body carbon each day (Heip et al. 1985). Harpacticoid copopodes are known to be an important link between primary producers and higher trophic levels (Coull 1999) and lower trophic levels, as bacteria are degrading the fecal pellets they produce (Jacobsen and Azam 1984). It is known that benthic macrofauna affect microbial processes in sediments by their burrowing and feeding activities (e.g. (Andersen and Kristensen 1988) and also impact meiofaunal organisms via predation and competition (Bell and Coull 1978). Moreover, megafauna will additionally affect the assemblage structure of meiofauna (Gallucci et al. 2008).

Notably, special emphasis should be put on changes of the meiobenthic communities, as meiofaunal organisms are represented in 22 of the 40 animal phyla

nowadays recognized and thus represent the dominant component among all benthic metazoans in all aquatic ecosystems (Higgins and Thiel 1988). At current state not much is known about the impacts of high CO_2 and low pH on ecosystem level, comprising all size classes thus, further investigations are needed, but it is clear, based on studies of single organisms or size classes that both positive and negative feedback mechanisms exist. This makes future predictions of the effects of rising CO_2 levels and extreme acidification on marine communities/ecosystem functioning difficult. Furthermore, evaluation on local- and regional- scale have to be integrated.

1.3.2 Resistance, resilience or death - responses to acidification and synergistic effects

One would assume that deep-sea organisms are highly fragile and clearly respond with negative feedback to ocean acidification but this is not generally true. Especially for calcifying organisms (corals, molluscs), limits were assumed to be set in the rate of calcification due to enhanced acidification. Several studies now revealed that some calcifiers could retain, or even increase net calcification under low pH conditions but certainly affiliated with increasing costs (muscle wastage) (Wood et al. 2008; Cohen et al. 2009; Ries et al. 2009). Mussels in the deep sea showed astonishing persistence to extremely low pH and managed to survive. However, the formation of only very thin shells increased the susceptibility to predation by crustaceans (Tunnicliffe et al. 2009). Nevertheless, the resilience of the bivalves to the acidic environment is highly unstable and only possible because predators can not cope with the low pH, indicating the existing diversity of species-specific responses within one ecosystem but also the tight interactions on whole community level. There is no universally valid threshold that sets the limit for life. It is gradually dependent on the magnitude, duration, and frequency of the altered environmental conditions. Multifactorial stressors combined (extreme temperatures, hypoxia, CO_2) will potentiate the impacts on organisms (Pörtner et al. 2005; Przeslawski et al. 2005; Rosa and Seibel 2008; Russell et al. 2009; Dissanayake and Ishimatsu).

In addition to the previously presented acidophiles, some neutrophile organisms are able to maintain organismal functioning, growth, and reproduction while resisting acidification. However, this remains species-specific and can not be extrapolated to functional groups. Differences in resistance are mainly based on species-specific morphology that helps to lower the effect of acidification. Comparative studies on corals

and molluscs indicated that when exposed to gradually high CO_2 concentrations some molluscs had an advantage over congeners because of their periostracum, an organic layer that protected the shells from dissolution (Rodolfo-Metalpa et al. 2011). The ability to resist acidification yet was weakened and disrupted in combination with elevated temperatures. This indicates that morphological aspects or adaptations may be sufficient to one stressor, but as soon as organisms are exposed to synergistic effects, they have to face functional constraints. At current state, human activities seem to challenge the capacity of ecosystems to cope with increasing levels of disturbance. It has become obvious that resilience, which has been defined by Holling (1973) as the magnitude of disturbance that a system can experience before it shifts into a different state with different controls on structure and function, has been worn out and that self-repairing capacity of ecosystem can no longer be taken for granted (Holling 1973; Folke et al. 2004). Several studies have illustrated, that ecosystems and their services can be transformed into a less productive system by human activities (c.f. Folke et al. 2004). These regime shifts are related to shifts in biodiversity and diversity of functional groups which are critical to the resilience and the generation of ecosystem services (Chapin et al. 1997; Luck et al. 2003). For instance, overexploitation of foremost herbivorous coral fish in the Caribbean, caused reefs to suffer a phase shift in ecosystem that entailed the expansion of sea urchin populations as key grazers on invading algae. Grazing of the sea urchins preserved the coral-dominance, albeit at low resilience. The sudden collapse of the sea urchins population through the outbreak and spread of a disease, led to a shift of an algal-dominated reef ecosystem (Knowlton 1992; Hughes 1994). Human activities today may cause loss of resilience through the removal of functional groups of species (as the reduction of the top down-controller, the herbivorous fish) and their response diversity which includes the loss of whole trophic levels (top-down effect). It may be triggered by overfishing, as in the latter study or by climate change, and is controlled by the alteration of the magnitude, frequency, and duration of disturbance regimes to which the biota are adapted to (Folke et al. 2004). The loss of resilience however, through combined and synergistic effects (hypercapnia, thermal stress, and hypoxia) of those disturbances (acidification, temperature shifts, oxygen minimum zones) may enhance the vulnerability of ecosystems to changes that previously could be damped. If ecosystem resilience can no longer be sustained, mortality rates will increase, inducing the establishment of impoverished or completely different ecosystem with different key species and thus a functional loss. This then may result in the loss of ecosystem services.

Therefore it is vital to look at whole communities and their responses to these changes on the long-run, as it was done in this PhD study.

1.4 Objectives

Over the last decades, social perception has changed and awareness rose towards the problems associated with global climate change and has resulted in the initiation of measures to counteract global change. As outlined above, research on global change topics and the generation and formulation of hypothesis and theories has led to several new approaches to elucidate and understand more about the combination of circumstances and interactions that drive changes in a high CO_2 ocean. Several international and interdisciplinary projects were launched with the "overall goal to advance understanding of biological, ecological, biogeochemical, and societal implications of ocean acidification" (www.epoca-project.eu) and combined expertise of many different research fields (e.g. "European Project on Ocean Acidification", EPOCA (recently ended), "Biological Impacts of Ocean Acidification", BIOACID). Research has made substantial progress in evaluating the effects of ocean acidification on marine organisms, but due to the complex oceanic system and negative concomitant effects through mitigation strategies originally supposed to counteract human-induced changes, new challenges have evolved. However, little is known about the potential consequences of CO_2 leakage in CCS projects on marine biota, and the importance to disentangle responses on community level has been realized. It remains an open question, however, whether and to what extent benthic communities of the deep sea respond to high CO_2 concentrations and low pH. Bacteria, meiofauna and macrofauna might show completely different responses but confounding effects of thermal stress, hypoxia or predation and competition may mask the CO_2 impact. Thus, it is critical to understand the interactions in the context of trophic connections. Given that bacteria dominate deep-sea sediments in terms of biomass and given that microbial processes dictate the biogeochemistry of deep-sea sediments e.g. in carbon cycling and remineralisation, it is crucial to better understand the impacts of low pH on the dynamics of bacterial communities (Jorgensen and Boetius 2007). This is crucial to better estimate environmental changes on the structure and function of the deep-sea ecosystem. Further understanding of the effects on the assemblage of meiofauna, with special focus on nematodes distribution and diversity, as they dominate meiofaunal communities, will substantiate predictions made for the future. Meiofauna comprise important key species in the benthic ecosystem as they link the trophic energy flow from bacteria to macrofauna and are both top-down and bottom-up controller. In addition, research on responses of the macrofauna and

megafauna as an important top-down control will promote understanding of ecosystem feedbacks.

The **overall aim** of this thesis was to improve the understanding of the effects of CO_2 on benthic communities in the deep sea, by investigating the geochemical properties of a naturally high CO_2 system and evaluating responses of all size classes that structure benthic deep-sea ecosystems. A natural laboratory, i.e., a CO_2 hydrothermal vent system, was used for this *in situ* approach to analyze long-term effects of high CO_2 concentrations on benthic communities.

The **specific objectives** were to disentangle effects of CO_2 and low pH on bacterial, meiofaunal, and macrofaunal abundance, distribution, and diversity from a set of contextual parameters and from natural variations of the benthic assemblages.

The **major questions** addressed in this thesis were:

1) **What are the geochemical properties of the investigated Yonaguni Knoll IV CO_2 hydrothermal system and how do long-term effects of hydrothermal CO_2 venting and low pH control the distributions of microbial processes in deep-sea sediments?**
Biogeochemical processes in deep-sea sediments are assumed to be affected by long-term exposure to high CO_2 concentrations and low pH. Despite reduced pH through CO_2 leakage around the vents, microbial processes were present, however only relative low rates were measured and theses were restricted to the upper 15 cm. Although capable to cope with very low pH, the depth limitation of microbial processes might indicate a threshold at where microbes are no longer resistant to these extreme high CO_2 concentrations **(Chapter I)**.

2) **Do we observe differences in the abundance, structure and diversity of deep-sea benthic bacterial communities exposed to naturally high CO_2 concentrations and a consequently low pH when compared to background sediments?** If bacteria are sensitive to low pH it should be reflected in the community structure through a reduction in abundance, and/or a shift towards more acid-tolerant species with concomitant reduction in sensitive bacterial types **(Chapter II).**

3) **What are the effects of high CO_2 and low pH on deep-sea macrobenthic and meiobenthic communities, and how do these affect the interconnection and interaction between both communities?**

The strong influence of the high CO_2 concentrations and a consequently low pH that alter the carbonate system of the seawater, resulting in reduced calcification rates is assumed to have severe effects especially on calcifying organisms. Nevertheless, non-calcifiers are likewise believed to be affected in terms of organism performance which will be reflected in a reduction of sensitive fauna and hence in a shift in community structure. It is further assumed that the regime shift will be amplified through alterations in predation and competition through the reduction of key species **(Chapter III).**

1.5 Material and Methods

1.5.1 The setting of the investigated "natural laboratory" - the Yonaguni Knoll IV hydrothermal vent system

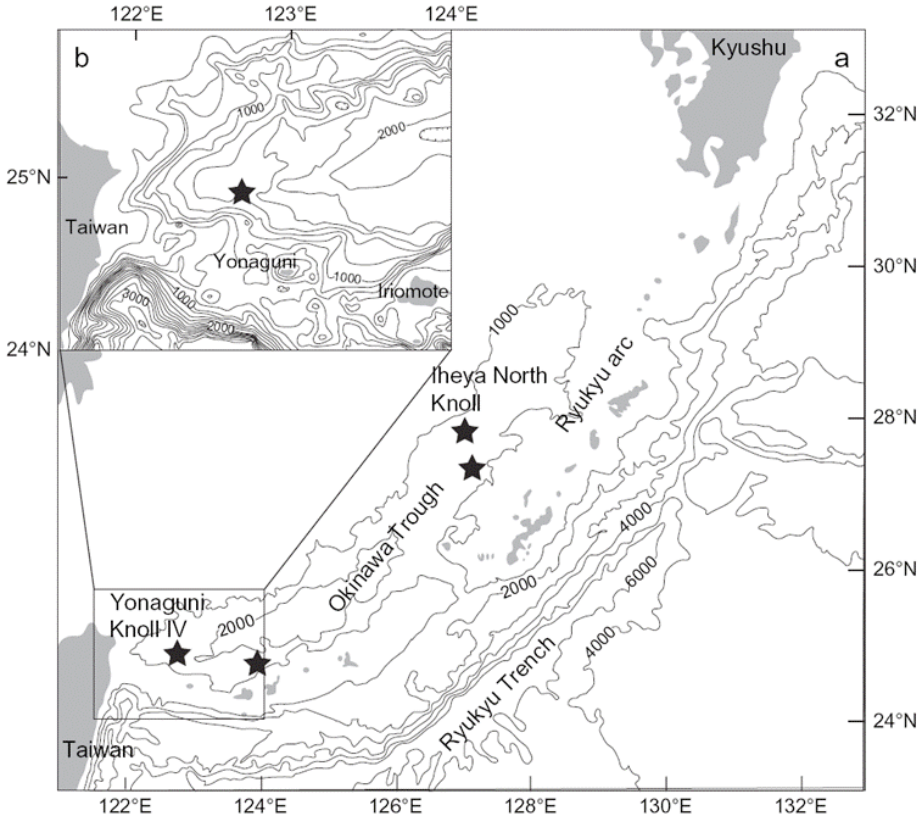

Figure 6 (a) Topography of the Ryuku arc and the adjacent Okinawa Trough backarc-basin indicating the locations of hydrothermal fields of the Yonaguni Knoll IV and at other places in the Okinawa Trough. (b) image enlargement of the southern part of the Okinawa Trough (adapted from Suzuki et al. 2008, Chung et al. 2000).

The Yonaguni Knoll IV vent field is located in the Okinawa Trough, which is an intracontinental back-arc basin behind the Ryuku arc-trench system and is related to the subduction of the Philippine Sea Plate under the Eurasian Plate (Fig. 6 a, b) (Suzuki et al. 2008). The Okinawa Trough is characterized by an extensional tectonic setting and is supposed to be in the rifting phase prior to the back-arc spreading (Sibuet et al. 1987). An eight km thick sedimentary layer covers the northern part of the seafloor basin, while the sediment layer in the southern part amounts to two km due to the supply of

terrigenous materials from the Asian continent (Sibuet et al. 1987). It was proposed that volcanic activity in the southern part of the Okinawa Trough essentially occurred as an early arc magmatism (Shinjo et al. 1999). The Yonaguni Knoll IV hydrothermal field is situated in the most southern part of the Okinawa Trough in an elongated valley with a length of 1000 m and a width of 500 m and lies adjacent to the Yonaguni Knoll IV (Suzuki et al. 2008). It is characterized by high-temperature fluid venting associated with sulfide-sulfate chimneys named Tiger, Lion, Swallow, and Chrystal ((Inagaki et al. 2006; Suzuki et al. 2008). Near the active black smokers (Lion and Tiger Chimneys), where vent fluids of up to 323°C are emitted, vapour-rich clear venting liquids, as well as small liquid CO_2 droplets were observed escaping from subsurface sediments (Inagaki et al. 2006). Furthermore, Inagaki et al. (2006) reported a liquid CO_2 lake below a 20–40 cm thick cover of sediment, approximately 50 m southward from the hydrothermal vents and a rise in *in situ* temperature from 3.9°C in the overlying bottom water to 9.9°C at 35 cm depth indicative of the migration of hydrothermal fluids through the sediment. Droplets that were released from the subsurface reservoir were composed of 98% CO_2 (Sakai et al. 1990; Inagaki et al. 2006). At a depth of 1380 m and accordant pressure, liquid CO_2 is less dense than water, thus the accumulation of the CO_2-lake is only possible with a solid ice-like hydrate cap, denser than seawater (formed when liquid CO_2 reacts with seawater), that keeps the liquid CO_2 in place (Sakai et al. 1990). The CO_2-rich fluid phase may have been separated by subsurface boiling of the hydrothermal fluids or by leaching of CO_2-rich fluid inclusions derived from interactions between volcanogenic sediments and pore water as during post-eruption interactions (Sakai et al. 1990). Konno et al. (2006) suggested, the observed liquid CO_2 on the seafloor to be formed through the following subseafloor processes (Fig. 7):

1) phase separation of high-temperature hydrothermal fluid emanating through the crust, 2) segregation of the vapour phase from the brine phase, 3) crystallization of CO_2-hydrate from the vapour phase due to cooling below 3.5°C within a closed system, and 4) melting of the CO_2-hydrate to produce liquid CO_2 (Konno et al. 2006).

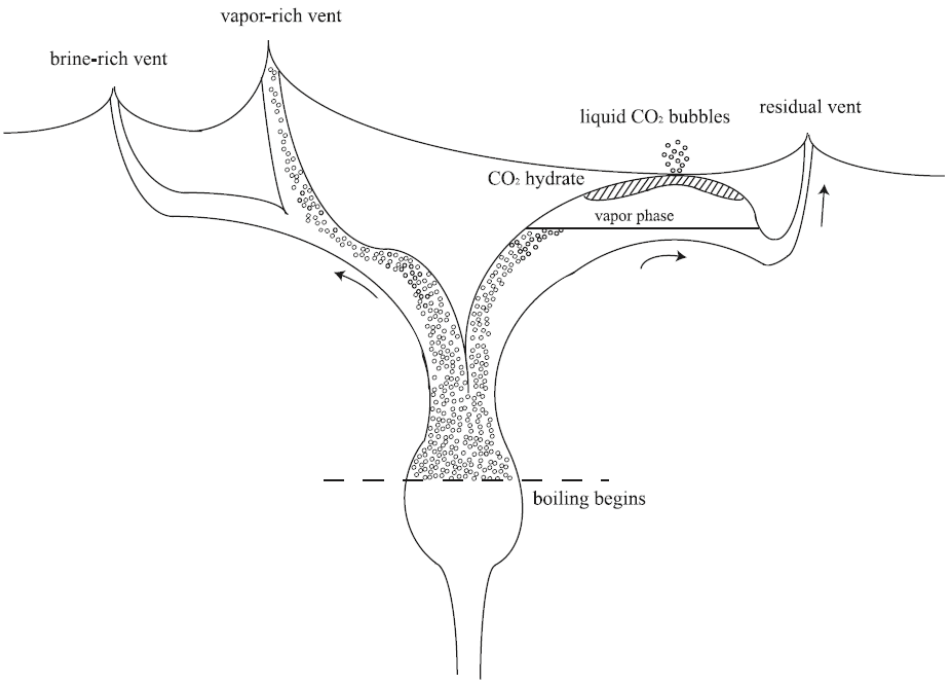

Figure 7 Schematic cross section of the CO_2 hydrothermal systems at Yonaguni Knoll IV hydrothermal site. The dotted line indicates the depth at where boiling starts (adapted from Konno et al. 2006).

1.5.2 Methods and tools used for the identification of faunal and bacterial diversity

For assessing bacterial diversity and community structure two different molecular techniques were applied: Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 454 Massively Parallel Tag Sequencing (MPTS), which will be briefly explained in the following. After that, extraction procedure and taxonomic identification of the meiofaunal and macrofaunal assemblages are described followed by a description of the extraction procedure and identification of nematodes.

1.5.2.1 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA is a so called community fingerprinting method to rapidly assess microbial diversity and community structure. It is cultivation-independent and therefore a time- and cost-effective method to rapidly process many samples, while still obtaining robust and reproducible patterns (Fisher and Triplett 1999). ARISA targets the intergenic transcribed spacer region (ITS) located between the 16S rRNA and 23S rRNA genes in

the rRNA operon. The length heterogeneity of the target ITS region (~300 - 1,200 bp) is used to generate a microbial community fingerprint (Fisher and Triplett 1999). Briefly described, the genomic DNA from an environmental sample is extracted and amplified in triplicates with primers targeting the ITS region, one being fluorescently labelled (Fig. 8). The resulting amplicons are cleaned and analyzed via capillary electrophoresis thereby fragment sizes are discriminated by comparison with an internal size standard. Each peak corresponds to one ARISA OTU (one or several phylotypes of the same length) (Crosby and Criddle 2003; Yannarell and Triplett 2005). ARISA profiles are analyzed and standardized by binning before further ecological interpretation (Cardinale et al. 2004; Hewson and Fuhrman 2006; Böer et al. 2009; Ramette 2009). Additional information and details on the technique and subsequent data processing can be found in (Böer et al. 2009).

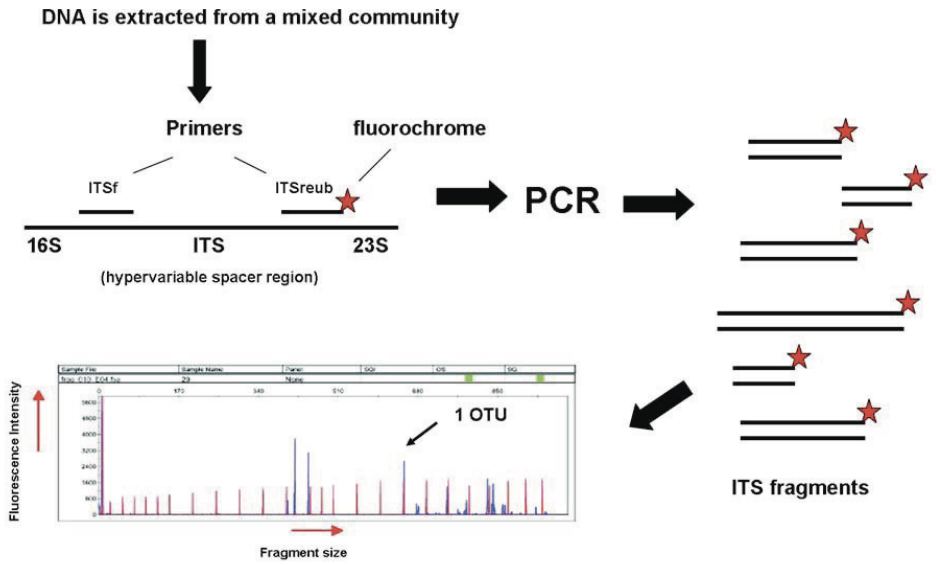

Figure 8 ARISA workflow (Böer 2008). Amplification of DNA produces fluorescently labelled fragments of different length of the ITS region which are detected and separated by capillary electrophoresis.

1.5.2.2 454 Massively Parallel Tag Sequencing

454 massively parallel tag sequencing is a high-throughput method to rapidly obtain a large number of sequences, exceeding traditional Sanger sequencing based on capillary electrophoresis (Margulies et al. 2005; Sogin et al. 2006). Margulies and colleagues (2005) were able to sequence 25 million sequences in a four-hour run. Sogin

and colleagues (2006) introduced a "tag sequencing" strategy that avoids nearly full length sequences of the 16S rRNA gene and is based on sequence tags from a hypervariable v4v6 region of the 16S rRNA gene (Sogin et al. 2006). Extracted DNA is ligated to specific primer-adapter complexes and amplified in order to create an amplicon library of the hypervariable v4v6 region (Fig. 9 A, B). DNA fragments (single strands) are bound to beads that are then emulsified in droplets of a PCR-reagents-oil mixture (Fig. 9 C). Amplification occurs within each droplet and every bead carries ten million copies of the originally attached DNA template. After the emulsion is broken, DNA strands are denatured and the beads carrying the DNA copies are brought into wells of a fibre-optic Pico Titer plate (Fig. 9 D). Smaller beads carrying immobilized enzymes that are required for pyrophosphate sequencing are placed into each well (Fig. 9 E). Briefly described, the actual pyrosequencing is processed by successively flowing bases (T, A, C, G) through the wells, while the incorporation of each base to the DNA sequence induces the release of light (Fig. 9 F). For further details on the process, see (Margulies et al. 2005).

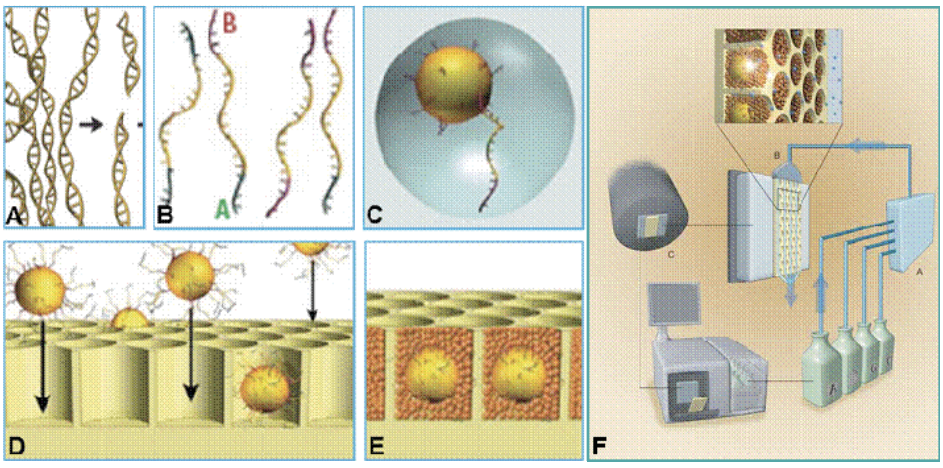

Figure 9 Overview of the 454 MPTS sequencing procedure. (A) Creation of an amplicon library of the hypervariable V6 region of the 16S rRNA gene and (B) the ligation of a V6-primer-adapter complex. (C) DNA sequence-adapter complex is attached to a bead which is captured in a droplet of a PCR-reagents-oil mixture to be amplified. (D) The bead with attached amplified templates is deposited into a well of a Pico Titer plate. (E) Smaller beads carrying immobilized enzymes required for pyrophosphate sequencing are added to each well. (F) Sequencing instrument: each base (T, A, G, C) is successively flowed and incorporation of bases induces a light signal (Figure modified from www.roche-applied-science and (Margulies et al. 2005).

1.5.2.3 Extraction and identification of benthic meiofauna and macrofauna

Onboard, macrofauna and meiofauna sediment samples were collected and stored in buffered 4% solution of formaldehyde. In the home laboratory, macrofauna samples were flushed with tab water and sorted into 5 major taxonomic groups: molluscs, polychaetes, crustaceans, echinoderms, and others and subsequently again stored in 4% solution formaldehyde. Macrofauna from meiofauna sediments was retrieved on a 1 mm sieve, which is commonly accepted as the upper limit for meiofauna (Soltwedel 2000). Both size fractions of the meiofauna samples were extracted by passing the sediment through a 1mm sieve subjacent a 32 μm to separate the macrofauna portion. For the retention of the meiofauna, a 32 μm sieve was used as lower limit, following other studies, e.g. (Carman et al. 2004; Van Gaever et al. 2006; Van Gaever et al. 2009).

The whole content of the formaldehyde-sediment mixture and the supernatant was poured on the sieves and the collected macrofaunal organisms were transferred in tubes of a final 4% solution formaldehyde and stained with Rose Bengal©. Sediment retained on the 32 μm sieve was again flushed with tab water and thoroughly rinsed to remove the gross of the sediment. Further centrifugation with Ludox was necessary. For futher description of the extraction procedure with Ludox, see (Heip et al. 1985).

Nematode identification is quite complex and the process is briefly described in the following.

From each sample, hundred nematodes were picked out randomly and mounted on glycerine slides using a slightly modified technique of (Seinhorst 1959). This technique is using a formalin-ethanol-glycerol mixture for making permanent slides of nematodes.

In the first step 50 nematodes each are transferred from the fixative into two embryo dishes (Fig. 10 A) containing a solution of 99% formol (4%) + 1% glycerine. The open embryo dishes are then placed in a desiccator (Fig. 10 B) containing an excess of 70% ethanol. The desiccator is placed into an oven heated up to 40°C – 45°C, for at least 12 hours. The formaldehyde is then displaced by ethanol. The glycerine is important so that the embryo dishes do not run dry since glycerine cannot evaporate rapidly and extracts ethanol, thus avoiding shrinkage to the nematodes. In the next step, the embryo dishes are dropwise filled with a solution of 95% ethanol (96%) + 5% glycerine and placed in a partly closed petridish while kept at 40°C for three hours until all ethanol has evaporated. The last solution added to the glasses, is a mixture of 50% ethanol (96%) + 50%

glycerine. Containers are then placed in a desiccator with dry silicagel and kept until nematodes are individually placed on glass slides.

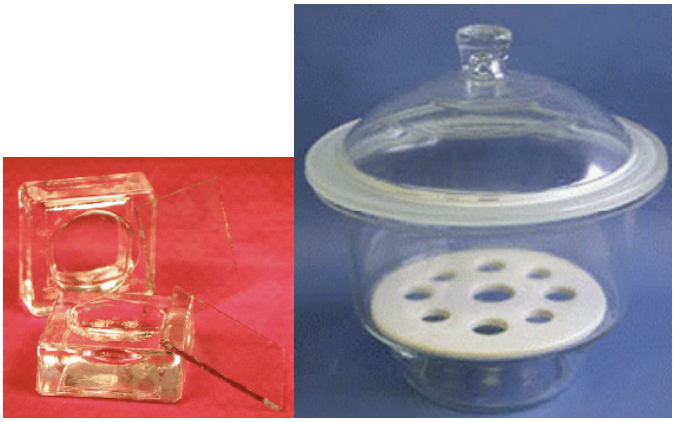

Figure 10 (A) embryodish (www.canemco.com), **(B)** desiccator (www.ginsbergscientific.com)

1.5.2.4 Procedure for permanent nematode slides

The glass slides are provided with a paraffin circlet. A drop of glycerine is placed in the middle of the circlet and then each nematode (10 nematodes for each slide) is placed on top of the glycerine drop and carefully pushed to the bottom of the slide. Then a cover slip is placed on top, gently heated and thus sealed.

1.6 Publication Outline

The following 3 chapters describe the effects of high CO_2 leakage from a natural subsurface seafloor CO_2 reservoir on 1) the biogeochemistry of deep-sea sediments, 2) the benthic bacterial community, 3) the benthic fauna including members of the meiofauna and macrofauna size classes. Chapter I provides the biogeochemical background on the effects of CO_2 leakage through deep-sea sediments and supplies a detailed look on the environmental data which formed the basis for the biological studies in Chapter II and III. Chapter II presents evidence that the composition of bacterial communities in CO_2 impacted deep-sea sediments is strongly altered by CO_2 seepage. Chapter III emphasizes that benthic fauna of the meiofauna and macrofauna size classes also show a significant shift of their composition as a consequence of high CO_2 and low pH. Samples originate from the ("Studies of marine CO_2-sequestration associated with a natural hydrothermal CO_2-system of the Northern West Pacific") (SUMSUN) cruise in March 2008 with RV SONNE and ROV QUEST (MARUM), and from the Japanese expedition „NT10-06 Leg 3" with RV Natsushima and ROV Hyper Dolphin (Jamstec) in April 2010 (Chapter I and II).

Chapter I: Life in extreme environments: Biogeochemical processes in CO_2 vented deep-sea sediments

Dirk de Beer, Matthias Haeckel, Judith Neumann, Gunter Wegener, Antje Boetius

(4.6. 2012 – in preparation for the Biogeosciences journal)

This study investigates the biogeochemistry of the sedimentary seafloor of the Yonaguni Knoll IV CO_2 hydrothermal vent system. Adaptability of microbes to extremely high subsurface CO_2 concentrations was indicated by measurable microbial processes in sediments saturated with CO_2. D. de Beer, M. Haeckel, and A. Boetius designed the study and performed sampling. Microsensor measurements were conducted by D. de Beer, pore water measurements were performed by M. Haeckel. Sulfate reduction, AOM measurements and cell count analyzes were done by G. Wegener, A. Boetius and J. Neumann. The manuscript was written by D. de Beer with input from all co-authors.

Chapter II: Effects of subsurface CO_2 leakage on deep-sea bacterial communities of the Yonaguni Knoll IV hydrothermal sediments (Okinawa Trough, 1350 m)

Judith Neumann, Alban Ramette, Dirk de Beer, Matthias Haeckel, Fumio Inagaki, Antje Boetius

(4.6.2012 – in preparation for ISME)

This study investigates the responses of bacterial communities in sediments vented by hydrothermal CO_2 in comparison to background sediments without venting. High CO_2 and low pH were found to cause substantial shifts in the bacterial community structure on the phylum to genus level. Several types of bacteria found in background deep-sea sediments were replaced with bacterial types that are typical for extreme environments including hydrothermal vent sites. The study was initiated by A. Boetius and F. Inagaki, sampling was done by A. Boetius. J. Neumann carried out all molecular and microbiological analyzes. Microsensor measurements (*in situ*) of CO_2, pH, sulfide and oxygen were conducted by D. de Beer. Pore water measurements were done by M. Haeckel. Statistical analysis of all data was done by J. Neumann and A. Ramette. The manuscript was written by J. Neumann with support and input from all co-authors.

Chapter III: Impact of high CO_2 leakage on macrobenthic and meiobenthic community structure of the Yonaguni Knoll IV hydrothermal system (Okinawa Trough, 1350)

Judith Neumann, Freija Hauquier, Fumio Inagaki, Antje Boetius, Ann Vanreusel

(4.6.2012 - in preparation for PLoS One)

This study analyzes how meiofauna and macrofauna communities change in composition and distribution in response to high CO_2 concentrations and low pH from a hydrothermal subsurface reservoir. Diverging effects between both size classes and across faunal taxa could be observed. Nematodes were the most abundant taxa in the meiofauna community, and showed a relatively low sensitivity towards high CO_2. The study was designed and sampling was done by A. Boetius and Fumio Inagaki. Taxonomic identification of the macrofauna and meiofauna was done by J. Neumann and A. Vanreusel. Nematode identification to genus level was performed by F. Hauquier and J. Neumann with support and input from A. Vanreusel. Statistical analysis was done by J. Neumann with input from A. Vanreusel. The manuscript was written by J. Neumann with input from all co-authors.

Participation in seminars and courses of the excellence graduate school "Global Change in the Marine Realm" (GLOMAR) provided an interdisciplinary and international framework for discussion on the consequences of climate change and the responses of the marine ecosystem in terms of diversity and function.

2.

Thesis chapters

Chapter I

Life in extreme environments: Biogeochemical processes in CO_2 vented deep-sea sediments

Life in extreme environments: Biogeochemical processes in CO_2 vented deep-sea sediments

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In preparation for submission to the Biogeosciences Journal
Manuscript version of 09.06.2012

Abstract

This study focused on biogeochemical processes and microbial activity in sediments of a natural CO_2 seepage area (Yonaguni Knoll IV hydrothermal system, Japan). The aim was to assess the influence of the geochemical conditions occurring in highly acidic and CO_2 saturated sediments on sulphate reduction (SR) and anaerobic methane oxidation (AOM). Porewater chemistry was investigated from retrieved sediment cores and *in situ* by microsensor profiling. The sites sampled around a sediment-hosted hydrothermal CO_2 leak were very heterogeneous in porewater chemistry, indicating a complex seepage pattern. Near the seeps liquid CO_2 was observed to emanate from the sediments, and the pH reached approximately 4.5 in a sediment depth > 6 cm, as determined *in situ* by microsensors. Whereas methane and sulphate were found in almost every sediment sample, down to a depth of 3 m, SR and AOM were restricted to the upper 7-15 cm bsf, although, neither the temperature, the low pH, nor availability of methane and sulphate could be limiting microbial activity. We argue that the extremely high subsurface concentrations of dissolved CO_2 (1000-1700 mM), through the ensuing high H_2CO_3 levels uncouples the proton-motive-force (PMF) and thus inhibits biological energy conservation by ATPase driven phosphorylation. This limits life to the surface sediment horizons above the liquid CO_2 phases, where less extreme conditions prevail. Our results may have to be taken into consideration for assessing the consequences for deep-sea deposition CO_2 on benthic element cycling and local ecology.

Introduction

The increase in atmospheric CO_2 will lead to global warming and acidification of the ocean. As one of the possible counter-measures it is considered to separate CO_2 from waste gas of large production units, such as powerplants and cement ovens, and to pump it in liquefied form into the deep seafloor where – depending on *in situ* pressure and temperature - it will become hydrate, liquid or supercritical. In the sub-seafloor reservoirs it is expected to be sequestered during the weathering of sedimentary carbonates and silicates (IPCC, 2005; House et al., 2006; Wallmann et al., 2008). This process of Carbon Dioxide Capture and Storage (CCS) would not bind CO_2 as such, but lead to neutralization of the acidified seawater. Besides economic costs and safety aspects, the consequences of such activities for biodiversity and element cycling in the deep sea are to be considered (Seibel and Walsh, 2001). Here we focused on the long-term effects of high CO_2 and low pH on biogeochemical processes in deep-sea sediments of a naturally CO_2-vented hydrothermal system. CO_2 emitted from hydrothermal sediments can be considered as natural analogue of leakage associated with CCS in the deep-sea floor. The interaction of liquid CO_2 with microorganisms and geochemical processes in deep-sea sediments is difficult to study otherwise because of the high pressure and steep gradients associated with point sources of CO_2 (Liu et al., 2010).

A main question of risk assessment is as to the effect of high CO_2 emissions on the functioning of marine ecosystems. Microbial processes dominate the biogeochemistry of deep-sea sediments (Reeburgh, 1983; Jørgensen and Nelson, 2004; Jørgensen and Boetius, 2007). When not limited by thermodynamics or transport, their function may be limited by kinetics due to physicochemical conditions in the habitat. Microbial kinetics can be repressed by e.g. toxic compounds (strong oxidants, heavy metals, uncouplers of membrane potentials), and high temperatures (>121°C) can degrade enzymatic function. However, highly adapted microorganisms have been found to populate extreme environments of extremely low or high pH, pressure, salinity, radiation. Thus these parameters do not limit life in general (Stan-Lotter and Fendrihan, 2012), yet it remains unknown if main functions such as aerobic and anaerobic remineralization and respiration of matter, autotrophy, methane oxidation can be maintained. Here we aimed at testing the hypothesis that such biogeochemical processes mediated by microorganisms can function in CO_2 saturated porewater of deep-sea sediments.

The Yonaguni Knolls are submarine volcanoes located in the southwestern end of Okinawa Trough, a back-arc spreading center (Suzuki et al., 2008). The Yonaguni Knoll IV hydrothermal system is one of the few sites on Earth known where liquid CO_2 leaks through

thick layers of terrigenous sediments supplied from the Asian continent (Sibuet et al., 1987). It comprises a sedimentary valley surrounded by large piles of rock debris, enclosing a string of active hydrothermal vents sites. A previous characterization of the hydrothermal fluids indicated the generation of liquid CO_2 by subsurface phase separation (Konno et al., 2006; Suzuki et al., 2008). Besides their high CO_2 content, the hydrothermal fluids exhibit wide variation in gas composition including H_2, CH_4, H_2S and NH_4. Previous studies have focussed on the distribution of bacterial and archaeal communities of the Yonaguni Knoll IV hydrothermal sediments and overlying bottom waters (Inagaki et al., 2006; Nunoura et al., 2010; Neumann et al., in prep.; Yanagawa et al., submitted). Here, we have investigated the biogeochemistry of the CO_2 vented sediments focussing on the distribution of microbial aerobic and anaerobic respiration. Combining *in situ* and *ex situ* analytical techniques, we investigated rates of benthic oxygen consumption, sulphate reduction (SR) and anaerobic oxidation of methane (AOM), to cover the most important redox processes in the CO_2-impacted system. These data form the basis for a discussion of the effects of low pH and high CO_2 on the biogeochemistry in subsurface deep-sea sediments, which are considered as potential CO_2 storage sites.

Methods

Sampling location and methods

Samples were taken in February-March 2008 during the RV Sonne 196 expedition of the project SUMSUN ("Studies of marine CO_2-sequestration associated with a natural hydrothermal CO_2-system of the Northern West Pacific") (Rehder et al., 2008). The area was revisited for a few additional samples during the Japanese expedition „NT10-06 Leg 3", with RV Natsushima and ROV Hyper Dolphin (Jamstec) in April 2010 (Table 1).

The Yonaguni Knoll IV hydrothermal field located in the Okinawa Trough (24°50.7´N, 122°42.0´E; 1,380-1,382 m water depths) (Table 1) is a sedimentary basin covered by volcanic rocks in its north-eastern part. It hosts several large hydrothermal vent chimneys along a North-South transect, named Lion, Tiger, Swallow, Carp and Mosquito Vents (Konno et al., 2006; Suzuki et al., 2008). A sedimentary venting site characterized by a few holes in the seafloor emitting hot fluids was discovered on the southern end of the hydrothermal field and named "Abyss vent" (Inagaki et al., 2006; Suzuki et al., 2008; Nunoura et al., 2010). Sampling sites were first visually explored by the ROV Quest (MARUM, University Bremen). *In situ* measurements were carried out close by the "Abyss vent" (< 10 m distance), some 10-50 m away from the Abyss vent, next to "Swallow chimney" characterized by abundant sulphur pavements, and 1 km

southwest of the hydrothermal system for "Background" samples. Sediment samples from the uppermost sediment horizons (top 20–30 cm) were taken either with a video-guided multicoring device (MUC, 10 cm diameter cores), with push-cores (PCs, 8 cm diameter cores) collected with the manipulator of the ROV, or with gravity cores (GC, 10 cm diameter, up to 3 m sediment depth). All sampling instruments were equipped with a POSIDONIA (Ixsea SAS) positioning system for targeted sampling of the same habitats. After recovery, the tubes containing sediment samples were transferred to a cold room that was cooled to *in situ* temperature (4°C). Afterwards, the cores were vertically subsampled with small subcore tubes.

Porewater extraction and chemical analyses

Porewater was extracted using a low-pressure squeezer (argon at 1–5 bar) at approximately *in situ* temperature of 4°C in the ship's cold room. While squeezing, the porewater was filtered through 0.2 µm cellulose acetate Nuclepore filters and collected in vessels. Onboard, the collected porewater samples were analyzed for their content of dissolved NH_4^+, H_2S, PO_4^{3-}, SiO_4^{4-}, Cl^-, Fe^{2+} (samples taken under anaerobic conditions in the glove bag), total alkalinity (TA) and (*ex situ*) pH. In addition, sub-samples were taken and stored at 4°C for further shore-based analyses (concentration and $\delta^{13}C$ isotope ratio of CH_4 and CO_2, metal cations, SO_4^{2-}, Br^-, I^-). In addition, 5ml of wet sediment were collected for porosity and solid phase CNS (carbon, nitrogen, and sulphur) analyses, and an additional 5 ml of sediment was suspended in 20 ml of 1 N NaOH for methane headspace analyses. These samples were stored at room temperature. Analyses of the gaseous, dissolved and solid porewater species followed standard chemical procedures ((Grasshoff et al., 1999); for specifications http://www.ifm-geomar.de/index.php?id=1858&L=1).

Microprofiling

Microsensors for O_2, H_2S, and pH were made and used as described previously (Revsbech and Ward, 1983; Jeroschewski et al., 1996; de Beer et al., 1997). The tip diameters were ca 20 µm, the response time (t_{90}) less than 3 seconds. A temperature sensor was used (Pt100, UST Umweltsensortechnik GmbH, Thüringen, Germany), with a length of 18 cm, a shaft and tip diameter of 3 mm and length of sensing element of 1 cm and a response time of ca 5 seconds. Microsensors for redox potential (ORP) were made from Pt wire of 50 µm diameter, fused in a glass capillary, leaving a length of 100 µm Pt exposed as sensing surface. After mounting the Pt surface was cleaned in 6 M HNO_3 for 10 minutes, and rinsed with seawater before calibration in standard redox buffers. All sensors were calibrated after mounting on the profiler, as described previously (Gundersen and Jorgensen, 1990; Wenzhöfer and Glud, 2002). The sensors were

mounted on the bottom of the titanium housing within a distance of maximally 11 cm. The titanium housing, containing amplifiers and a computer for data-acquisition and motor control, could be moved vertically by a high precision motor, with a smallest stepsize of 12.5 μm.

The slope of the pH calibration was taken as during the calibration (at least 55 mV per pH unit), however, usually an off-set was observed in the bottom water, which was corrected for with the pH determined from Niskin bottle samples from 50 cm above the sediment surface. The O_2 sensors were 2-point calibrated *in situ*, by using the signal in the bottom water and in the anoxic zones of the sediments. The bottom water O_2 concentration was measured by Winkler titration from the same Niskin bottles. The detection limit was ca. 0.5 μmol L^{-1}. Total sulphide was calculated from the local pH and H_2S concentrations (Jeroschewski et al., 1996), using a pK value for sulphide of 6.92, as calculated from the local temperature and salinity (Millero et al., 1988). The H_2S sensors had detection limits for H_2S of ca. 1 μmol L^{-1}. Thus, the detection limit for total sulphide ($H_2S+HS^-+S^{2-}$) was ca. 10 μmol L^{-1} at pH 8 (bottom water and top sediment), and close to 1 μmol L^{-1} at pH 4.5 (in the deeper sediments). Care was taken that the sensor tips were placed in one horizontal plane with an inaccuracy of maximally 1 mm, so that the resulting profiles were aligned. Each step included a waiting time of 4 seconds before a reading was made, consisting of the average of 4 samplings in a time interval of 4 seconds. For profile analysis, the surface was taken as reference point (depth = 0), defined by the steepest slope of the oxygen profile (Gundersen and Jorgensen, 1990). Negative depths indicate positions above the surface. The profiler was pre-programmed to measure vertical profiles, with steps of 250 μm, over a depth of 17 cm. The profiler was positioned at the seafloor by a ROV. The profiling program was started by a switch on the profiler. After finishing a profile, the profiler could be repositioned and restarted. For use in the laboratory microsensors were mounted on a motor-driven micromanipulator.

Measurements of Total Benthic Oxygen uptake (TOU) and fluxes of other porewater constituents

The TOU was determined with a cylindrical benthic chamber module as previously described (Felden et al., 2010) operated by the ROV QUEST. Briefly, the stirred chamber (radius 9.5 cm) enclosed a seafloor area of 284 cm^2 together with 10–15 cm (equivalent to 4–6 liters) of overlying bottom water. A valve in the chamber lid ensured the release of overpressure while placing the chamber gently into the sediment avoiding any disturbance of the sediment surface. A temperature sensor was installed inside the chamber to measure heat flux during the incubation, as well as a reference temperature sensor outside of the chamber. An Aanderaa oxygen optode was mounted in the chamber lid to continuously monitor the oxygen concentration in the

enclosed water. A two-point calibration of the reading of the optode was performed. The reading at zero O_2 concentration was taken on board at *in situ* temperature. Values for the bottom water O_2 concentration were determined *in situ* at the seafloor. The respective oxygen concentration of the bottom water was determined by Winkler titration of water samples or from *in situ* readings with other oxygen sensors. In addition to the sensor readings, five water samples were taken with 50-ml syringes at pre-programmed time intervals to determine the fluxes of dissolved methane, silicate and ammonium. All chemical analyses followed standard procedures (Grasshoff et al., 1999), TOU and the other fluxes was calculated from the linear regressions of concentration vs. time over the area of the sediment enclosed by the chamber.

Methane oxidation and sulphate reduction rates

Sediment cores for measurements of methane oxidation and sulphate reduction were subsampled on board with three replicates per sample site. The rates were measured according to (Treude et al., 2005). Briefly, either 25-ml $^{14}CH_4$ (dissolved in water, 2.5 kBq) or 5-10 ml carrier-free $^{35}SO_4^{2-}$ (dissolved in water, 50 kBq) were injected in 1-cm intervals into the subcores (whole core injection method; (Jørgensen, 1978)). The sediment was incubated in the dark at *in situ* temperature for 12–48 h. After the incubation, the reaction was terminated by cutting 1 cm sections of the sediment cores into the respective fixative for further analysis in the home laboratory.

Results

Visual observations

The Yonaguni Knoll IV working area comprises an approximately 1 nautical mile wide sedimentary valley surrounded by rocky slopes in the west and northeast. A number of hydrothermal chimneys are located roughly on a line in NW-SE-direction (Supplementary Fig. 1). In addition to the mineral chimneys through which hot fluids > 300°C escape, we observed pavements of sulphur and amorphous SiO_2 precipitates, associated with holes and cracks in the underlying soft sediments from which hot fluids and liquid CO_2 emanated. The seafloor area surrounding the vents and seeps as well as sulphur pavements was characterized by an absence of bottom dwelling megafauna and lacked typical features of bioturbation, burrows and other traces of life. Only immediately at vents, dense accumulations of chemosynthetic fauna was observed, including the mussel *Bathymodiolus platifrons,* the shrimp *Alvinocaris longirostris,* and the crab *Shinkaia*

crosnieri. However, no mats of giant sulphide oxidizing bacteria typical for sulphide emitting hydrothermal vents and hydrocarbon seeps were observed on the rocks or the seafloor. The sedimentary seafloor was flat and featureless, i.e. not marked by pockmarks or ebullition holes away from the vents.

In the absence of such morphological indications for seepage, our sampling strategy was to follow a spatial gradient from the active sedimentary CO_2 leak "Abyss vent" and the sulphur pavement around "Swallow chimney". When pushcores were pulled out of the sediments by the ROV arm, it was observed several times by the ROV camera that large amounts of liquid CO_2 escaped from the sampling hole, in the form of droplets. This phenomenon was restricted to the < 20 m vicinity of the vents. The main investigated site was Abyss vent, marked by a couple of round openings of 10 cm diameter from which hot CO_2-rich water (> 60°C) was ejected. In addition some gravity corer (GC) and multi-corer samples (MUC) were obtained from Swallow Chimney, ca. 100 m north of Abyss vent. Swallow Chimney emitted relatively cold fluids dominated by liquid CO_2.

In situ bottom water measurements by a profiler mounted to a MUC equipped with a camera and towed at 2 m above the seafloor showed a reduced pH (< 7.45) and elevated CO_2 concentrations (> 0.03 mM) across the investigated area surrounding the vents. No free H_2S was detected in the bottom waters. In a distance of > 400 m away from the vents and seeps, a visual change of the seafloor features were recorded, including the appearance of large benthic megafauna (*Anthozoa* and *Echinodermata*), as well as numerous burrows, worm tubes and other biological features. Here, pH raised above 7.5 and CO_2 concentrations dropped to 0.025 mM in the bottom waters. We selected such an area about 1 km SW of the vents to study background biogeochemical processes unaffected by CO_2 leakage (Supplementary Fig. 1). Oxygen concentrations in the bottom water were low across the valley from the reference area to the CO_2-vented area (0.08 mM) and did not show a gradient towards the vents.

Microprofiles

In situ microprofiles of pH, oxygen, sulphide, redox and temperature at three different sites were measured in 2008 (Fig. 1a-c) and two in 2010 (Fig. 1d, e). The distances of the five profile sites from the Abyss vent were 0.5 m, 10 m, 50 m, 200 m and 1 km (the reference site). The oxygen sensors broke during the measurements at 0.5 m and 50 m from Abyss vent, all other microsensors functioned. The reference site showed an oxygen penetration of 8 mm, hardly a decrease in pH, sulphide was absent, the redox potential and temperature remained that of the seawater in the upper 12 cm (Fig. 1e). The oxygen concentration in the bottom water, measured with *in situ* sensors and retrieved water samples, was in 2008 and 2010 approximately 0.08 mM.

The sites closer to the vent showed increasing effects of the seepage. The oxygen penetration decreased to 5 mm at the site of 200 m distance from the vent (Fig. 1d) and 1 mm at < 10 m distance (Fig. 1b). Sulphide was detected in sediments at 50 m distance (Fig. 1c) and the sulphide profiles became increasingly steeper closer to the vent. Remarkably, they were almost perfectly linear, indicating high transport rates, but relatively low sulphide production in the upper 5 cm. The redox potential decreased rapidly to negative values at the sites closer than 50 m from the vent. The CO_2 sensors showed a drastic increase in signal with depth, however, the sensors could not follow the extreme CO_2 concentrations and the signals could not be used for quantification. The pH profiles became steeper close to the vent, showing a pH of ca 4.5 below 6 cm bsf, at sites closer than 50 m from the vent.

Accordingly, the fluxes of oxygen and sulphide increased when approaching the vents (Table 1). At the site 10 m from Abyss vent the diffusive oxygen flux was almost ten times the sulphide flux, thus sulphide was not the only electron donor fuelling microbial respiration in the sediments. Porewater profiles showed elevated concentrations of sulphide, methane, ammonium (Fig. 2, 3), manganese and iron, which may all enhance microbial oxygen consumption at the seep site. Their concentrations could not be quantified due to the outgassing upon retrieval on deck, but all were highly elevated compared to the background site.

Also, the microprofiles measured in retrieved cores taken near the vent sites were drastically changed due to outgassing. The sulphide was mostly stripped from the sediments into the water column in the cores, and after retrieval the pH values in the sediments were around 7 from surface to the bottom of the core (data not shown).

Geochemistry

As described above, several of the MUC, PC and GC cores sampled close to the vent site were extremely gaseous and were outgassing for 10 to 20 minutes after arrival on deck. This outgassing induced artefacts, e.g. it reduced the amount of CO_2, methane and sulphide in the porewaters, and increased the pH in the sediments. Upon retrieval, some cores precipitated a white amorphous silicate phase into the bottom water overlying the cores.

Yet, the geochemistry still showed substantial effects of CO_2 leakage and hydrothermalism in sediments retrieved close to the vent sites (Figs. 2 and 3). Generally, CO_2 seepage in association with elevated transport of hydrothermal fluids was accompanied by steep porewater profiles and high total alkalinity, low sulphate, and elevated concentrations of sulphide, silicate, ammonium and methane. The subsurface porewater retrieved from the vents showed increased concentrations of methane (> 1 mM, 3 out of 5 sites sampled), sulphide (> 2 mM) and total alkalinity (> 60 meq/l) (Figs. 3 and 4). Pore fluids around the vent sites were also strongly

enriched in dissolved silicate (up to 2 mM) but depleted in sulphate, which is typical for hydrothermal fluids. The sediments around Swallow Chimney were extensively covered with volcanic rock debris and sediments could only be recovered by gravity coring. Generally, the collected porewaters exhibited also very high total alkalinity, elevated sulphide and dissolved silicate (Fig. 4), but little methane. Another difference compared to Abyss vent included an extreme enrichment in NH_4, which is potentially produced by high-temperature degradation of organic matter at large sediment depths. Secondly, the chlorinity was lower (< 450 mM) than at the reference site and Abyss vent (~545 mM). This could be due to gas hydrate formation but also originate from a Cl^- depleted fluid rising from greater depth. Concentrations of K^+, Na^+, B, Li^+, Mg^{2+}, and Ca^{2+} differed from seawater and were typical for sediment-hosted hydrothermal vents (data not shown). More reactive species like PO_4^{-3}, I^- and Mn^{2+} were higher than in the seawater, but the concentration distributions were chaotic and showed no clear correlation with seepage. Generally, the porewater profiles were surprisingly heterogeneous, indicating a complex spatial scaling of subsurface transport of hydrothermal fluids and liquid CO_2, as well as intense reactions with the surface sediments.

Microbial respiration rates and fluxes of porewater constituents

The benthic chamber measurements generally demonstrated decreasing effects of seepage with distance from the seeps (Table 3). The total oxygen consumption was an order of magnitude higher at and near the vent sites compared to the reference. Ammonium fluxes gradually decreased with distance. Methane, DIC and Si effluxes were generally much higher near the vents than the reference, but, remarkably, lower at 10 m distance than at 25 m distance. This irregular trend underlines the heterogeneity of the area. None of the sites investigated here with chamber measurements leaked sulphide to the overlying bottom water.

Also, the rates of sulphate reduction (SR) and anaerobic methane oxidation (AOM) were highly heterogeneous (Fig. 5, 6 and 7). Areal rates in cores, taken less than 40 cm apart, could differ an order of magnitude in SR and AOM rates, as well as in methane concentrations (the standard deviations are not shown for clarity). Whereas the variability defeats accurate quantification at limited spatial replication, trends can be seen. Generally both SR and AOM rates were higher close to the vents than at the distant sites > 20 m, thus constituents of the hydrothermal fluids fuelled these microbial processes despite the high CO_2 concentrations and the low pH associated with the vents. However, measurable rates of SR and AOM were limited to the upper 7-15 cm in retrieved cores (Fig. 7). Accordingly, AOM or SR could not be detected in gravity cores from Abyss vent and Swallow chimney, which were sampled in 50 cm intervals, starting at 50 cm bsf (data not shown). Integrated SR rates were 6 x 10^{-8} mol m^{-2} s^{-1} for all sites

with high CO_2 leakage (reaching 60 meq L^{-1} total alkalinity > 15 cm sediment depth) around Abyss vent and Swallow chimney; 0.07 x 10^{-8} mol m^{-2} s^{-1} for the sites with low CO_2 leakage and were negligible at the reference site. The average of all areal rates of AOM (0.8 mol x 10^{-8} m^{-2} s^{-1}) in CO_2 vented sediments near seeps was almost a magnitude lower than the SR rates. AOM was below detection limit away from the vents and at the reference site. This suggests that most SR is driven by other e-donors provided in the hydrothermal vent fluids. Indeed, in the core with the highest SR (MUC 10) little methane was detected. The sulphide fluxes, measured with microsensors (Table 2), were close to the areal SR rates at the vent. But at more remote sites the diffusive fluxes were much higher than the areal SR rates, indicating that most of the sulphide diffusing to the sediment surface does not originate from SR in the upper sediments but was transported with hydrothermal fluids. Indeed, the highest sulphide concentration was found in a deep GC core.

Discussion

The occurrence of liquid CO_2 in a natural marine setting has been firstly observed in the Jade hydrothermal field, Okinawa Trough (Sakai et al., 1990), and was subsequently studied also at other backarc systems at water depths between 1200 and 1700 m, such as the NW Eifuku hydrothermal field in the Mariana Arc, and the Yonaguni Knoll IV hydrothermal system (Okinawa Trough), with respect to geology, hydrochemistry and biology of these extreme environments (Inagaki et al., 2006; Konno et al., 2006; Lupton et al., 2006; Suzuki et al., 2008; Tunnicliffe et al., 2009; Nunoura et al., 2010). This is the first study of the effect of hydrothermally-induced liquid and supercritical CO_2 venting on the biogeochemistry of deep-sea sediments.

We observed a large heterogeneity of the porewater geochemistry, transport processes and rates, which may present clues to the complex geology of the area. CO_2 is brought in supercritical form from large depth to the sediment surface where it condenses to liquid CO_2 and CO_2 hydrates (Konno et al., 2006). Spontaneous release of liquid CO_2 was only observed from the rocky vent chimneys, but not from the sedimentary seafloor. Here, emission of liquid CO_2 was caused only by disturbance of the surface seabed, e.g. by penetrating the top 20 cm with a temperature probe or with sampling cores. This indicates lateral and upward migration of CO_2 rich fluids capped by cold surface sediments and porewaters as well as by mineral precipitates (Inagaki et al. 2006).

Several sites in < 25 m distance to the vents studied here leaked substantial amounts of dissolved CO_2 with the upward rising fluids. Thus, the CO_2 is present in the sediments in supercritical phase, as hydrates and as liquid, as well as dissolved in porewater. The supercritical and liquid CO_2 phase is less dense than porewater whereas the CO_2 hydrates have a higher density (and hence will not float up) making the system meta-stable. Supercritical- and liquid CO_2 may finger upwards through the sediments, and may tend to creep inside the sediments from the subsurface hydrothermal reservoirs laterally through the valley. Finally, convection cells will develop in sediments very close to the seeps, where upward flow close to the hot seeps is compensated by penetration of seawater at some distance around the seeps. The diameter of such cells is thought to be several meters (Haeckel and Wallmann, 2008). Detailed observations on circulation cells in this area cannot be scope of this paper, and will be discussed separately.

In addition, our results indicate that the fluid composition is highly variable between vents and seeping sediment sites, especially in the methane and ammonium content. Despite the local variability of heat, fluid, CO_2 and energy transport, it is obvious that CO_2 leakage around the

vent systems reduces the pH of the sedimentary environment, and co-migrates with methane-, sulphide- and ammonium-rich hydrothermal fluid. The Yonaguni Knoll IV hydrothermal fluids also contain hydrogen (Konno et al. 2008). These potential electron donors for microbial reactions are products of thermal degradation of organic matter. In addition, alkalinity is increased by CO_2-induced weathering of silicates (Wallmann et al., 2008). It was shown that in sediments of productive continental margins the CO_2 produced during methanogenesis is almost completely converted into HCO_3^- via reactive silicates + CO_2 => clays + HCO_3^- + SiO_2 + metal cations (thus alkalinity) and that this is attributed to the weathering of reactive silicates in anoxic sediments (Wallmann et al., 2008). In the sediments at Yonaguni Knoll IV, CO_2 concentrations are orders of magnitude higher as indicated by the low pH values observed (pH < 6), than in the sediments investigated by Wallmann et al. (2008). As dissolved CO_2 is the primary substrate this process with ensuing alkalinity release will be drastically enhanced at the extremely high CO_2 levels in the seep area, as confirmed by the high subsurface concentrations of SiO_2.

The gravity cores showed that methane and sulphide are present down to several meters bsf, thus probably originating from hydrothermal reactions at larger depth. The interfacial fluxes of sulphide were much higher than the SR rates, thus most of the sulphide diffusion to the seafloor originates from hydrothermal processes, and microbial production by SR is only a small fraction of the sulphide budget. This is also clear from the almost linear sulphide profiles, which indicate the dominance of transport, and the absence of significant sulphide conversions. Interestingly, sulphate was consistently present, even at larger depths in the GCs and even at the hydrothermally vented sites (Fig. 4).

Although methane, numerous other potential electron donors, and sulphate are present deep in the sediments, no sulphate reduction was detected below 15 cm depth, and only relatively low SR and AOM rates were measured at the vents compared to other methane-rich hydrothermal vents and seeps not impacted by CO_2 leakage (Felden et al., 2010; Biddle et al., 2012). An important question is as to what restricts microbial anaerobic respiration to the upper 7-15 cm, despite the availability of chemical energy. Experiments carried out with sediments collected from the Abyss vent and Swallow Chimney showed the potential for SR up to temperatures of 60°C (Yanagawa et al., submitted). From extrapolation of the interfacial gradients based on the temperature profiles measured with the microprofiler, we estimate that 60°C is reached at 2.5 m, 0.8 m and 0.6 m bsf at the locations 50 m, 10 m and 0.5 m from Abyss vent, thus lethal temperatures are only reached far below the upper 15 cm horizon and should not limit microbial activity here. Then, possibly, the low pH could limit AOM and SR. However, the experiments of Yanagawa et al. (submitted) showed that SR was not inhibited at a pH value of 4.5 or even at pH 3. Importantly, a main difference of the *ex situ* incubation conditions with

those *in situ* are the CO_2 levels to which the microbial communities are exposed. It should be noted that at the low ambient pH, well below the pK_1, almost all DIC will be in the form of CO_2. *In situ*, at pressures of > 130 atmospheres, dissolved CO_2 concentrations in equilibrium with CO_2 hydrate, liquid, or supercritical CO_2 may reach 1000-1700 mM (Duan and Sun, 2003; Duan et al., 2006) much higher than to the < 0.02 mM in seawater at atmospheric pressure in equilibrium with the atmosphere.

Liquid and supercritical CO_2 are highly powerful solvents of apolar compounds, thus may dissolve cytoplasmic membranes. As droplets of liquid CO_2 were consistently observed upon retraction of pushcores by the ROV arm around Abyss vent, liquid CO_2 reaches probably close to 15-30 cm bsf. Deeper in the sediments, where the temperature exceeds the critical point of CO_2 (31°C, 74 bar), supercritical CO_2 is present. The liquid and supercritical CO_2 only occupy a fraction of porespace, as many ionic species were found in concentrations common for seawater. CO_2 may be present in the form of droplets, or bubbles that are retained in the low-permeability sediments. However, also in the porewater near zones of liquid CO_2 life will be unlikely. The extremely high CO_2 levels may well be toxic, as protonated CO_2 (free H_2CO_3) will act as uncoupler of membrane potentials. Most probably, microbial activity is completely repressed below 15 cm sediment depth, where dissolved CO_2 can be considered to be close to equilibrium with liquid and hydrated CO_2. At 1 atm, 0.259% of the CO_2 is hydrated as H_2CO_3 (Wissbrun et al., 1954) this fraction will increase to 0.36% due to the pressure of 130 atm at the Yonaguni seafloor (Ellis, 1959). In surface seawater in equilibrium with the atmosphere (CO_2 < 0.02 mM) the concentration of H_2CO_3 is less than 0.05 μM, therefore the substance is mostly disregarded. However, in the CO_2 saturated Yonaguni sediments the concentration of dissolved CO_2 will reach 1000-1700 mM and H_2CO_3 will be proportionally high. This apolar compound can pass through membranes, dissipate the proton motive force and disrupt the cytoplasmic pH homeostasis. Uncouplers lead to full PMF loss at < 0.1 μM to 10 μM concentrations (Terada, 1990) while ambient H_2CO_3 concentrations near liquid CO_2 are in the order of 3 mM. Whereas microbial life can adapt to many well known parameters that make a habitat 'extreme' such as high temperature, low pH and high pressure, such uncoupling processes disturb the basic energy needs of the cells. Against such stresses adaptations are impossible. It may well be that microbial cells and preserved DNA are found in deeper sediments. These cells will be, however, inactive or dead. Further experimental work will be needed for testing the hypothesis of uncoupling effects of extremely high CO_2 levels in high pressure reactors.

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Figures and Tables

Figure 1 *In situ* microprofiles of oxygen, pH, sulphide, ORP (redox potential) and temperature, measured during two cruises in 2008 (Figs. a-c) and in 2010 (Figs d and e), at different distances to the Abyss vent. The distances are indicated in the plots, the reference was taken approximately 1 km southwest of the vents. The positions can be found in Table 1. All gradients clearly increase towards the vent.

Figure 2 Porewater chemistry profiles obtained from sediments retrieved by the MUC. The positions are given in Table 1. The reference data are indicated with black symbols, obtained from a core approximately 1 km southwest of the vents. The other cores are from the Abyss vent area. MUC 10 is the only core among all sediments sampled that showed sulphate depletion, due to a high proportion of sulphate-free hydrothermal fluids.

Figure 3 Porewater chemistry profiles obtained from sediments retrieved by the PC. The positions are given in Table 2. The reference data are indicated with black symbols, obtained from a core approximately 1 km southwest of the vents. PC 1, 5, 24 and 33 were taken within 2 m from Abyss vent, PC 11 about 10 m off the vent, and PC 14 about 50 m NE of the vent, and PC 2 2010 approximately 200 m east of the vent.

Figure 4 Porewater chemistry profiles obtained from sediments retrieved by the PC. The positions are given in Table 1. The reference data are indicated with black symbols, obtained from a core approximately 1 km southwest of the vents. GC 9 was obtained 50 m south of Abyss vent GC 1 and 3 were taken near Swallow Chimney.

Figure 5. AOM rates measured using $^{14}CH_4$ tracer on retrieved MUC and PC cores. The positions are given in Table 1. MUC 3 (black symbols) was obtained approximately 200 m SE of the Abyss vent. MUC 8 was near Swallow Chimney, MUC 10, PC 23 and 24 were taken near Abyss vent. Although the scatter was large and rates low despite relatively high methane and sulphate concentrations, AOM activity was detectable in the upper 7 cm.

Figure 6 SR rates measured using the $^{45}SO_4^{2-}$ tracer method on retrieved cores. The positions are given in Table 2. MUC 3 was the same as in Fig. 5; PC 1 2010 was taken about 1 km south of Abyss vent. PC 21 and 29, and MUC 10 were from the Abyss vent area, MUC 8 from

Swallow Chimney, and PC 2 2010 at approximately 200 m east from Abyss vent. The SR activities are restricted to the upper 10-13 cm.

Figure 7 SR rates measured using the $^{45}SO_4^{2-}$ tracer method, *in situ*. The profile is the average of data from 3 cores. The deployment location was approximately 10 m NW of Abyss vent. As reference (black symbols) *ex situ* data are presented from a PC taken about 1 km south of Abyss vent. The SR activities are only significant above 7 cm bsf.

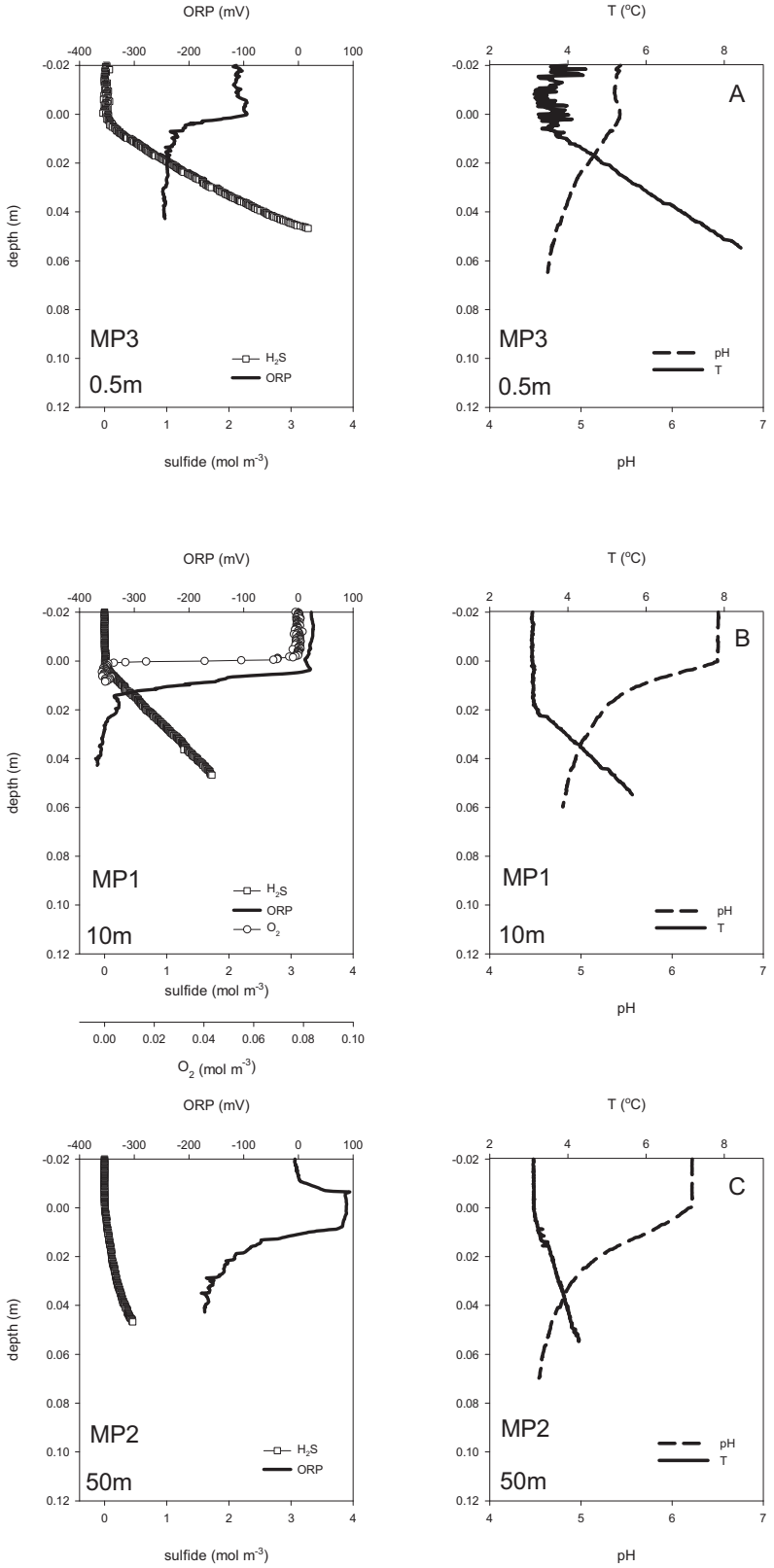

ORP (mV)
T (ºC)
depth (m)
MP3
0.5m
H2S
ORP
pH
T
A
sulfide (mol m-3)
pH
MP1
10m
O2
B
O2 (mol m-3)
MP2
50m
C

Figure 1

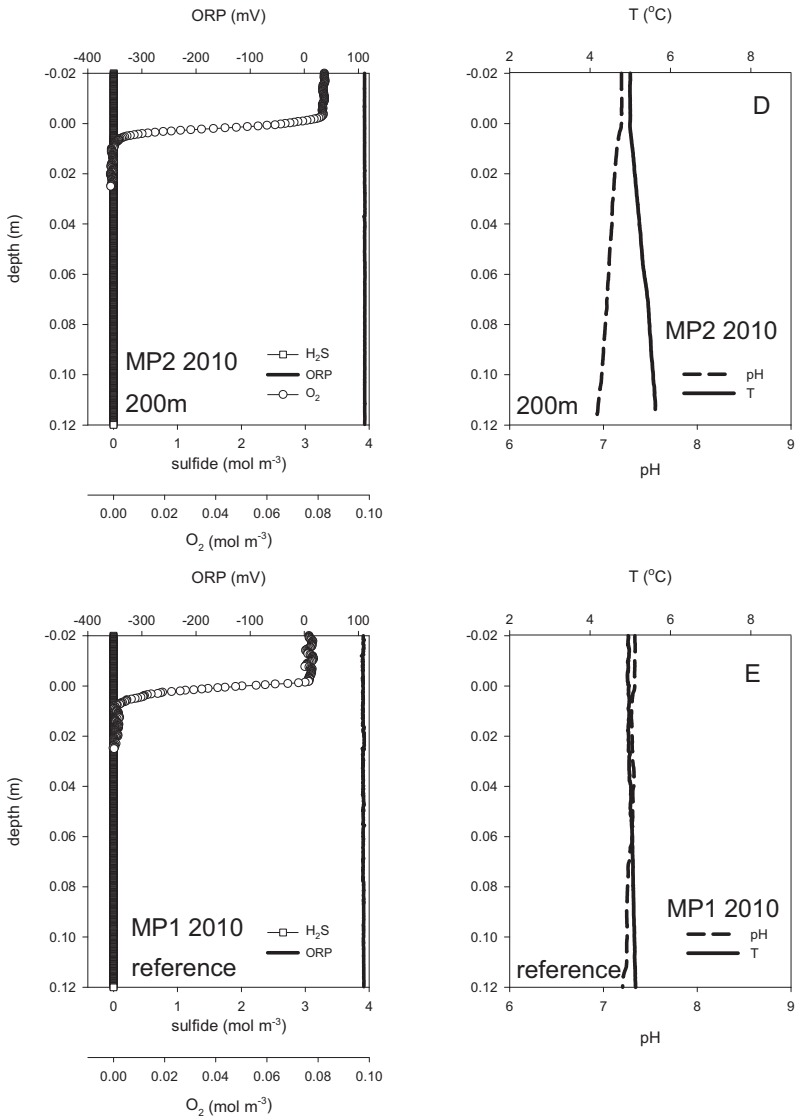

ORP (mV)
T (°C)
depth (m)
MP2 2010
200m
H2S
ORP
O2
sulfide (mol m-3)
O2 (mol m-3)
D
pH
T
MP1 2010
reference
E

Figure 1

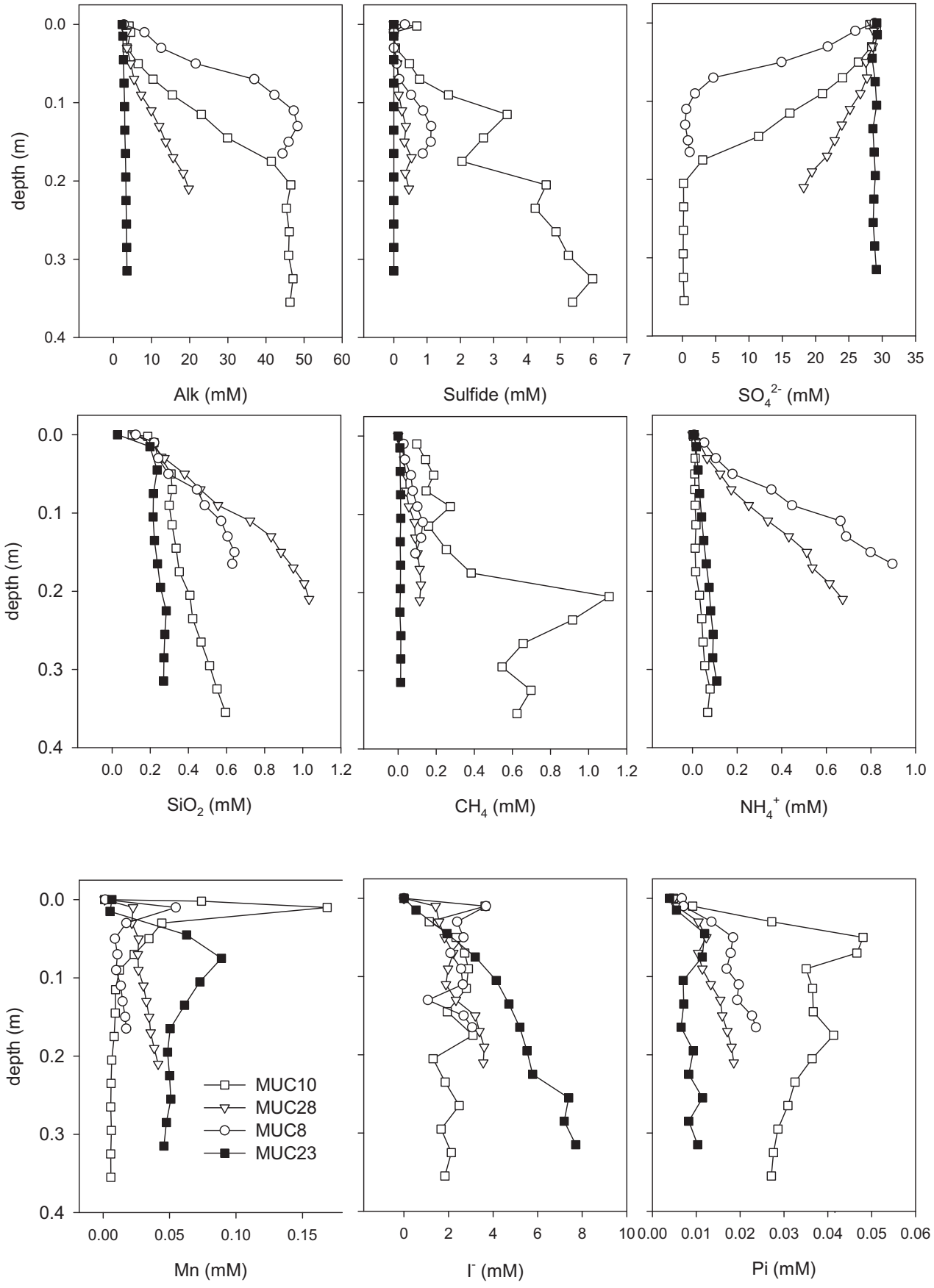

depth (m)
Alk (mM)
Sulfide (mM)
SO_4^{2-} (mM)
SiO_2 (mM)
CH_4 (mM)
NH_4^+ (mM)
Mn (mM)
I^- (mM)
Pi (mM)
MUC10
MUC28
MUC8
MUC23

Figure 2

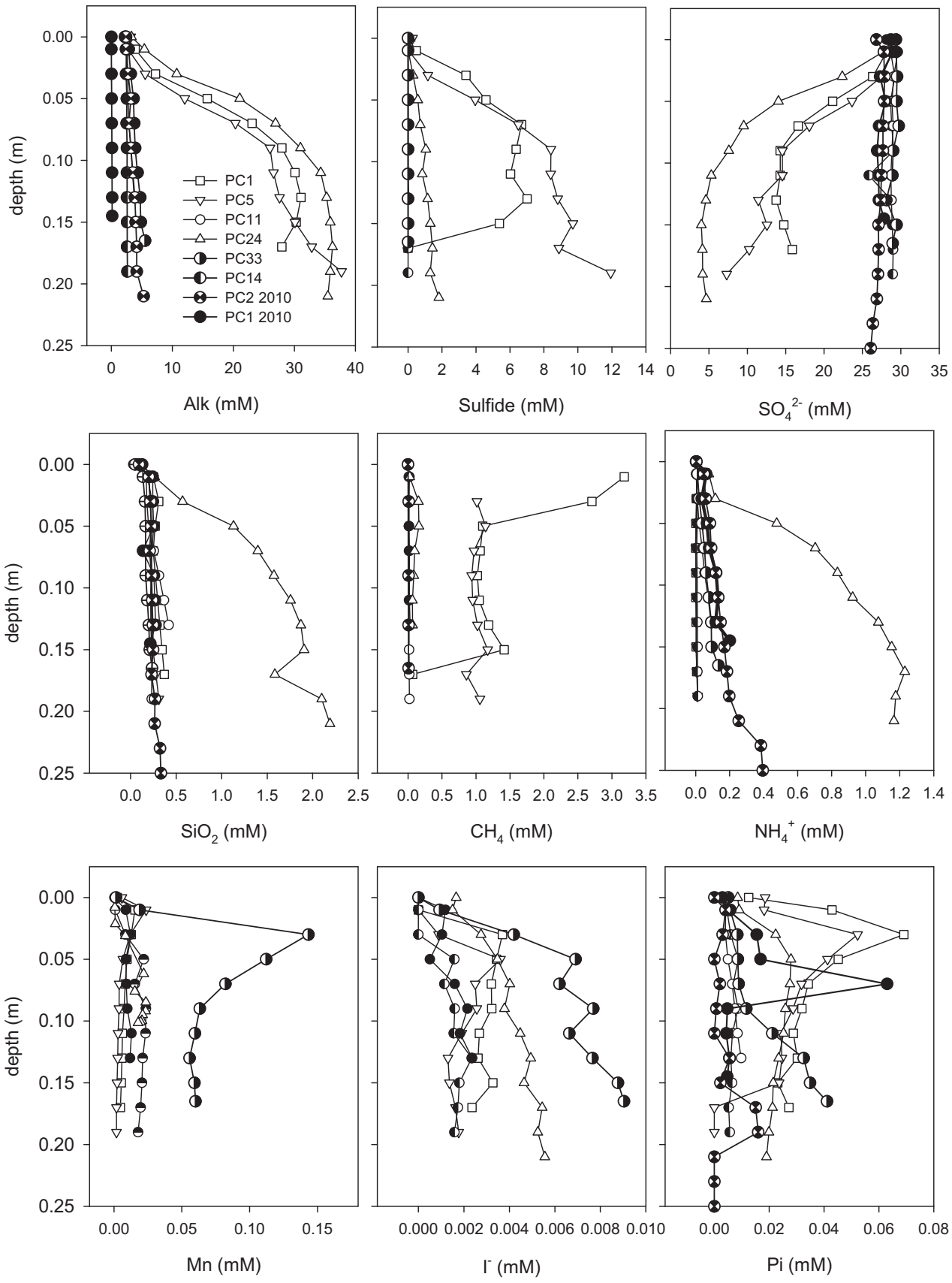

depth (m)
0.00
0.05
0.10
0.15
0.20
0.25
PC1
PC5
PC11
PC24
PC33
PC14
PC2 2010
PC1 2010
0 10 20 30 40
Alk (mM)
0 2 4 6 8 10 12 14
Sulfide (mM)
0 5 10 15 20 25 30 35
SO_4^{2-} (mM)
0.0 0.5 1.0 1.5 2.0 2.5
SiO_2 (mM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
CH_4 (mM)
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
NH_4^+ (mM)
0.00 0.05 0.10 0.15
Mn (mM)
0.000 0.002 0.004 0.006 0.008 0.010
I^- (mM)
0.00 0.02 0.04 0.06 0.08
Pi (mM)

Figure 3

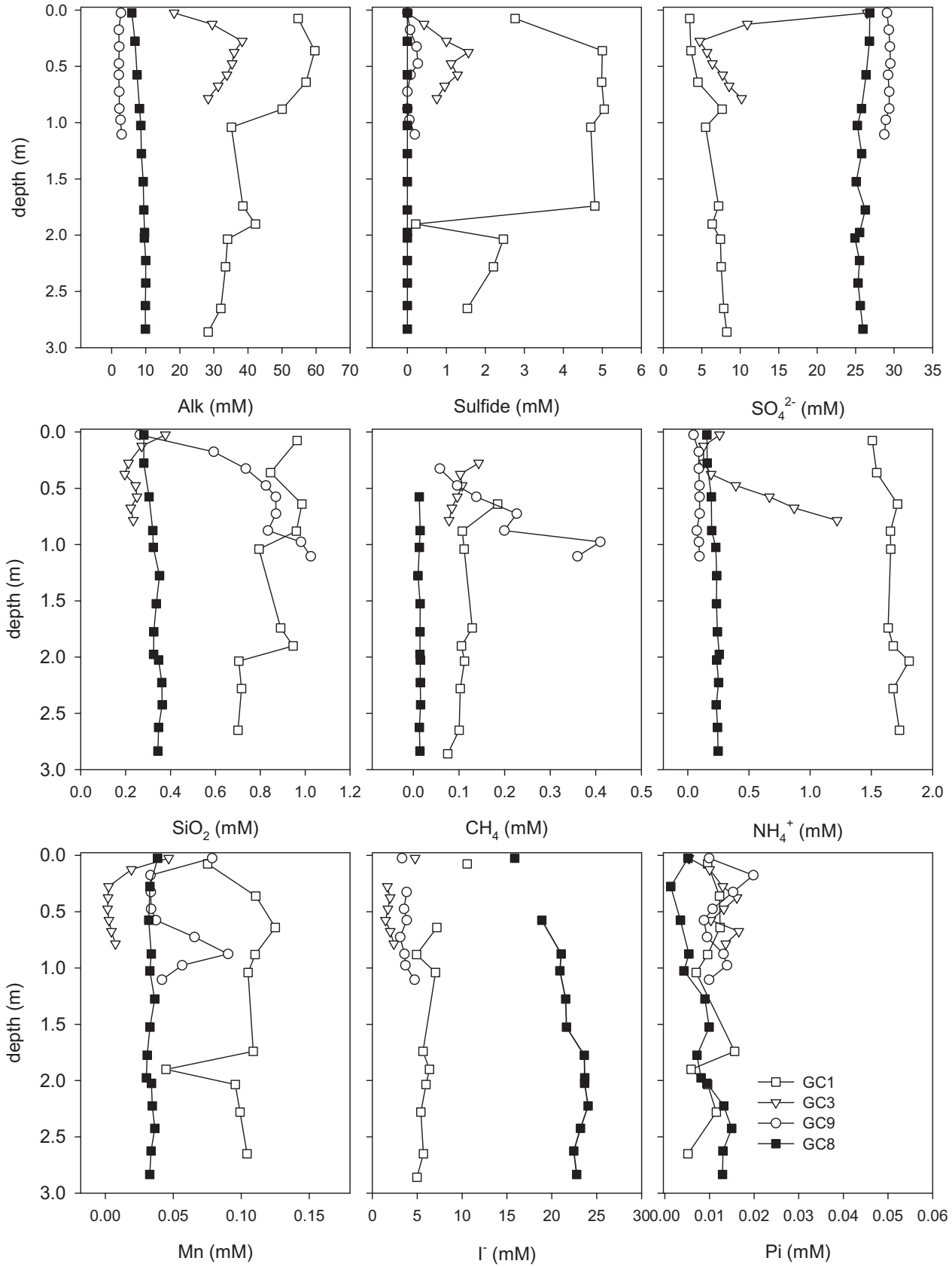

depth (m)
Alk (mM)
Sulfide (mM)
SO4 2- (mM)
SiO2 (mM)
CH4 (mM)
NH4 + (mM)
Mn (mM)
I- (mM)
Pi (mM)
GC1
GC3
GC9
GC8

Figure 4

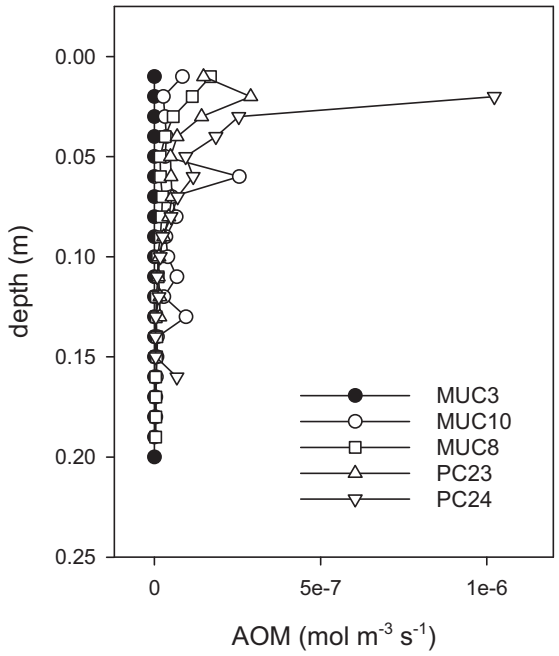


Figure 5

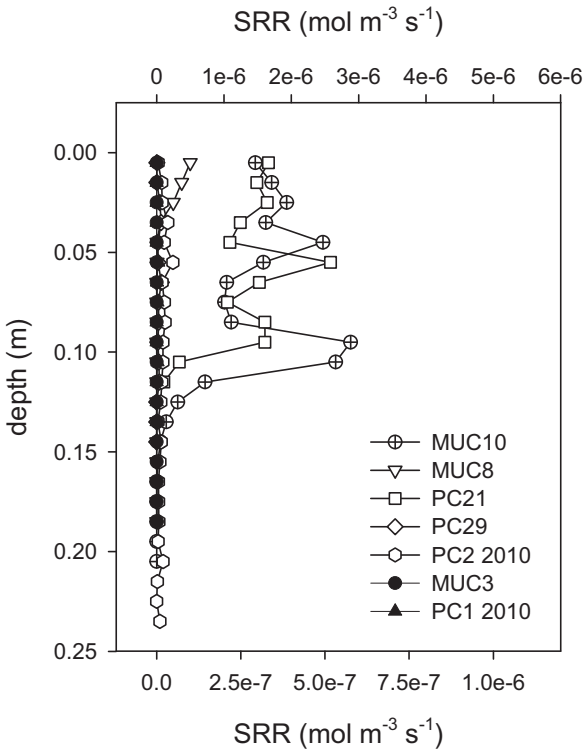


Figure 6

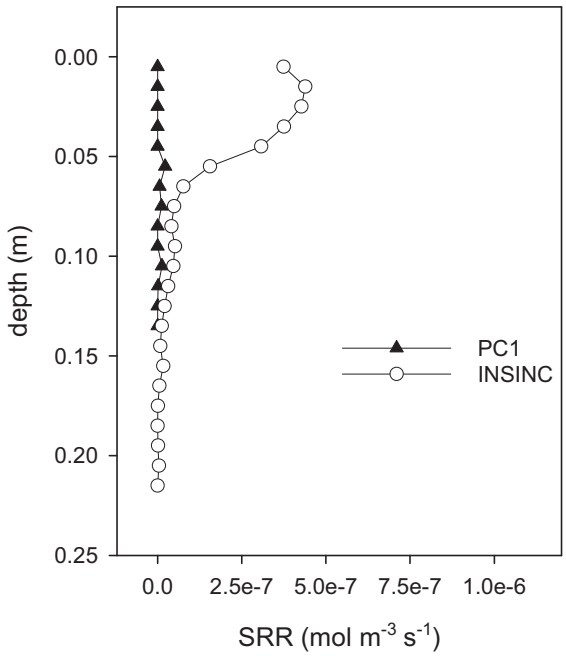


Figure 7

Table 1 Station list with sampling dates and locations.
(http://www.pangaea.de/ddi?retr=events/Sonne/SO196.retr&conf=events/CruiseReportHTML.conf&title=Station+list+of+cruise+SO196&format=html)

cruise name	device type	no	date	N	E	depth (m)
SO196	MP	1	13.03.2008	24 50.781	122 42.0307	1382
SO196	MP	2	13.03.2008	24 50.782	122 42.0251	1381
SO196	MP	3	13.03.2008	24 50.826	122 42.0842	1365
SO196	PC	1	13.03.2008	24 50.781	122 42.0273	1382
SO196	PC	5	13.03.2008	24 50.781	122 42.0273	1382
SO196	PC	21	13.03.2008	24 50.781	122 42.0273	1382
SO196	PC	23	13.03.2008	24 50.781	122 42.0273	1382
SO196	PC	11	16.03.2008	24 50.783	122 42.0347	1380
SO196	PC	14	16.03.2008	24 50.783	122 42.0360	1380
SO196	PC	24	16.03.2008	24 50.780	122 42.0285	1383
SO196	PC	29	16.03.2008	24 50.784	122 42.0365	1380
SO196	PC	33	16.03.2008	24 50.7820	122 42.0290	1383
SO196	MUC	3	08.03.2008	24 50.827	122 42.086	1372
SO196	MUC	8	08.03.2008	24 50.838	122 41.992	1362
SO196	MUC	10	09.03.2008	24 50.791	122 42.020	1392
SO196	MUC	23	17.03.2008	24 50.355	122 41.736	1324
SO196	MUC	28	21.03.2008	24 50.781	122 42.028	1394
SO196	GC	1	12.03.2008	24 50.841	122 42.003	1382
SO196	GC	3	15.03.2008	24 50.851	122 42.019	1383
SO196	GC	8	20.03.2008	24 50.341	122 41.726	1320
SO196	GC	9	20.03.2008	24 50.774	122 42.043	1399
SO196	BC	203, 1	16.03.2008	24 50.7831	122 42.0303	1380
SO196	BC	203, 2	16.03.2008	24 50.7806	122 42.0266	1382
SO196	BC	206, 1	21.03.2008	24 50.4789	122 41.8599	1347
SO196	BC	206, 2	21.03.2008	24 50.7757	122 42.0383	1381
NT10_06	MP	1	17.04.2010	24 50.515	122 41.882	1355
NT10_06	MP	2	17.04.2010	24 50.790	122 42.051	1379
NT10_06	PC	1	17.04.2010	24 50.515	122 41.882	1355

MP= microprofiler, PC=pushcore, MUC=multicore, GC=gravity core, BC=benthic chamber

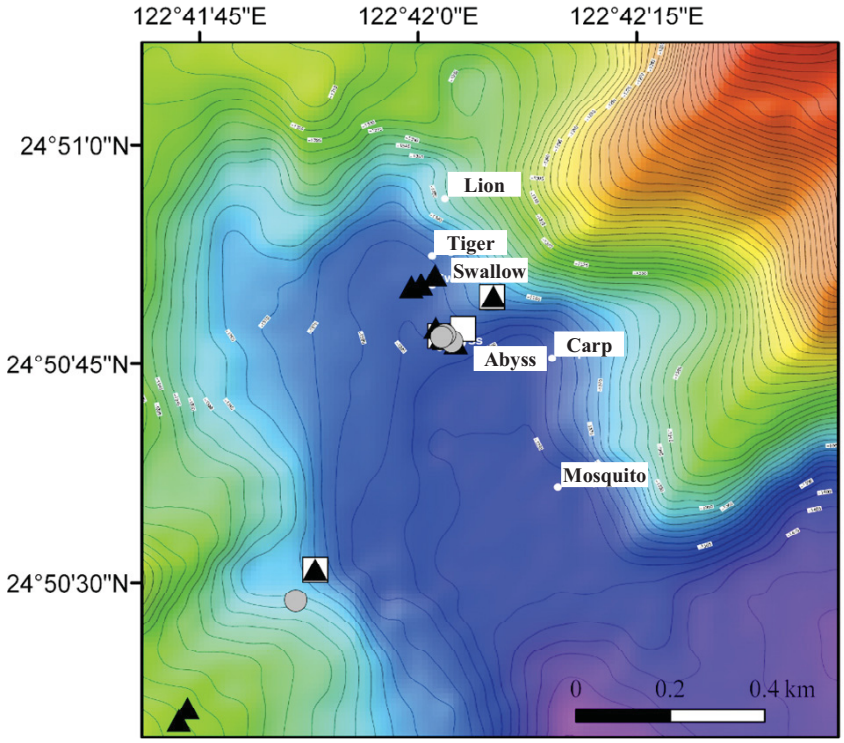
Table 2. Fluxes of oxygen and sulphide near the sediment-water interface, calculated from the microprofiles using Fick's law of diffusion, the average integrated areal SR and AOM measured in MUC and PC taken in and near seeps. The distances are between the microprofiler and the Abyss vent. n.d. not determined.

Distance to Abyss vent:	O_2 (mol m^{-2} s^{-1})	Sulphide (mol m^{-2} s^{-1})	SR (mol m^{-2} s^{-1})	AOM (mol m^{-2} s^{-1})
Reference 1km (MP1 2010)	-2×10^{-8}	0	0.002×10^{-8}	0
200m (MP2 2010)	-2.3×10^{-8}	0	n.d.	n.d.
50m (MP2)	#	1.6×10^{-8}	0.07×10^{-8}	0
10m (MP1)	-2.1×10^{-7}	3.5×10^{-8}		
0.5m (MP3)	#	5.8×10^{-8}	6×10^{-8}	0.07×10^{-8}

Table 3. *In situ* fluxes obtained from the benthic chambers near Abyss vent. n.d. not determined

Distance to vent:	O_2 (mol m^{-2} s^{-1})	CH_4 (mol m^{-2} s^{-1})	DIC (mol m^{-2} s^{-1})	Si (mol m^{-2} s^{-1})	NH_3 (mol m^{-2} s^{-1})	Dive, Chamber number
0.5 m	-4.6 x 10^{-8}	4.6 x 10^{-8}	300 x 10^{-8}	14 x 10^{-8}	12 x 10^{-8}	203, 2
10 m	n.d.	0.07 x 10^{-8}	30 x 10^{-8}	5.8 x 10^{-8}	5.8 x 10^{-8}	203, 1
25 m	-4.6 x 10^{-8}	0.6 x 10^{-8}	115 x 10^{-8}	52 x 10-8	0.7 x 10^{-8}	206, 2
Reference (1km)	-0.9 x 10^{-8}	0	n.d.	0	0	206, 1

Supplementary Information



Supplementary Figure 1 Map of the Yonaguni Knoll IV hydrothermal system with sampling sites. Symbols are black triangles for sediment samples, white squares for *in situ* microprofiler measurements and grey circles for *in situ* flux measurements with the benthic chamber.

Chapter II

Effects of subsurface CO_2 leakage on deep-sea bacterial communities of the Yonaguni Knoll IV hydrothermal sediments (Okinawa Trough, 1350 m)

Effects of subsurface CO_2 leakage on deep-sea bacterial communities of the Yonaguni Knoll IV hydrothermal sediments (Okinawa Trough, 1350 m)

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Abstract

The sedimentary hydrothermal vent system of Yonaguni Knoll IV (1350 m, Southern Okinawa Trough) is a natural laboratory to study the effects of CO_2 leakage from the seafloor on the diversity and community composition of benthic deep-sea bacteria. This study focused on high CO_2 seepage through deep-sea sediments, and the effects on the diversity and structure of bacterial communities inhabiting the surface seafloor (top 0-10 cm). The distribution of bacterial populations was compared across different locations ranging from strongly CO_2 vented sites to background conditions, within an area of 3 km^2. The sampling sites grouped into three categories of CO_2 impact, 1) high CO_2 seepage, characterized by pH as low as 5-6 at the surface seafloor and strong alteration of the liquid and solid phase of the sediment matrix; 2) low-intermediate CO_2 seepage mostly confined to the subsurface (> 5 cm); and 3) background conditions, indicated by absence of CO_2 venting. We determined changes in benthic bacterial community composition across this natural gradient in CO_2 leakage and pH using Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 454 massively parallel tag sequencing as fingerprinting techniques. CO_2 impact on bacterial diversity was observed from phylum to genus and individual tag sequence level. Bacterial richness and community composition changed significantly between the background and CO_2 impacted sites. No negative CO_2 effect on cell densities and microbial respiration rates was observed in the surface sediments, indicating selection of adapted bacterial types, which were able to profit from the co-migrating chemical energy in the vent fluids. Members of the acidophilic *Deltaproteobacteria* and hydrothermal vent *Epsilonproteobacteria* increased in sequence abundance with high CO_2 leakage. In contrast, the sequence abundance of *Alphaproteobacteria* and *Planctomycetes* decreased, indicating that some deep-sea bacterial taxa are repressed by CO_2 venting.

Keywords: benthic bacteria / bacterial diversity / hydrothermal vents / CO_2 leakage / pH effects / acidophiles

Introduction

The ocean absorbs 25% of the CO_2 emissions from human activities, and hence is an important CO_2 sink (Sabine et al. 2004). As this atmospheric CO_2 dissolves in the surface seawater, it forms carbonic acid, decreases the concentration of carbonate ions, lowers the seawater pH and hence causes "ocean acidification". Effects of seawater acidification by increasing concentrations of carbonic acid on marine life are diverse and include calcification and growth rates of organisms (Orr et al. 2005; Fabry et al. 2008; Kuffner et al. 2008; Doney et al. 2009), dissolution of the shells of calcifying organisms (Riebesell et al. 2000; Tunnicliffe et al. 2009), changes of benthic community structure (Hall-Spencer et al. 2008; Widdicombe and Spicer 2008), larval survival (Dupont et al. 2008), among many other types of responses.

Among the proposed measures against increasing CO_2 emissions to the atmosphere - and its many unwanted effects such as long-term ocean acidification - are mitigation strategies such as the Carbon Capture and Storage (CCS) technique, including offshore submarine CO_2 injection into the seabed. At first sight it seems an attractive idea to store excess CO_2 from industrial processes back into depleted oil- and gas reservoirs to balance carbon emission. It has been estimated that such submarine reservoirs could store a few million to billion tons of CO_2, depending on reservoir size and the geological formation. However, risks of this technique include amongst others the leakage of highly concentrated CO_2 from subsurface reservoirs to the seafloor and hydrosphere. Whereas predicted levels of CO_2 increase by atmospheric CO_2 uptake for the next 100 years could reach 1000 ppm (seawater pH 7.5 at atmospheric pressure; 0.1-0.2 mM CO_2), subseafloor CO_2 leakage in the deep sea could locally acidify the environment to CO_2 levels of hundred thousands of ppm (> 100 bar; > 300 mM CO_2), exerting much higher CO_2 stresses as commonly investigated by ocean acidification research. Few studies have used experimental exposure to high CO_2 at the seabed (Barry 2003; Barry et al. 2004; Carman et al. 2004; Thistle et al. 2005; Fleeger et al. 2006; Thistle et al. 2006, 2007; Fleeger et al. 2010), and a few others were carried out *in situ* using natural gradients in pH and CO_2 concentrations associated with hydrothermalism and gas seepage in the marine environment (Inagaki et al. 2006; Hall-Spencer et al. 2008; Tunnicliffe et al. 2009). Such natural point sources of CO_2 emission to the marine environment provide long-term spatial gradients in CO_2 for the assessments of ecological

effects of leakage from subseafloor CO_2 injection. High CO_2 pressures are difficult to apply and maintain experimentally (Barry et al. 2004), hence field observations along defined gradients around high CO_2 vents are highly valuable.

Despite their important function in the cycling of nutrients, still very little is known on potential high CO_2 effects on microbial communities. Acidophilic bacteria, thriving at a pH < 3 are known from many phylogenetic groups of bacteria, but these have mostly been isolated from low pH environments formed by metal- or sulfur-leakage. The direct effect of high CO_2 concentrations on bacterial metabolism, adaptation and selection is not known. The few data available from experimental studies of ocean acidification effects on e.g. growth rates, enzymatic activity and community turnover did not show consistent, direct effects of acidification (Grossart et al. 2006; Liu et al. 2010; Joint et al. 2011; Weinbauer et al. 2011 and literature therein). Of course, effects will depend on spatial and temporal scales of exposure to high CO_2, as well as other co-varying factors such as temperature or oxygen (Hutchins et al. 2009 and literature therein). Especially long-term effects of high CO_2 on complex natural communities are difficult to observe and predict. Accordingly, it remains a major scientific challenge to study and simulate potential threats of ocean acidification for ocean life (Cicerone et al. 2004; Riebesell et al. 2010).

At the high hydrostatic pressure of deep-sea environments, CO_2 concentrations e.g. in hydrothermal vent fluids can reach hundreds of mM (Konno et al. 2006). Only four natural deep-sea locations with such emissions from subsurface reservoirs are known today, all of them are associated with back-arc hydrothermalism (Mariana arc and Okinawa Trough; (Sakai et al. 1990; Konno et al. 2006; Lupton et al. 2006)). One of these sites, the Yonaguni Knoll IV hydrothermal field, has been previously targeted for studies of bacterial and archaeal diversity patterns associated with hydrothermalism, located in the basin of the Southern Okinawa trough (Inagaki et al. 2006). Here, subsurface liquid CO_2 is generated by hydrothermal activity and migrates through the seabed to the surface seafloor. In this study, we investigated the spatial distribution of CO_2 fluxes through surface sediments and associated pH levels as well as biogeochemical processes using *in situ* techniques. Secondly, we sampled the benthic bacterial communities from locations characterized by different levels of CO_2 impact using the high-resolution fingerprinting technique Automated Ribosomal Intergenic Spacer Analysis (ARISA) in combination with 454 tag sequencing of

ribosomal genes. The aim was to describe effects of CO_2 leakage on bacterial community structure and function, and to identify those bacterial taxa repressed or selected by high CO_2 levels in sedimentary deep-sea settings.

Materials and Methods

Sample collection and site description

Samples were taken in February-March 2008 during the *RV Sonne* 196 expedition of the project SUMSUN ("Studies of marine CO_2-sequestration associated with a natural hydrothermal CO_2-system of the Northern West Pacific") (Rehder et al. 2008). The area was revisited for a few additional samples (Background data) during the Japanese expedition „NT10-06 Leg 3" with RV Natsushima and ROV Hyper Dolphin (Jamstec) in April 2010 (Table 1). The Yonaguni Knoll IV hydrothermal field located in the Okinawa Trough (24°50.7´N, 122°42.0´E; 1,380-1,382 m water depths) (Table 1) is a sedimentary basin covered by volcanic rocks in the northeastern part, and hosting several hydrothermal vent chimneys along a North-South transect. The vent chimneys are named Lion, Tiger, Swallow, Carp and Mosquito Vent (Fig. 1a) (Inagaki et al. 2006). In addition, a sedimentary venting site characterized by a few holes in the seafloor emitting hot fluids was discovered on the southern end of the hydrothermal field and named "Abyss vent" (Inagaki et al. 2006; Suzuki et al. 2008; Nunoura et al. 2010). In this study, sampling sites were first visually explored by the *ROV Quest* before targeted, combined sampling for *in situ* biogeochemistry and molecular studies. *In situ* measurements were carried out close by the Abyss vent (< 10 m distance; "High CO_2 seepage"), some 10-50 m away from the vents ("Low CO_2 seepage") and 1 km southwest of the hydrothermal system for "Background" samples. Sediment samples from the uppermost sediment horizons (top 20–30 cm) were taken either with a video-guided multicoring device (TV-MUC, 10 cm diameter cores) or with push-cores (PCs, 8 cm diameter cores) collected with the manipulator of the ROV. The sampling instruments were equipped with a POSIDONIA (Ixsea SAS) positioning system for targeted sampling of the same habitats. After recovery, the tubes containing sediment samples were transferred to a cold room that was cooled to *in situ* temperature (4°C). Afterwards, the cores were

vertically subsampled with small subcore tubes, and samples were immediately processed on board. Subsamples for DNA analysis were immediately frozen and stored at -20°C, subsamples for cell counts were fixed in 4% formaldehyde/seawater.

In situ microsensor profiles

In situ biogeochemical measurements with a profiling module equipped with microsensors for O_2, H_2S, pH, redox potential, temperature, and CO_2 were conducted at the seafloor bottom water interface of one high (at Abyss vent) and one low CO_2 seepage site. The measurements for the background site were accomplished *ex situ*. The microsensors had a spatial resolution of 25 μm. The CO_2 and pH profiles were measured by commercial minisensors (Microelectrodes LTD, Ottawa). The CO_2 sensor was calibrated in acidified seawater (pH 3), flushed with N_2, and to which defined aliquots of CO_2 saturated seawater were added. The pH sensors were calibrated with standard buffers at ambient temperature. The pH in the bottom water, sampled by Niskin bottles, was used to correct for the pressure effect. The redox sensor was made of a platinum wire fused into glass, with an exposed tip of ca. 50 μm. Before use it was cleaned with ethanol and distilled water, then kept for 15 minutes in 6 M HNO_3. The O_2 and H_2S sensors were made as described previously (Revsbech and Ward 1983; Jeroschewski et al. 1996). The H_2S microsensor was calibrated in acidified seawater (pH 3) to which aliquots of 1 M Na_2S were added. At each calibration step a sample was taken, in which sulfide was quantified using the Cline method. The O_2 microsensors were *in situ* calibrated from the profiles, using the bottom water concentration (sampled with Niskin bottles) and the 0 signal was taken from the stable subsurface sediment signal.

In situ oxygen fluxes

In situ oxygen fluxes were determined with a cylindrical benthic chamber module as previously described (Felden et al. 2010). The benthic chamber was operated by the ROV next to the profiler incubations, and the water height inside the chamber was determined by visual observation with the ROV camera system. The stirred chamber (radius 9.5 cm) enclosed a seafloor area of 284 cm^2 together with 10–15 cm (equivalent to 4–6 liters) of overlying bottom water. A valve in the chamber lid ensured the release of overpressure while placing the chamber gently into the sediment avoiding any disturbance of the sediment surface. An Aanderaa oxygen optode mounted in the chamber lid continuously monitored the oxygen concentration in the enclosed water. A two-point calibration of the reading of the optode was performed. The reading at zero O_2 concentration was taken on board at *in*

situ temperature. Values for the bottom water O_2 concentration were determined *in situ* at the seafloor.

Porewater geochemistry

Porewater was extracted using a low-pressure squeezer (argon at 1–5 bar) at approximately *in situ* temperature of 4°C in the ship's cold room. While squeezing, the porewater was filtered through 0.2 µm cellulose acetate Nuclepore filters and collected in vessels. Onboard, the collected porewater samples were analyzed for their content of dissolved NH_4^+, H_2S, PO_4^{3-}, SiO_4^{4-}, Cl^-, Fe^{2+} (samples taken under anaerobic conditions in the glove bag), total alkalinity (TA) and (*ex situ*) pH. In addition, sub-samples were taken for further shore-based analyses (concentration of CH_4 and CO_2, metal cations, SO_4^{2-}, Br^-, I^-, B^{3+}, Mn^{2+}, Sr^{2+}, $CaCO_3$, and alkalinity). In addition, 5 ml of wet sediment were collected for porosity and solid phase CNS (carbon, nitrogen, and sulfur) analyses, and an additional 5 ml of sediment was suspended in 20 ml of 1 N NaOH for methane headspace analyses. These samples were stored at room temperature. Analyses of the gaseous, dissolved and solid porewater species followed standard chemical procedures ((Grasshoff et al., 1999); for specifications http://www.ifm-geomar.de/index.php?id=1858&L=1; and DeBeer et al. in prep; Chapter 2.I this thesis)

Sulfate reduction

Sulfate reduction rates (SRR) were measured according to previously published methods (Felden et al. 2010). Briefly, SRR was obtained from the turnover of ^{35}S-sulfate radiotracer injected in 1 cm intervals into intact sediment cores. Sediments were incubated for 12 hours under anaerobic conditions and at *in situ* temperature. Reactions were terminated with ZnAc. Further analyses were done at the home laboratory.

Cell counts

The total number of single cells was determined by the AODC (Acridine Orange Direct Count) method (Meyer-Reil 1983; Boetius and Lochte 1996). Cores were subsampled vertically with smaller cores (Ø 28 mm), sliced into 1 cm sections. Samples were fixed in a 4% formaldehyde/seawater mixture and stored at 4°C. The AO-staining and further

processing was done in the home laboratory. For each sample, a minimum of two replicate filters and 30 grids per filter were randomly counted.

Microbial community analyses

On board, sediment cores for DNA analysis were sliced in 1 cm intervals, down to at least 10 cm sediment depth and stored at -20°C for further analysis in the home laboratory. For each sample, community DNA was extracted from 1g sediment with The Ultra Clean Soil Isolation Kit (MO BIO, Carlsbad, California, U.S.A.), using the "alternative protocol" for high DNA yield and finally eluting the purified DNA in 1 × TE buffer. Changes in bacterial community structure were estimated by ARISA (Fisher and Triplett, 1999). PCR reactions were conducted in triplicate and PCR-amplified fragments were discriminated by capillary electrophoresis after purification with Sephadex G-50 Superfine (Sigma Aldrich, Munich, Germany). ARISA profiles were analyzed using the GeneMapper Software v 3.7 (Applied Biosystems). Binning of the ARISA data was done according to Böer et al. (2009).

Furthermore, DNA was amplified using primers targeting the hypervariable V4-V6 region of the bacterial 16S rRNA gene (597 base pairs long), and which included 454 Life Science's A or B sequencing adapter according to Sogin et al. (2006). Pyrosequencing was performed on a Genome Sequencer 20 system (Roche, Basel, Switzerland) at 454 Life Sciences (Branford, CT, USA) by primer extension (Margulies et al., 2005), yielding on average 400-bp long fragments after removing primer sequences. Data from the 454 MPTS were retrieved from the publicly available 'Visualization and Analysis of Microbial Populations Structure (VAMPS)' website (http://vamps.mbl.edu/). Sequences were taxonomically assigned by an automatic annotation pipeline (Sogin et al., 2006), using several known databases (Entrez Genome, RDP, SILVA). Here, analyses were based on a definition of operational taxonomic units (OTU) as unique (i.e. two sequences belong to two different OTU_{unique}) when they differed by 3% sequence identity to keep a consistent definition throughout (Sogin et al. 2006; Huse et al. 2008).

Statistical analyses

Statistical analyses of changes in bacterial community structure and patterns of community variation based on ARISA fingerprints and contextual parameters (Tab. 2; also

see DeBeer et al., in prep, see Chapter 2.I of this thesis) were carried out as described in (Böer et al. 2009) and (Gobet et al. 2010). The environmental variable data set (Table 2), subdivided into the categories vent fluids, weathering, mineralogy, sulfur species, sediment depth and space, latter being expressed as a polynomial of degree three to represent more complex spatial patterns (Legendre and Legendre 1998), were forward selected to reduce co-variation in the following analyses. The measurement of pH for every sample could not be accomplished, therefore we considered space and weathering as proxies for the acidification effect as a result of high CO_2 concentration. Environmental parameters were normalized to reduce statistical error except for Cl^{-1}, and porosity. Other porewater parameter such as alkalinity, B, Ca^{2+}, H_2S, K^+, Li^+, NH_4^+, SiO_4^{4-}, Sr^{2+} were $\log_{10}$ transformed; and $CaCO_3$, TN, TS were arcsine transformed (Ramette 2009). Overall and individual fractions of explained community variation were assessed for significance using simple and partial redundancy analyses with 1000 permutations of the data (Borcard et al. 1992).

Results

Visual observations and bottom water measurements

The seafloor of the Yonaguni Knoll IV system (Figure 1) was mapped with the ROV QUEST and a TV-guided MUC. Visual observations of geological and biological seafloor features showed three different zones of sedimentary habitats 1) background seafloor with various species of echinoderms, and many traces of bottom dwelling fauna (Fig. 1b, c), featureless brownish deep-sea sediments devoid of worm tubes and burrows (Fig. 1d), whitish silicate precipitates and sulfur pavements (Suzuki et al. 2008) associated with cracks in the seafloor emitting hot CO_2-enriched fluids or liquid CO_2 (Fig. 1e,f at Abyss vent). No bacterial mats were recognizable on the seafloor in the whole area of Yonaguni. The background zone > 1 km away from the vents showed a bottom water pH > 7.5 (< 0.08 mM CO_2). No gradient in bottom water oxygen concentrations was observed across the entire valley from the Background area to the vent sites, these were low throughout (ca. 80 μM O_2). *In situ* measurements of bottom water pH and CO_2 in the vicinity of the vents showed decreased pH (< 7.4) and increased (> 0.1 mM) CO_2 already at around 300 m distance. The CO_2 content in the bottom waters further increased towards the vent area, to millimolar concentrations around Abyss vent. End member concentrations of the Abyss vent fluids (at 60-90°C in the holes) were similar to those emitted from other vents in the area, previously determined with ca. 90 mM CO_2, 1 mM CH_4, 2-3 mM SiO_2, > 1 mM NH_4 (Nonoura et al. 2010); ca 1 mM hydrogen and no sulfate (Konno et al. 2006; Suzuki et al. 2008).

In situ pH profiles and biogeochemistry

In situ microsensor profiles (DeBeer et al. in prep; see Chapter 2.I, this thesis) showed that around Abyss vent ("High CO_2 seepage", 0.5-10 m distance), the highly concentrated CO_2 seeping through the seafloor induced an substantial decrease in pH already at surface seafloor from pH 5.5 down to 4.5 at > 6 cm sediment depth. To reach such low pH requires an *in situ* CO_2 concentration of 30-300 mM in the surface porewaters at the *in situ* temperature 4-8°C and pressure (13.8 MPa). Below 15 cm sediment, CO_2 is most likely saturated (DeBeer et al. in prep, Chapter 2.I, this thesis). In the "Low CO_2 seepage" area, pH

values were higher in the top 2 cm (pH 6-7.2) and reached levels of pH 5-7 at 6-10 cm sediment depth. In the Background area, no pH gradient was recorded with sediment depth. Based on this characterization of the CO_2 gradient, we sampled all three zones in replicate with push cores and TV-guided multiple corers (Fig. 1a; Tab. 1) to investigate microbial diversity in the surface sediments (top 10 cm layer). At all three sites, the *in situ* temperature was 4°C to maximally 8°C (close to the Abyss vent), oxygen penetration into the sediment was limited to the top few mm layer, but pH, CO_2 content and chemical composition varied with distance to the vent zone (Tab. 2).

In the entire 200 m zone around Abyss vent (Low and High CO_2 seepage), geochemical porewater and mineral composition was highly variable (DeBeer et al. in prep; see Chapter 2.I, this thesis). The chemistry of the surrounding of Abyss vent ("High CO_2 seepage", 0.5-10 m distance) was characterized by high alkalinity, silicate, methane, sulfide, ammonium and phosphate; and low sulfate, calcium carbonate and total organic carbon (Tab. 2). Hydrogen concentrations were not measured, but potentially as high as methane as known from earlier studies of vent fluids (Konno et al. 2006). A second category of samples, grouped as "Low CO_2 seepage" (generally 20-200 m away from Abyss vent) showed also influences of CO_2 seepage and mixing with vent fluids, but to a lesser extent. In this category, also substantial alkalinities were recorded, but much less potential chemical energy in the form of sulfide, methane and ammonium (Tab. 2). The Background site > 1 km off Abyss vent (Fig. 1, Tab. 2) showed a typical porewater and solid phase composition for continental margin deep-sea sediments, and did not contain methane, ammonium or sulfide in the porewaters. Oxygen penetration was 8 mm, compared to 1-2 mm at the CO_2 vented sites.

In situ rates of microbial activity were measured by benthic chamber incubations and by microsensor profiling in all three zones (Tab. 3). Total oxygen consumption at the Background zone was 0.8 mmol m^{-2} d^{-1}. As the porewaters did not contain any chemical energy in the form of methane, sulfide and ammonium, we assume that the aerobic respiration in the Background area was entirely fueled by organic matter from particle sedimentation. In comparison, total oxygen consumption of the CO_2 impacted sites was clearly elevated with 4 mmol m^{-2} d^{-1}, most likely from microbial consumption of chemical energy transported with the vent fluids. Anaerobic microbial sulfate reduction rates showed

elevated rates at the High CO_2 seepage sites of on average 5 mmol m^{-2} d^{-1}, compared to 0.06 mmol m^{-2} d^{-1} at the Low CO_2 seepage, and 0.002 mmol m^{-2} d^{-1} at the Background site (Tab. 3). Microbial sulfate reduction rates matched sulfide fluxes measured in the surface sediments, but not the anaerobic oxidation of methane, which explained only 1-10% of sulfate reduction rates, pointing to other electron donors in the vent fluids (Tab. 2).

Cell counts

Cell numbers were integrated across 3 different sediment layers (0-5, 5-10 and 10-15 cm) for comparison within and between the three different habitats (Fig. 2). In all zones, cell numbers decreased with increasing sediment depth. Significant differences were recorded between the Background and the High Seepage sites, of which the latter showed higher numbers even in the deepest sediment zone. The integrated cell counts for the Background $1.8 \pm 0.1 \times 10^{14}$ cells m^{-2} were similar as for Low CO_2 Seepage $2.0 \pm 0.7 \times 10^{14}$ cells m^{-2}, but both were lower than the High CO_2 Seepage sites $2.8 \pm 0.2 \times 10^{14}$ cells m^{-2}.

Microbial Community Fingerprinting

The DNA fingerprinting method ARISA, which targets the length polymorphism of amplified bacterial ITS1 regions, was used for a comparison of Operational Taxonomic Units of abundant bacterial types ($OTUs_A$). From a total number of different $OTUs_A$ of 416, 70% were common to all sites. However, the absence-presence analysis of all OTUs integrated over the top 10 cm of sediment showed that $OTUs_A$ richness was reduced by 20% between Background and the High CO_2 seepage sites (Fig. 3). While $OTUs_A$ richness increased slightly with sediment depth from 191 at 0-1 cm, to 199 at 4-5 cm, and 201 at 9-10 cm at the Background site, at the Low Seepage sites the highest number of $OTUs_A$ was found at depth 4-5 cm (181, 202, 184; respectively). At the High CO_2 Seepage sites, $OTUs_A$ decreased with increasing sediment depth (170, 161, and 144).

In addition, a substantial replacement of $OTUs_A$ was detected between High Seepage sites and the other locations: A comparison of relative numbers of unique $OTUs_A$ between all locations showed that the High Seepage sites hosted the highest proportion of unique populations (14-18% $OTUs_A$). When comparing Background sequences with all of the CO_2-impacted sites, only 1% of the sequences were unique to the Background, in comparison to 15% at the CO_2 vented sites.

Patterns of beta-diversity as recorded by ARISA across the three different habitats were visualized by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrices between samples and their communities (Fig. 4). This plot shows the dissimilarity-distance of bacterial communities between the three habitats. The community dissimilarity between sites increased with increasing CO_2 effect (Fig. 4). Bacterial communities in Background samples showed a much higher similarity to each other compared to those of the Low and High CO_2 Seepage sites. The differences between bacterial communities were significant as confirmed by ANOSIM tests (Background / Low CO_2 Seepage, ANOSIM $R = 0.434$; $p < 0.001$, and Background / High CO_2 seepage; $R = 0.212$, $p < 0.001$). Community differences between Low CO_2 Seepage and High CO_2 seepage were also significant ($R = 0.151$, $p = 0.001$). Moreover, significant differences were observed between sediment depth-related bacterial community patterns. Communities in the 0-2 cm surface horizon significantly differed to the communities hosted in the subsurface layers, which were more impacted by CO_2 (0-2 / 4-5, ANOSIM $R = 0.093$, $p = 0.023$; 0-2 / 9-10, $R = 0.231$; $p < 0.001$; 4-5 / 9-10 $R = 0.023$, $p = 0.452$).

As a next step, we used 454 massively parallel tag sequencing of bacterial OTUs to identify changes across different taxonomic levels from bacterial phylum to the genus level, and also with regard to rare types (here defined as singletons, i.e. taxonomic units occurring only once in the data set (Fig. 5). Total sequence reads obtained were 235,731 (101,513 different sequences; Table 4), of which 84% were singletons (defined as sequence types occurring only once in the entire data set). Common sequences found in all samples accounted for only 0.003%. At the 3% clustering threshold, of all 79,263 different OTU types ($OTUs_{454}$); 62,921 were singletons comprising 79% of the total $OTUs_{454}$. The proportion of singleton types was highest at the low CO_2 impacted site, and lowest at the Background (Tab. 3). In all habitats, decreasing numbers of singletons were found with increasing sediment depth. Only 0.08% of all $OTUs_{454}$ types were common to all sites, comprising 0.03% of all sequence reads. The highest sequence reads of any common OTU was detected at the High CO_2 Seepage site and belonged to the (*Proteobacteria*).

The six most sequence abundant phyla of the Background zone were: *Proteobacteria* (especially the classes *Gammaproteobacteria, Deltaproteobacteria,* and *Alphaproteobacteria*), *Planctomycetes*, *Acidobacteria, Bacteroidetes, Chloroflexi,* and *Actinobacteria* (Fig. 5, Table S1). Overall, differences between the Background and Low CO_2 seepage sites were confined

mostly to shifts in sequence abundances. In comparison, the High CO_2 seepage site showed different abundances even at the phylum level. Highest sequence read abundances were detected for the *Epsilonproteobacteria*, *OP9*, and *Spirochaetes* (Fig. 5). In contrast, *Alphaproteobacteria, Planctomycetes, Actinobacteria,* and *Acidobacteria* decreased from the Background to the CO_2 impacted sites. At higher taxonomical resolution the High CO_2 seepage sites hosted more acidophiles including members of the family *Thermodesulfobacteriaceae* (the genus *Caldimicrobium*), the family *Caldisericaceae* (genus *Caldisericum*) and the family *Acidithiobacillaceae* (genus *Acidithiobacillus*). Table 5 shows examples of winner and loser types with regard to CO_2 impact on sequence abundance. In the *Chloroflexi*, *Alpha-* and *Betaproteobacteria*, several types were strongly reduced in sequence abundances with increasing CO_2 impact. In contrast, members of the *Epsilon-* and *Deltaproteobacteria* clearly increased with increasing sequence abundance.

Analysis of environmental factors structuring community composition

A statistical variation partitioning approach was used to disentangle environmental factors potentially responsible for structuring bacterial communities of the different habitats (Fig. 6 and 7). The effect of 16 different chemical parameters (Tab. 2), space (transformed to third-degree polynomials), and sediment depth were tested in a multivariate regression approach. The variation in community structure was mostly explained by pore water composition and space, of which the pure fractions explained 20%, and 13% of the ARISA OTU variation, respectively. Altogether, about 70% of the total variation in community structure was explained by these categories which all reflected the effect of venting by subsurface fluids rich in CO_2 and chemical energy. Accordingly, pure sediment depth effects (i.e. without chemical gradients) were not significant (1%).

Next we used a forward selection procedure to group the environmental parameters further into categories, namely those associated with mineralization processes, hydrothermal fluid composition, and those directly linked to high CO_2 emissions (Fig. 7, Table 2).

According to our spatial sampling design with a central area of high CO_2 emission (Abyss vent), an intermediate CO_2 emitting area (Low CO_2 seepage), and a more distant Background location, we used space as another proxy for CO_2 venting, by a polynomial transformation of geographic coordinates of all sampled sites. Weathering (9%), space (9%), and vent fluid composition (7%), explained most of the variation followed by mineralogy

(2%), sediment depth (2%), and sulfur species (1%), with all six factors being significant ($p = 0.001$, except sulfur species $p = 0.013$).

Discussion

Through a reduction in seawater pH and the high levels of CO_2 concentrations, some important microbial processes may be depressed. Decreased microbial nitrification and ammonium oxidation rates for example were reported with a complete inhibition of nitrification at pH 6 (Huesemann et al. 2002; Beman et al. 2011). Further, a reduction in ocean pH could lead to a shift in ammonia (NH_3) favoring ammonium ($NH4^+$) and thus triggering eutrophication effects (Turley et al. 2006).

This study investigated the effect of high levels of natural CO_2 seepage through deep-sea sediments on benthic bacterial respiration and community diversity. Previous investigations have already shown that members of the Bacteria and Archaea can populate areas of high CO_2 emission causing low pH < 5 (Inagaki et al. 2006; Nunoura et al. 2010; Yanagawa et al. submitted). Here we investigated in detail changes in community structure and respiration from background conditions to CO_2-vented sediments above liquid CO_2. While differences in temperature and oxygen supply were negligible between sites, the natural gradient of *in situ* CO_2 from subsurface vent fluids mixing with porewaters covered 1-2 orders of magnitude because of the high solubility of CO_2 at high pressure (DeBeer et al., Chapter 2.1, this thesis). The vent fluids also transported chemical energy such as hydrogen, methane, ammonium and iron (Tab. 2; (Konno et al. 2006; Nunoura et al. 2010)), mimicking a scenario of leakage of CO_2 and hydrocarbon-enriched subsurface fluids as from Carbon Capture and Storage in submarine gas reservoirs.

Previous studies at this site have shown, that main functional groups such as sulfate reducers (Yanagawa et al. submitted), sulfide and methane oxidizers (Inagaki et al. 2006; Nunoura et al. 2010) occur in areas affected by high CO_2, but have indicated that their activity may be considerably lower than in cold seeps and hydrothermal vents at neutral pH (Inagaki et al. 2006). By measurements of community sulfide production, aerobic and anaerobic respiration (sulfate reduction (SR); anaerobic oxidation of methane (AOM)), we

detected microbial activity in the vicinity of the vent sites characterized by high CO_2 emissions and co-migration of methane, hydrogen and other potential energy sources for microbial metabolism (Tab. 3). However, compared to cold and hot methane seeps at neutral pH, microbial activity of the high CO_2-high CH_4 sites was at the low end of rates known from deep-sea sediments of active cold seeps and hot vents (Tab. 3). As previously shown by Inagaki et al. (2006); especially AOM rates were atypically low given the high methane content of the vent fluids, with up to 0.06 mmol m^{-2} d^{-1}. Also, total oxygen consumption of the benthic communities appear significantly impacted, with an order of magnitude lower rates, compared to other energy-rich deep sea ecosystems (Sahling et al. 2002; Treude et al. 2003; Sommer et al. 2006; Felden et al. 2010; Lichtschlag et al. 2010; Holler et al. 2011; Grünke et al. 2012).

In contrast, the comparison of total cell counts showed no negative effect of high CO_2 levels on total microbial biomass in surface sediments (top 15 cm; Fig. 2). Cell numbers were even elevated at the High CO_2 seepage site with counts of several 10^9 cells ml^{-1} (Inagaki et al. 2006; Nunoura and Takai 2009), it was observed that the sulfur-rich surface pavements around Abyss vent contain high cell numbers (> 10^9 cells cm^{-3}), declining rapidly towards the zone of liquid CO_2 (~10^7 cells cm^{-3}) (Inagaki et al 2006, Yanagawa et al. submitted). Of course, the chemical energy delivered to the microbial communities around the vent sites may support higher cell abundances compared to the Background site fueled by sedimentation of organic detritus. However, much higher total cell numbers compared to the High CO_2 seepage site are found at active cold seeps vented with neutral pH fluids such as Hydrate Ridge (Knittel et al. 2003; Boetius and Suess 2004) and Haakon Mosby Mud Volcano (Niemann et al. 2006), due to a high contribution by methanotrophic archaea and their sulfate reducing bacterial partner forming dense consortia (Knittel and Boetius 2009). Yanagawa et al. (submitted) showed accordingly, that such typical anaerobic methanotrophic consortia were missing from the high CO_2 settings around Abyss Vent, but could nevertheless show substantial methane-fueled sulfate reduction rates at very low pH.

Adaptations of bacteria to acidic environments include pumping protons out of the intracellular space to keep the cytoplasm at or near neutral pH, or the production of acid-stable proteins (Menzel and Gottschalk 1985). To date, many natural and anthropogenic environments are known, where bacterial communities thrive at pH < 4. These are often characterized by low diversity and selection of adapted, acidophilic types (e.g. Rio Tinto,

(Gonzalez-Toril et al. 2003); acidic hydrothermal vents, (Nakagawa et al. 2006)). In contrast, very little is known on specific adaptations to low pH environments caused by high CO_2 concentrations (> 20 mM, up to > 1000 mM) at high pressure. At low pH and high pressure dissolved carbon dioxide dominates the carbonate system. Extremely high CO_2 levels may be toxic, as protonated CO_2 could uncouple membrane potentials, affect the uptake and hydrolysis of organic compounds and other physiological reactions. On the other hand, the chemical energy contained in the rising CO_2 rich vent fluids could support energy-costly microbial adaptations to high CO_2 - low pH and favor certain types of bacteria such as acidophilic chemoautotrophs, methanotrophs and sulfate reducing bacteria.

Interestingly, our study showed that the increase in total cell abundance from the Background setting to the High CO_2 vented area was accompanied by a loss and replacement of $OTUs_A$ (Fig. 3). The ARISA fingerprinting method analyzes shifts in community structure of the most abundant populations, with a taxonomic resolution between genus and family (Gobet et al. in prep.). This could be a result of a selection pressure of high CO_2 in combination with chemical energy from vent fluids for acidophilic vent populations, as described earlier in other studies of microbial community profiles across hydrothermal vent chimneys (e.g. Nunoura and Takai 2009). Secondly, we found a clear effect of high CO_2 seepage on beta-diversity of the bacterial community (Fig. 4). We assume that beta-diversity was increasing most probably due to the disturbances by CO2 venting on the background community, together with an increased availability and accessibility of niches provided for opportunistic acidotolerant or acidophilic bacteria by the hydrothermal gradient both in temperature and supply of biogeochemical species. Accordingly, the analysis on differences in beta-diversity by means of non-metric multidimensional scaling (Fig. 4) revealed a high similarity for the Background communities, but substantial dissimilarities between communities sampled from CO_2 impacted environments. An in depth analysis of the $OTUs_A$ showed that this dissimilarity was mostly due to changes in relative abundances of types, as well as the appearance of types not represented in the Background area, and unique to either Low Seepage or High Seepage sites. Hence, both CO_2-impacted sites show a much higher degree in heterogeneity amongst communities, fitting the heterogeneity in chemical composition of porewater fluids and minerals (Tab. 2; DeBeer et al. Chapter 2.I; this thesis).

When testing the correlation of environmental data with the distribution of $OTUs_A$ we found that parameters describing processes of weathering, vent fluid composition or distance to vents (polynomials of geographic positions = space) (Table 2, Fig. 6 and 7) explained most of the variation in community structure. The environmental parameters grouped under chemistry, space and sediment depth were correlated with each other, but also the analysis of pure effects showed a substantial influence of chemistry (CO_2 + other species) and space (Fig. 6). The factors grouped under "weathering" (e.g. alkalinity, calcium carbonate, silicate), space and vent fluids explained together a high proportion of variations in community structure (Fig. 7).

The high resolution of OTU fingerprinting by 454 massive tag sequencing allowed a further investigation of community patterns (Sogin et al. 2006; Huse et al. 2008). No substantial difference in total sequence reads, total sequences, or richness of OTU_{454} (3% cluster) was detected between sites, if at all, sequence and OTU_{454} (3% cluster) richness increased with CO_2 leakage. However, most of the apparently higher richness was explained by the number of singletons at the CO_2-impacted sites. This may hint to a disturbance effect from varying CO_2 fluxes and chemical heterogeneity (Tab. 4). Earlier studies of extreme environments have shown that heterogeneities in vent fluid composition can affect microbial communities in terms of abundance, diversity, and composition (Polz and Cavanaugh 1995; Nakagawa et al. 2005b; Nakagawa and Takai 2006; Nunoura and Takai 2009).

While the sequence abundance of the dominant phyla was rather similar between Background and Low CO_2 seepage site, substantial changes were observed at the High CO_2 area (Fig. 5a). Based on the acidification by high CO_2 levels we found an increase in acidophilic bacteria (e.g. *Firmicutes*) and typical opportunistic vent phyla such as *Epsilonproteobacteria* from the Background towards the High CO_2 area (Fig. 5a, Tab. S1). Several types of bacteria were favored by CO_2 venting and the provision of chemical energy, whereas many member of the Background community became rare (Tab. 5). *Alphaproteobacteria* as well as *Planctomycetes*, which were present both at the Background as well as at Low CO_2 seepage, decreased in sequence abundance with proximity to the vents. Also *Acidobacteria,* and *Actinobacteria* which are known to be key phyla found in marine deep-sea sediments, declined in sequence abundance (Dang et al. 2009). *Epsilonproteobacteria*, however, increased in sequence reads towards the High CO_2 area. Similarly, Huber et al. (2003) found an increasing abundance and diversity of *Epsilonproteobacteria* following a volcanic eruption

causing changes in the geochemical properties of the investigated vent environment. The ecological importance and adaptability to extreme environments of the class *Epsilonproteobacteria* has increasingly been recognized, particularly with respect to biogeochemical and geological processes (Nakagawa et al. 2005a). Table 5 shows such examples of bacterial types of similar functional groups, which are selected by differences in CO_2 leakage. For example, neutral pH sulfur oxidizers include e.g. several *Thiobacillus* species and the genera *Beggiatoa*, *Thioploca*, *Thiothrix* (Nealson 1997), which were absent from the High CO_2 seepage site. Typical for acidic, H_2 rich vent fluids are e.g. *Sulfurimonas* (Takai et al. 2009), which were clearly selected by the low pH and likely presence of H_2 in the subsurface fluids around Abyss vent.

Conclusions

Field studies and observation of deep-sea ecosystems naturally seeping CO_2 are important as natural analogues for ecological risk assessment of CO_2 leakage from submarine Carbon Capture and Storage sites. Especially with regard to the use of exploited hydrocarbon reservoirs as submarine storage sites, ecological effects are likely driven by a co-migration of CO_2 and other compounds such as CH_4, H_2, H_2S and NH_4^+. Hence, locally and as a result of long-term leakage, ecological effects may not be obvious as a mere decline in microbial cell numbers, or community diversity. However, we observed evidence for a general decline in respiration rates (oxygen consumption, sulfate reduction, anaerobic oxidation of methane) compared to other pH neutral seep and vent systems. Furthermore, typical dominant bacterial phyla of deep-sea sediments declined in sequence abundance and richness. Other types were selected and favored by the combination of high CO_2 and supply of chemical energy, e.g. in the form of sulfide. A striking consequence of CO_2 seepage was a substantial increase in community dissimilarity within the vented areas, and the increase in rare types of bacteria (Singletons). This may serve as an important factor to consider in environmental assessment surveys of impacted deep-sea sites. Further investigations are needed to assess the consequence of the alteration of bacterial community composition and evenness for higher trophic levels, and for the ability of benthic microbial communities to

control emission of hydrocarbons from exploited hydrocarbon reservoirs potentially used as carbon storage sites.

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Figures and Tables

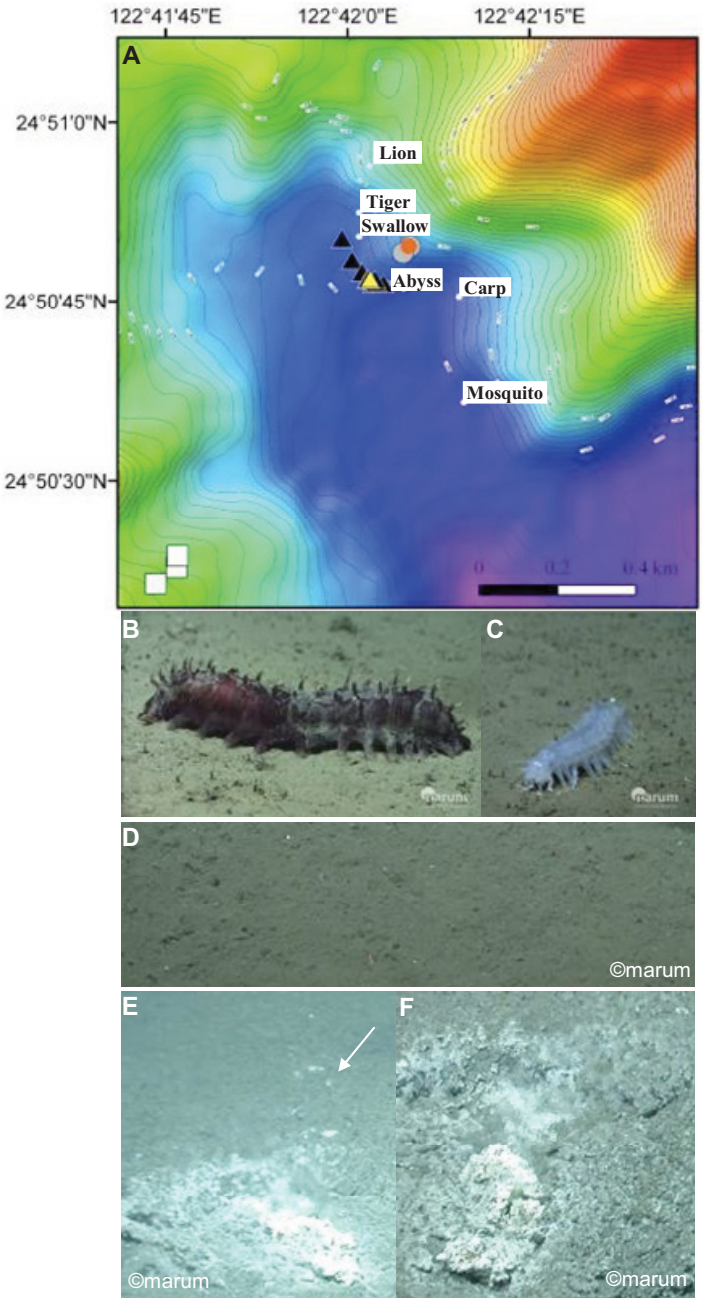

Figure 1 (A) Overview of the Yonaguni Knoll IV hydrothermal vent system. Symbols for sediment samples for the Background, High CO_2 seepage and Low CO_2 seepage site are white squares, black triangles and grey circles, respectively. Yellow triangle and red circle for *in situ* microprofiler measurements at High and Low CO_2 seepage site, respectively. (B and C) Background site with biogenic seafloor structures and sediment-feeding deep-sea holothurians *Laetmogone violacea* and *Pannychia moseleyi*, respectively. (D) Low CO_2 seepage site lacking biogenic structures and traces of epifauna. (E, F) Fluid flow and liquid CO_2 droplets emanating from sediments of the High CO_2 seepage site (Abyss vent).

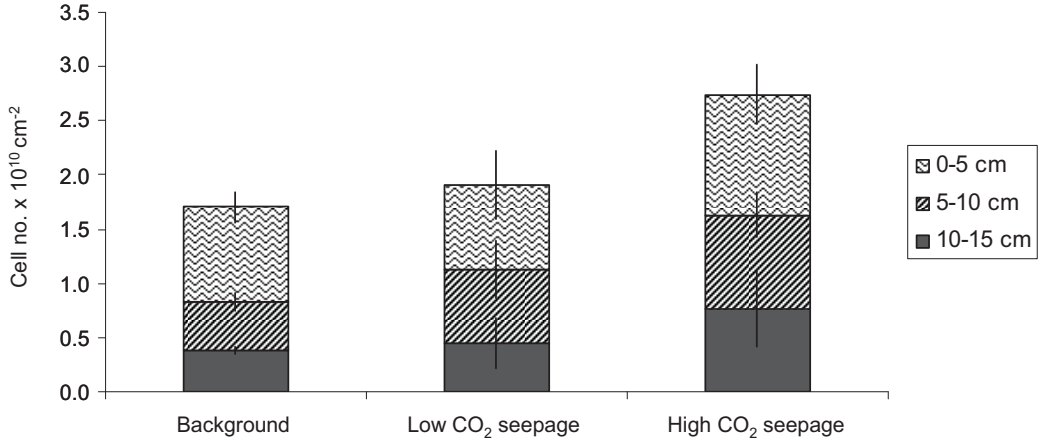

Figure 2 Total microbial cell numbers integrated over 15 cm sediment depths. Error bars indicate standard deviation (n = 3)

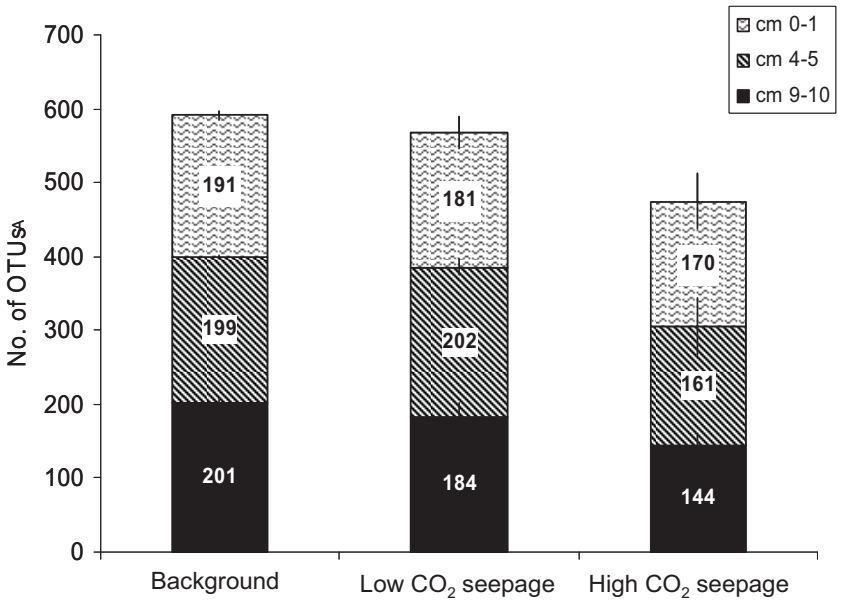

Figure 3 OTUs$_A$ richness as determined by ARISA of three sediment depth horizons of the Background (n = 3 sites), Low CO_2 seepage (n = 2 sites), High CO_2 seepage (n = 7 sites). Error bars indicate standard deviations. Significance tested with Wilcoxon-Mann-Whitney-Test. Significantly different are: Horizon 0-1 cm, Background and High CO_2 seepage, P = 0.03; Low CO_2 seepage and High CO_2 seepage, P = 0.05. Horizon 9 10 cm, Background and High CO_2 seepage, P = 0.02. Kruskal-Wallis-Test: Background and High CO_2 seepage, P = 0.03.

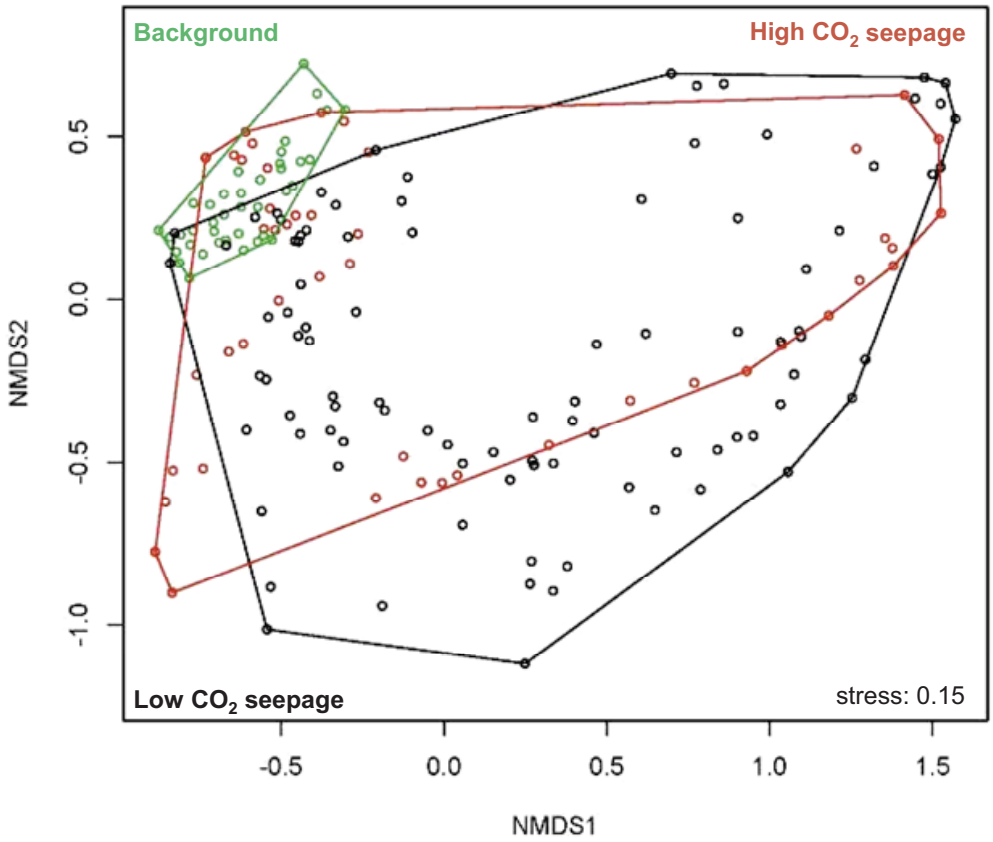

Figure 4 Non-metric multidimensional scaling plot for community structure based on Bray-Curtis distance metric for community dissimilarity. One circle refers to one community sample of a specific site and sediment horizon.

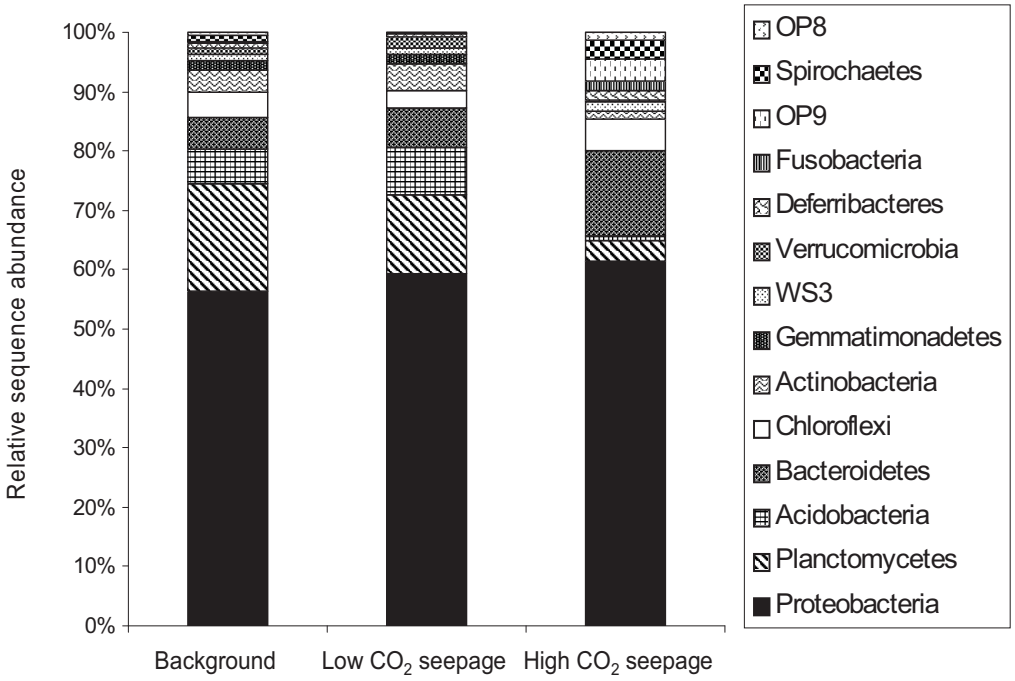

Figure 5a Proportions of sequence tags for the different habitats integrated over 10 cm sediment depth. All phyla with absolute sequence numbers > 1000 tags for at least one site were included. Total no. of tags for each site: Background (54,669), Low CO_2 Seepage (85,139), High CO_2 Seepage (80,228). Background, Low CO_2 Seepage, High CO_2 Seepage n = 1.

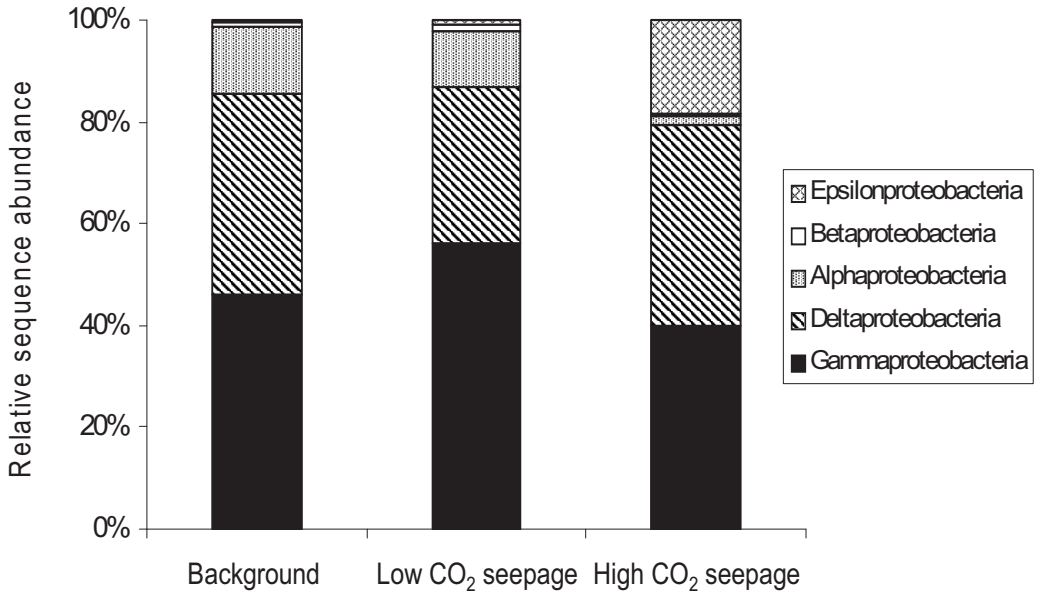

Figure 5b Proportions of sequence tags of the *Proteobacteria* classes integrated over 10 cm sediment depth. Total no. of tags for each site: Background (30,849), Low CO_2 Seepage (50,599), High CO_2 seepage (49,232).

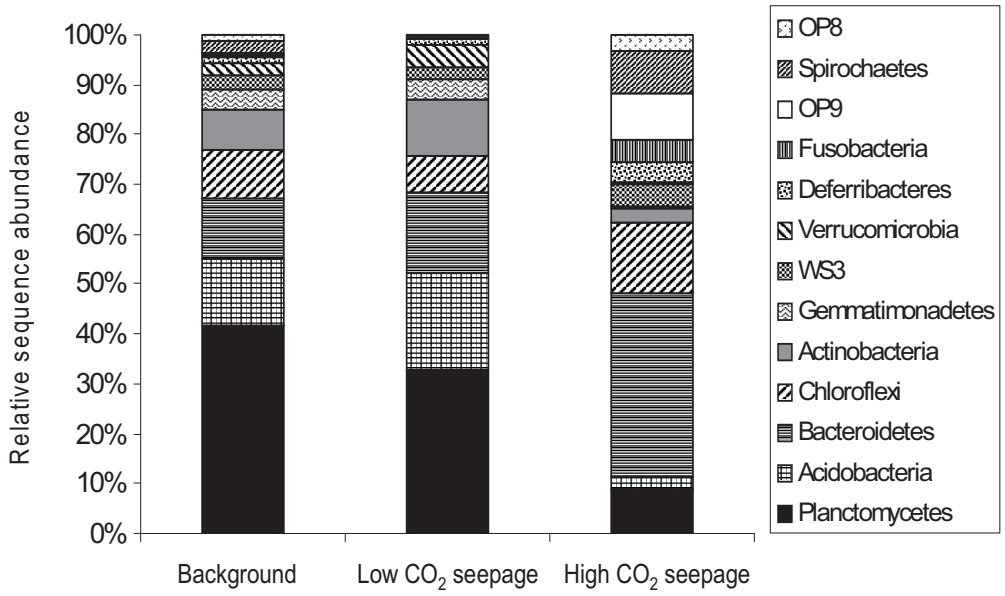

Figure 5c Proportions of sequence tags for all phyla (except *Proteobacteria*) with sequence numbers > 1000 tags for at least one site integrated over 10 cm sediment depth. Total no. of tags for each site: Background (23,820), Low CO_2 Seepage (34,540), High CO_2 seepage (30,996).

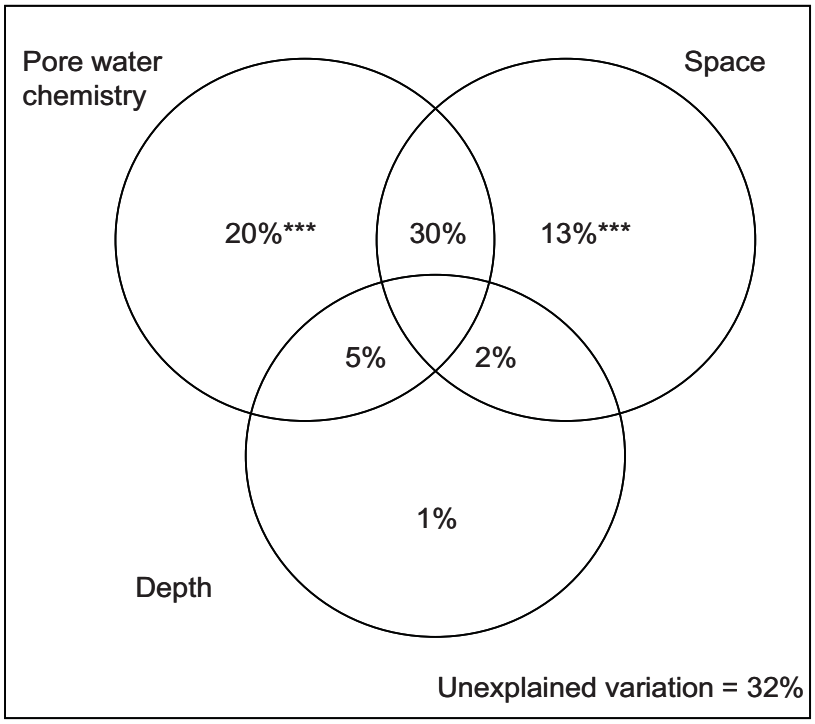

Figure 6 Variation partitioning of the bacterial community structure (ARISA analysis) into either pure or combined effects of pore water chemistry (16 parameters; see Table 3), space (distance to seep, transformed to 8 polynomials), and sediment depth (0-28 cm). The pure effect of pore water and space was significant: $p < 0.001$.

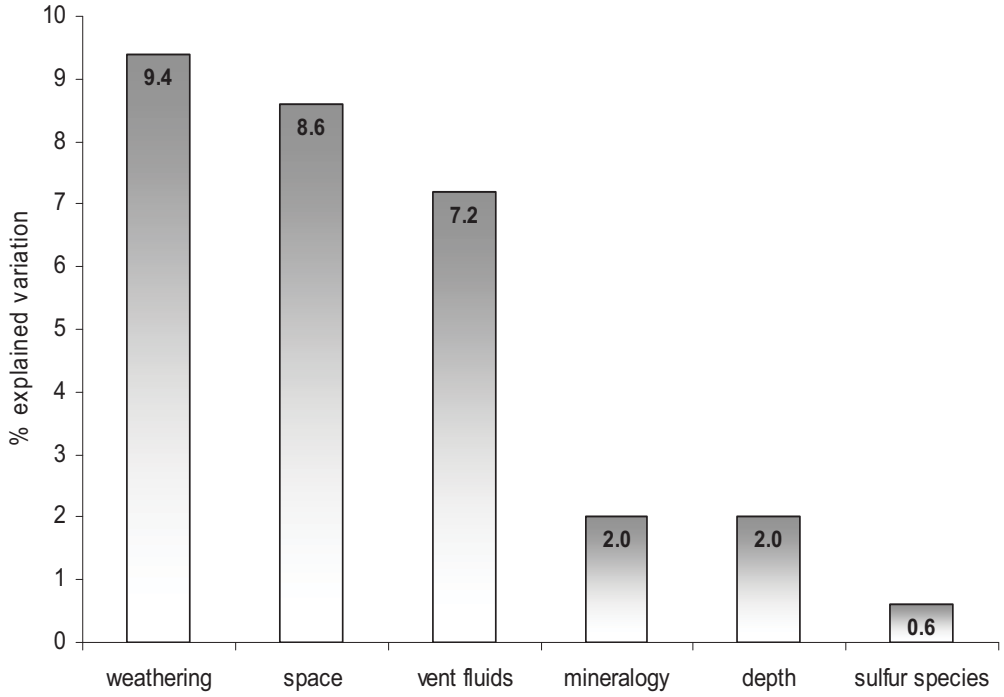

Figure 7 Variation partitioning of the ARISA data set with 6 different explanatory variables: categorized pore water groups, space, and depth. Overall model and all fractions significance: P = 0.001.

Table 1 Samples obtained for molecular analyses at the three habitats. Samples were used for following analyses: [A] cell counts, [B] 454 MPTS, [C] ARISA. [D] marks those samples used for the variation partitioning analysis. The event label refers to the metadata identifier of the World Data Center PANGAEA (www.pangaea.de). PC 20 and 33 were from Dive 201 and 203, respectively. Further replicate samples from the same stations were obtained for porewater analyses (DeBeer et al. in prep, Chapter 2.I this study)

Site	Station	Date	Latitude	Longitude	Depth [m]	Event label	Device type
Reference	MUC 23[ABCD]	3/18/2008	24.8393	122.6956	1324	SO196_65	Multicorer
	MUC 24[C]	3/18/2008	24.8396	122.6961	1323	SO196_66	Multicorer
	MUC 25[C]	3/18/2008	24.8399	122.6961	1318	SO196_67	Multicorer
	DIVE_1111_PC9[A]	4/17/2010	24.8419	122.6980	1355	NT10_06	Push corer
Low CO_2 seepage	MUC 3[ABCD]	3/9/2008	24.8471	122.7014	1372	SO196_15	Multicorer
	MUC 20[C]	3/17/2008	24.8470	122.7013	1318	SO196_55	Multicorer
High CO_2 seepage	MUC 7[C]	3/9/2008	24.8468	122.7001	1384	SO196_19	Multicorer
	MUC 8[C]	3/9/2008	24.8473	122.6998	1362	SO196_20	Multicorer
	MUC 10[CD]	3/10/2008	24.8465	122.7003	1392	SO196_25	Multicorer
	MUC 12[C]	3/14/2008	24.8463	122.7006	1385	SO196_37	Multicorer
	MUC 14[C]	3/14/2008	24.8463	122.7008	1385	SO196_39	Multicorer
	MUC 16[C]	3/17/2008	24.8463	122.7007	1365	SO196_49	Multicorer
	MUC 19[CD]	3/17/2008	24.8464	122.7006	1387	SO196_54	Multicorer
	MUC 28[CD]	3/22/2008	24.8464	122.7005	1394	SO196_95	Multicorer
	PC 20[BCD]	3/13/2008	24.8464	122.7005	1382	SO196_30_PUC20	Push corer
	PC 33[ACD]	3/16/2008	24.8422	122.6975	1383	SO196_44_PUC33	Push corer

Table 2 Ranges of porewater and solid phase composition used in the variation partitioning analyses. Ranges refer to the 0-10 cm sediment depth horizon.

Site	Background	Low CO_2 seepage	High CO_2 seepage
TAlk (meq/l)*‡	2 - 3	3 - 4	5 - 30
B (mM)*‡	0.39 - 0.40	0.40 - 0.41	0.42 - 0.43
Br^- (µM)*	1026 - 1037	857 - 822	863 - 897
Ca^{2+} (mM)*‡	10	10	10 – 11
Cl^- (mM)‡	544	544 - 545	543 - 547
Fe^{2+} (µM)*	0.8 - 8	2.4 - 170	3- 45
I^- (µM) *	0.5 - 4	0.2 - 1.1	2.1 - 3
H_2S (mM)‡	0.0	0.0	0.00 - 2.2
Mg^{2+} (mM)*	24	54	51-53
Mn^{2+} (µM)*	5 - 73	3 – 29	27-50
K^+ (mM)‡	9.6 - 10.1	9.8	9.9 - 10.7
Li^+ (µM)*‡	24	25	31 - 107
NH_4^+ (µM)*‡	16 - 40	8 – 20	32 - 327
SiO_4^{4-} (µM)*‡	198- 215	204 - 302	228 - 705
SO_4^{2-} (mM)*	29	27 – 28	12-28
Sr^{2+} (µM)‡	85 - 86	87	84-87
$CaCO_3$ (wt%)‡	3.3 - 4.1	0.2-1.6	0.02 - 0.01
TN (wt%)‡	0.12 - 0.13	0.11 - 0.14	0.14 - 0.15
TS (wt%)*‡	0.1 - 0.2	0.1 - 0.2	2.9 - 6.1
Porosity*‡	0.7 - 0.8	0.7 – 0.8	0.6 - 0.7
TC (wt%)	1.3	1.0 - 1.1	0.8 - 0.9
Ex situ CH_4 (mM)*	0.01	0.02 – 0.03	0.28 – 0.5

Abbreviations read as follows: TAlk, total alkalinity in porewater; B, dissolved boron compounds (mainly $B(OH)_3$ and $B(OH)_4^-$); TC, total carbon in solid phase; TN, total nitrogen in solid phase; TS, total sulphur in solid phase.

* Parameter used in variation partitioning (Figure 6), ‡ Parameter used in variation partitioning (Figure 7). Categories variation partitioning Figure 7: vent fluids (NH_4^+, Li^+, K^+, B, Cl^-), mineralogy (Porosity, TN, TS), weathering (TAlk, Ca^{2+}, Sr^{2+}, $CaCO_3$, SiO_4^{4-}), free sulfide (H_2S).

Table 3 Fluxes and turnover rates in comparison to other seeps and vents. Fluxes calculated from the microprofiles and benthic chamber, the average integrated areal SR and AOM measured in MUC and PC taken in (High CO_2 seepage) and near seeps (Low CO_2 seepage) and at a reference (Background). The distances are between the microprofiler, benthic chamber and the Abyss vent. n.d. not determined.

		mmol m^{-2} d^{-1}					
Site	distance to vent (m)	AOM	SR	TOU	DOU	H_2S flux	Reference
High CO_2 seepage	0.5	0.1	5			5.0	this study
	10				18.1	3.0	this study
Low CO_2 seepage	25		0.1				this study
	50					1.4	this study
Background	1000	0	0.0	0.8	1.7	0	this study
Hydrate Ridge Bacterial mats		99[a]	32[a]	48[b]	n.d.	60[c]	[a]Treude et al. 2003; [b]Sommer et al. 2006; [c]Sahling et al.2002
HMMV Bacterial mats		9.8[d]	14.2[d]	107.5[d]	33.7[e]	11.6[e]	[d]Felden et al., 2010; [e]Lichtschlag et al., 2010
Nyegga Bacterial mats		n.d.	113.6[f]	n.d.	15.8[f]	8.2[f]	[f]Grünke et al. 2012
Storegga Bacterial mats		n.d.	23.2[f]	n.d.	15.6[f]	7.6[f]	[f]Grünke et al. 2012
Guaymas hydrothermally influenced sediment			50[g]			50 - 150[h]	[g]Holler et al. 2011; [h]Winkel et al. in prep.

Table 4 454 massively parallel tag sequencing reads for the sites Background, Low CO_2 Seepage, and High CO_2 seepage and three different depths horizons. Percentages in parentheses.

Sites	**Background**	**Low CO_2 Seepage**	**High CO_2 seepage**	**Total**
Sum reads **(total tag reads)**				235,731
Different sequences				101,513
Common sequences (%)				8 (0.003)
singleton sequence reads (%*)	50,176 (25)	79,198 (40)	69,531 (35)	198,905 (84)
Sum reads 0-1cm	20,659	30,554	30,668	
Sum reads 4-5cm	24,391	29,317	24,030	
Sum reads 9-10cm	15,016	30,296	30,800	
No. of different seq. 0-1cm	18,893	28,501	27,260	
No. of different seq. 4-5cm	22,170	27,460	21,112	
No. of different seq. 9-10cm	13,550	28,181	26,364	
singleton reads per 0-1cm	17,901	27,419	26,174	
singleton reads per 4-5cm	21,006	26,449	20,166	
singleton reads per 9-10cm	12,799	27,091	24,908	
Different **OTU 3%**				79,263
Singleton OTUs (%)				62,921 (79)
Common OTUs (%)				64 (0.08)
No. of different OTUs 0-1cm	6,208	17,450	9,778	
No. of different OTUs 4-5cm	6,682	16,751	8,692	
No. of different OTUs 9-10cm	3,224	16,881	7,504	
OTU (3%) singletons[#] (%)	7,826 (12)	38,369 (61)	16,726 (27)	
singleton OTUs 0-1cm	4,288	14,866	7,768	
singleton OTUs 4-5cm	4,620	14,253	6,932	
singleton OTUs 9-10cm	1,765	14,211	5,528	

* from total singleton reads, # from total OTU singletons

Table 5 Selected bacterial sequence types assigned by 454 massively parallel tag sequencing representing the shift in the bacterial community structure with CO_2 impact. Numbers are sequence reads.

Phyla	***Class***	***Order***	***Family***	***Genus***	**Background**	**Low CO_2 seepage**	**High CO_2 seepage**
Chloroflexi	*Anaerolieae*	*Anaerolineales*	*Anaerolinaceae*	*Bellilinea*	497	329	303
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Rhodobium*	354	279	29
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	270	121	7
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfonema*	143	25	6
Proteobacteria	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*	77	57	8
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Prosthecomicrobium*	76	68	4
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Filomicrobium*	71	41	3
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Bradyrhizobium*	10	0	3
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurimonas*	79	56	3495
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulforhopalus*	48	9	2782
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfocapsa*	34	106	1014
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurovum*	28	282	5470
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromusa*	14	49	1611
Thermotogae	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*Kosmotoga*	0	0	66
Proteobacteria	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosomonas*	253	651	19
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Nitrospinaceae*	*Nitrospinaceae*	57	269	0
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Thiothrix*	21	203	3
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geopsychrobacter*	19	161	4
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	*Ectothiorhodospiraceae*	*Thioalkalispira*	10	97	12
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Wenxinia*	0	12	0

Supplementary Material

Supplementary Table 1a Numbers of sequence reads shown in Figure 5a

Phyla	**Background**	**Low CO_2 seepage**	**High CO_2 seepage**
Proteobacteria	30,849	50,599	49,232
Planctomycetes	9,911	11,309	2,786
Acidobacteria	3,204	6,741	739
Bacteroidetes	2,901	5,617	11,392
Chloroflexi	2,318	2,456	4,398
Actinobacteria	1,960	3,905	910
Gemmatimonadetes	895	1,433	129
WS3	661	798	1,321
Verrucomicrobia	572	1,521	218
Deferribacteres	357	430	1,207
Fusobacteria	114	42	1,344
OP9	21	16	2,913
Spirochaetes	612	166	2,602
OP8	294	106	1,037

Supplementary Table 1b Numbers of sequence reads shown in Figure 5b

Class	**Background**	**Low CO_2 seepage**	**High CO_2 seepage**
Gammaproteobacteria	14274	28310	19721
Deltaproteobacteria	12140	15641	19461
Alphaproteobacteria	3967	5572	840
Betaproteobacteria	356	735	35
Epsilonproteobacteria	112	341	9175

Supplementary Table 1c Numbers of sequence reads shown in Figure 5c

Phyla	**Background**	**Low CO_2 seepage**	**High CO_2 seepage**
Planctomycetes	9911	11309	2786
Acidobacteria	3204	6741	739
Bacteroidetes	2901	5617	11392
Chloroflexi	2318	2456	4398
Actinobacteria	1960	3905	910
Gemmatimonadetes	895	1433	129
WS3	661	798	1321
Verrucomicrobia	572	1521	218
Deferribacteres	357	430	1207
Fusobacteria	114	42	1344
OP9	21	16	2913
Spirochaetes	612	166	2602
OP8	294	106	1037

Chapter III

Impact of high CO_2 concentrations on macrobenthic and meiobenthic community structure in deep-sea sediments of the Yonaguni Knoll IV hydrothermal system (Okinawa Trough, 1350 m)

Impact of high CO_2 concentrations on macrobenthic and meiobenthic community structure in deep-sea sediments of the Yonaguni Knoll IV hydrothermal system (Okinawa Trough, 1350 m water depth)

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Abstract

The Yonaguni Knoll IV hydrothermal vent system is a unique deep-sea ecosystem where liquid CO_2 rises from subsurface reservoirs and mixes with porewater or is vented to the hydrosphere. This CO_2 leakage causes acidic pH values at the seafloor and its overlying bottom waters. We examined metazoan macrofaunal and meiofaunal communities at three variedly CO_2-influenced habitats characterized as pH neutral (Background), moderately acidified (Low CO_2 seepage), and profoundly acidified (High CO_2 seepage). Abundance, distribution patterns and richness of macro- and meiobenthic communities were quantified. Macro- and meiofauna showed different responses to elevated CO_2 concentrations in porewaters. The macrofauna was dominated by polychaetes, crustaceans and gastropods in the Background area with lower abundances at the CO_2 impacted habitats. In contrast, high abundances of juvenile bivalves were detected in the High CO_2 seepage habitat. Meiofauna communities were dominated by nematodes, and were similar in abundance and structure at the background and Low CO_2 seepage areas, but differed substantially at High CO_2 seepage. One specific nematode genus *Thalassomonhystera* dominated all three habitats and showed highest densities at High CO_2 seepage, along with several other nematode taxa. Our study of a CO_2-leaking deep-sea ecosystem shows substantial effects of seafloor acidification on macrofauna and meiofauna community composition, such as local losses and replacement of CO_2-sensitive taxa. Ecosystem surveys for the assessment of potential ecological risks of Carbon Capture and Storage in the deep seabed must include high-resolution biodiversity studies in relation to biogeochemical gradients.

Keywords: Macrofauna, meiofauna, nematodes, hydrothermal vent, high CO_2, extreme environment, Carbon Capture and Storage (CCS)

Introduction

The global average atmospheric CO_2 concentration increases by more than 3% per year, and may reach levels of > 1000 ppmv until the year 2100 (IPCC 2007; Le Quéré et al. 2009). Ocean and atmosphere exchange large amounts of CO_2, and the ocean is a major sink for atmospheric carbon dioxide, causing its acidification with yet unknown effects for marine life and human well-being (Gattuso and Hansson 2011a). Furthermore, among several other greenhouse gas mitigation strategies, the ocean seabed is considered as a sink to reduce anthropogenic CO_2 emissions by Carbon Capture and Storage (CCS) techniques, such as the injection of liquid CO_2 in depleted subsurface oil and gas reservoirs. This technique bears risks such as the leakage of CO_2 to the surface seafloor and hydrosphere, potentially causing a severe local reduction in pH with yet unknown ecological consequences.

Acidification of seawater by dissolving CO_2 can affect marine life in many ways. It can alter organism performance due to effects on basic metabolic functions such as ion transport, membrane potential and enzyme activities. Changes in the pH of body fluids can lead to an increased energy expenditure for buffering, affecting productivity and mortality of animals (Pörtner et al. 2004). Furthermore, calcifying organisms such as coccolithophores, corals, molluscs and echinoderms face altered formation and dissolution rates of calcite and aragonite (Doney et al. 2009). At the community level, acidification could hence change rates of photosynthesis, calcification and nitrogen fixation of phytoplankton and hence influence food-webs and particle export from the surface to the ocean floor (Riebesell et al. 2000; Levitan et al. 2007; Lohbeck et al. 2012).

However, ocean acidification research of the past decade has also shown that assessments and predictions of ecological effects of high CO_2 and low pH at the community and ecosystem level are difficult because of the wide range of species-, life-cycle and habitat-specific biological responses (Pörtner and Farrell 2008). Most studies of ecological acidification effects have focused on phyto- and zooplankton, corals and macroalgae (Gattuso and Hansson 2011b), and only few were yet concerned with sedimentary benthic habitats (Widdicombe et al. 2011). Marine sediments have a strong buffering potential for increases in CO_2 within the range predicted for the next 100 years. Furthermore, natural CO_2 and pH fluctuations in marine porewaters can be very large, from processes such as advective fluid transport, organic matter remineralization, and anaerobic microbial metabolism. However, as the submarine seabed is considered for injection and storage of CO_2 from CCS, the risk of local leakage of CO_2 on benthic communities must be assessed.

At very high CO_2 levels, benthic organisms could respond by tolerance, compensation, migration or death (Barry 2003). Such responses could cause substantial shifts in community structure and function, depending on the level and duration of exposure of the benthic communities to high CO_2. So far, two approaches have been used to study ecological effects of high CO_2 leakage (equivalent to molar concentrations of CO_2 in seawater). Earlier studies looking at the potential ecological risks of CCS have used mesocosm studies, including experimental *in situ* impact studies at the deep sea floor, to assess the responses to high CO_2 and/or low pH of megafauna (Barry 2003; Barry and Drazen 2007), or of meiofauna (Barry et al. 2004, 2005; Carmann et al. 2004; Thistle et al. 2005; Fleeger et al. 2006; Watanabe et al. 2006; Thistle et al. 2006; Kurihara et al. 2007; Thistle et al. 2007; Ricketts et al. 2009; Bernhard et al. 2009; Fleeger et al. 2010). This approach allows controlling the duration and level of exposure to high CO_2, as well as to minimize environmental heterogeneity and cofounding effects. However, it serves mostly to study short-term effects (days to months) on organism performance and mortality, but is limited with regard to long-term effects such as animal migration, species turnover and adaptation.

Another approach to study *in situ* effects of elevated CO_2 concentrations on benthic community ecology is the targeted sampling of naturally CO_2-leaking environments such as shallow (Hall-Spencer et al. 2008; Fabricius et al. 2011) and deep-sea (Inagaki et al. 2006; Tunnicliffe et al. 2009; Nonoura et al. 2010, Yanagawa et al. submitted) hydrothermal vents. This allows assessing long-term effects such as selection processes and community succession, but is often complicated by cofounding effects such as the co-migration of chemical energy in CO_2-rich vent fluids. Also, it remains a technological challenge to assess *in situ* CO_2 concentrations, pH and other environmental parameters at the respective pressure and temperature regimes. In any case, because of the much higher solubility of CO_2 at high pressure, *in situ* studies at the deep-sea floor are the only way to assess long-term ecological effects of the risks associated with CCS for deep-sea benthic community structure and function (Barry et al. 2011).

Here we report the results from a study on benthic metazoan macrofauna and meiofauna communities at one of the few known natural CO_2-leaking deep-sea sedimentary systems: the Yonaguni Knoll IV hydrothermal vent system. The Yonaguni Knoll IV hydrothermal vent system is part of a back-arc spreading center and comprises a sedimented valley with many CO_2-leaking hydrothermal vents, surrounded by volcanic rock debris (Inagaki et al. 2006; Konno et al. 2006). Our main question was as to the distribution and structure of macro- and the meiofauna communities along a natural gradient from very high CO_2 concentrations (20-60 mM, pH < 5) in the sediments, to background concentrations. Therefore we identified three habitats including a

Background area, and an area of low and high CO_2 seepage where both meiofauna and macrofauna communities were characterized and quantified in broad taxa categories. Special emphasis was put on nematode identification. Generally, nematodes are among the most abundant metazoan taxa in the deep-sea environment and are therefore considered an important indicator for habitat heterogeneity (Vanreusel et al. 2010b). Despite their widespread and frequent appearance, still little is known on their ecology and biogeography. We characterized differences in terms of benthic community densities and composition in order to investigate the following research questions: (1) to what extent differ the benthic communities along a natural CO_2 gradient, and (2) do different taxa show different responses to these gradients; we hypothesized that mainly calcified taxa will show a strong response to the highest seepage concentrations, and that high CO_2 causes a reduction in densities and diversity of deep-sea taxa of both size-classes.

Material and Methods

In spring 2008 samples were taken during the RV Sonne 196 expedition for the project SUMSUN "Studies of marine CO_2-sequestration associated with a natural hydrothermal CO_2-system of the Northern West Pacific" (Rehder and Schneider von Deimling, 2008). Sediment samples for macrofauna and meiofauna quantification were taken along a CO_2 gradient from a background area to the source vents. We explored the Yonaguni Knoll IV hydrothermal field located in the Okinawa Trough (24°50.7´N, 122°42.0´E; 1,380-1,382 m water depths, Inagaki et al. 2006) by using the ROV Quest (MARUM) for videographic surveys and for biogeochemical analyses (DeBeer et al. Chapter 2.I, this thesis). For retrieving sediment samples either push cores (80 mm core diameter) were operated by the ROV, or a TV-guided, GPS navigated Multiple Corer (MUC, 60 mm (meiofauna) and 100 mm (macrofauna) core diameter) was used. Three different habitats were sampled according to the biogeochemical pre-characterization: a Background area (no CO_2 flux), an area with Low CO_2 seepage (low CO_2 flux), and another one surrounding a focused CO_2 vent with High CO_2 seepage (high CO_2 flux). This vent is known as "Abyss vent" and has been previously characterized in biogeochemical and microbiological studies (Inagaki et al. 2006; Konno et al. 2006; Nunoura et al. 2010; Yanagawa et al. submitted). The *in situ* biogeochemical measurements used to characterize the CO_2 and pH gradient at the seafloor and the bottom water interface were done with a profiling module equipped with microsensors for O_2, pH, temperature and CO_2 (methods provided in DeBeer et al., in prep;

Chapter 2.I this thesis). For each of the three habitats at least three replicates were taken (see Fig. 1). As it was difficult to replicate the intermediate CO_2-impacted habitat "Low CO_2 seepage" because of the lack of morphological or geographical indicators at the seafloor, pseudo-replicates from a multi core deployment (MUC 3) were included in the analysis. Sampling station description and coordinates are listed in Table 1 and Figure 1.

Sample treatment

Sediment cores were cut into horizons of 1 cm thickness (0-5 cm section), 2.5 cm (5-10 cm section), and a final layer of 5 cm (10-15 cm). Macrofauna samples were sieved onboard through a 1 mm sieve, brought into plastic bottles and filled with 4% buffered formaldehyde/seawater. Meiofauna samples were directly preserved in 4% buffered formaldehyde/seawater without sieving.

Meiofauna extraction

The complete content of one sample was poured on a 1 mm sieve placed above a 32 µm-sieve and washed with tap water. Initial washing was done to remove the gross of sediments. The > 1 mm fraction was directly transferred into plastic bottles filled up with formalin right after washing, to a final concentration of 4% formaldehyde/seawater. The extraction of the meiofauna was done by density gradient centrifugation using Ludox (a colloidal silica polymer) as a flotation medium (Heip et al. 1985). Subsequently, the samples were stained with Rose Bengal solution and stored at a final concentration of 4% formaldehyde/seawater.

Macrofauna, meiofauna and nematode identification

Macrofauna and meiofauna identification was based on comparative morphological identification and taxonomic classification of the groups (Table 2). For nematode identification 100 nematodes from the uppermost sediment layer were picked out randomly and 10 at a time were mounted on a glycerin microscope slide for classification to genus level. For one site of each habitat, densities were determined in all depth layers. As > 80 % of the communities were restricted to the upper two cm layers and no habitat specific depth gradient was observed, the further meiofauna analyses were confined to the top 2 cm (Vincx et al. 1994).

Statistical analysis

Non-metric multidimensional scaling (nMDS) ordination was applied using the Bray-Curtis similarity index to measure dissimilarity between the Background, Low CO_2 seepage, and

High CO_2 seepage habitat with regard to meiofauna composition. The stress value gives a measure for the goodness-of-fit for the nMDS ordination a low value of < 0.2 indicates good ordination of the data (Paine 1966). One-way analysis of similarity (ANOSIM) was carried out to test for significant differences in the community structure between the habitats. All analyses mentioned above were performed with the free software PAST (PAleontological STatistics, v. 1.89; (Hammer et al. 2001).

Results

Visual observations and biogeochemical characterization of habitats

The three habitats sampled for the assessment of meiofauna and macrofauna distribution patterns were characterized in DeBeer et al. in prep (Chapter 2.I; this thesis) and in Neumann et al. in prep. (Chapter 2.II; this thesis). Briefly, based on Aanderaa oxygen optodes measurements, oxygen concentrations in the bottom waters above the sediment showed all similar depleted values (around 80 μmol l^{-1}), Oxygen penetration into the sediments was slightly deeper at the Background compared to the seepage areas, but was restricted to a few mm. CO_2 concentrations increased substantially from the Background area > 1 km away towards the active vent chimneys (Fig. 1). The seafloor area surrounding the CO_2-emitting Abyss vent (< 300 m) was characterized by an absence of bottom dwelling megafauna and lacked typical features of bioturbation, burrows and other traces of life. Around Abyss vent (0.5-20 m distance; High CO_2 seepage (HS) habitat) we saw whitish-yellowish sulfur precipitates at the seafloor, and observed emission of hot CO_2 rich fluids as well as liquid CO_2 bubbles, the latter especially upon disturbance of the seafloor by coring. The Low CO_2 seepage (LS) habitat (20-50 m distance to Abyss vent) was also characterized by an absence of biogenic seafloor traces, but no escape of liquid CO_2 was observed upon disturbance. In the top 10 cm of seafloor sediments, CO_2 concentration decreased with distance from the Abyss vent. Within the High CO_2 seepage habitat, low pH values of around 5 were measured in the surface sediments, and CO_2 saturation was reached below 15 cm sediment depth (DeBeer et al. in prep, Chapter 2.I, this thesis). At the Low CO_2 seepage habitat, surface sediments had a pH of 6.2 decreasing to 4.9 at 15 cm. At the Background (B), an *in situ* pH of 7.5 was measured throughout the top 15 cm. Due to increasing subsurface hydrothermalism with decreasing distance to Abyss vent, temperatures of the top 0-5 cm depth horizon differed from the Background to the High CO_2 seepage sites as follows: $T_{(Background)}$= 4.9-5.0 °C, $T_{(Low\ CO2\ seepage)}$ = 3.1-5.4 °C, $T_{(High\ CO2\ seepage)}$ = 3.6-7.9 °C. Increasing availability of chemical

energy (e.g. methane, sulfide, ammonium) with increasing proximity to the vent caused 50% higher bacterial cell abundances around the vent compared to the Low CO_2 seepage area and the Background (Neumann et al. in prep, Chapter 2.II), which showed typical average cell counts for deep sea sediments around a 1000 m water depth (Jørgensen and Boetius 2007).

Meio- and macrobenthic densities and community composition

Of all three habitats, meiofaunal densities showed highest numbers at the Low CO_2 seepage habitat, mainly caused by a significant increase of nematodes compared to the Background area (Fig. 2 a, b). Total meiofaunal densities at the Low CO_2 seepage habitat (2,146 ± 317 ind. cm^{-2}) differed significantly from the Background (829 ± 191 ind. cm^{-2}) and High CO_2 seepage (785 ± 350 ind. cm^{-2}) habitats based on ANOVA ($F = 23.62$, p (same) < 0.001) and post-hoc comparison (Tuckey's pairwise comparison: $p = 0.0015$ (LS and B) and $p = 0.0013$ (LS and HS)). Nematodes accounted for > 80% of the meiofauna organisms at all sites (Table 2). Successively, the further most abundant taxa in the sediments were copepods (including nauplii), as well as polychaetes. Most meiofauna taxa were present at all sites (15 out of 27) except for echinoderms and gnathostomulids, which were only found at the Background, while aelosomatids, cnidarians, cumacaeans, and nemertines were only present at the High CO_2 seepage habitat, although in very low abundances. Tunicates were exclusively found at the Low CO_2 seepage habitat. Amphipods, gastropods, halacaroids, priapulids, and syncardia were present at the Background and High CO_2 seepage habitat, but apparently excluded from the Low CO_2 seepage area (Table 2 a-c). Unique to the Low CO_2 seepage habitat was a relatively high abundance of kinorhynchs (Table 2 b, all kinorhynch specimen are novel species, personal communication B. Neuhaus, Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin).

Of all habitats, the variation in meiofaunal densities was highest at the High CO_2 seepage habitat as confirmed by ANOSIM R. This was attributed to one of the three replicates sites samples, namely MUC 10, which revealed elevated taxa richness compared to the low richness of the other samples taken at the High CO_2 seepage habitat (PC 20 and MUC 19). The high variation within the High CO_2 seepage habitat was visualized by nMDS (Fig. 3). Differences in community composition were also significant between the Background and Low CO_2 seepage site ($p < 0.03$), however they were not recorded by the classical Shannon Wiener and Hill's biodiversity indices (Tab. 2)

In contrast to meiofauna density, average metazoan macrofaunal densities were almost equal for the Background (15,218 ± 934 ind. m^{-2}) and the High CO_2 seepage habitat (15,300 ±

8,990 ind. m^{-2}), but showed a low at the Low CO_2 seepage habitat (7,830 ± 6,267 ind. m^{-2}). However, large variations between taxa were observed. Polychaetes densities were significantly higher at the Background (7,456 ± 1,609 ind. m^{-2}), compared to Low CO_2 seepage (4,488 ± 5,399 ind. m^{-2}), and High CO_2 seepage (2,190 ± 664 ind. m^{-2}) according to Levene's test for homogeneity of variance, based on means: $p = 0.024$; Welch F test: $p = 0.0036$. Echinoderms were only present at the Background and not at the CO_2-impacted sites. Molluscs had maximum numbers at High CO_2 seepage, but this was caused by the high densities of juvenile bivalves (7,727 ± 6,135 ind. m^{-2}; Fig. 4). These bivalves were identified as normal deep-sea taxa, with no indication of a specific vent association (personal communication R. Janssen, Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt am Main). In contrast, molluscs at the Background comprised mostly gastropods and solenogaster. Hence, macrofaunal community composition varied strongly between the habitats but also within the sites sampled at the seepage habitats (Fig. 4).

Nematode diversity

All three sites were colonized by one specifically dominant nematode genus. The genus *Thalassomonhystera* accounted for more than 15% for the Background, and almost up to 40% for the Low CO_2 seepage and High CO_2 seepage habitats (Fig. 5, Fig. 6). At the Background, 25-26 other nematode genera were additionally found, respectively. At Low CO_2 seepage only 19-23 other genera occurred, and at High CO_2 seepage, diversity was even more reduced to 12-17 nematode genera. The five most abundant nematode genera in addition to *Thalassomonhystera* were identified as *Leptolaimus, Daptonema, Microlaimus, Terschellingia,* and *Desmodora* (Fig. 7). Proportions of the nematode genera differed among the sites: besides *Thalassomonhystera,* also *Daptonema, Leptolaimus, Microlaimus, Halomonhystera,* and *Halichoanolaimus* dominated the Background. The community was almost identical to the Low CO_2 seepage habitat, but instead of *Desmodora* (Background), *Halomonhystera* was more abundant. *Halalaimus* was among the topmost abundant genera at the High CO_2 seepage habitat, as well as *Terschellingia* (Table 3).

Discussion

Meio- and macrobenthic densities and community composition

Results of the meiofauna classification and quantification showed a significant change in community composition (beta-diversity), but not in taxa richness as recorded by typical diversity

indices (Tab. 2). In general, meiobenthic densities of the Background area of the Yonaguni Knoll IV valley (601-1,064 ind. 10 cm^{-2}) were similar or slightly increased when compared to average densities found in similar water depths of worldwide continental margins recorded so far (Soltwedel 2000). Yet, meiobenthic studies of the northwest subtropical regions of the Pacific are underrepresented (Vanreusel et al. 2010b) and comparison is difficult in terms of mesh size and water depth (Grove et al. 2006). Relatively high meiofaunal abundances in non-vented background sediments may be evoked by increased seasonal primary production in this region (Iseki et al. 2003). As samples were taken in March, sedimentation of particulate organic matter may be responsible for enhanced densities. Meiobenthic densities (Fig. 2b) at Low CO_2 seepage exceeded typical values three-fold (1,849-2,480 ind. 10 cm^{-2}), and even at the High CO_2 seepage habitat values were augmented (up to 1,094 ind. 10 cm^{-2}) compared to average values for similar water depths found at different study sites worldwide (Soltwedel 2000). The peak in meiofaunal density at Low CO_2 seepage is difficult to explain, as typical indicators of food availability such as organic matter content and bacterial cell counts did not show the same trend. We propose that it may be caused by reduced macrofaunal predation and competition (Fig. 2a) at the CO_2-impacted habitats, especially the Low CO_2 seepage sites.

In contrast to meiofauna densities, abundance of typical macrofauna taxa such as polychaetes and crustacea was highest at the Background site and decreased with increasing CO_2 impact (Fig. 2b). Our study did not include higher taxonomic resolution of macrofauna taxa, but we found some evidence that macrofaunal community composition changed with CO_2 impact. Macrofauna polychaetes and echinoderms appeared most sensitive to CO_2 leakage, as their numbers decreased from the Background to the High CO_2 seepage habitat by 50%, or to 0%, respectively. Crustacean taxa also changed between habitats, with cumaceans dominating the High CO_2 habitat. However, the presence of abundant juvenile deep-sea bivalves detected only at the High CO_2 seepage sites elevated densities of macrofauna to comparable numbers as at Background. The bivalves were identified to the families *Yoldiidae* and *Thyasiridae*, typical deep-sea representatives (personal communication R. Janssen, Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt am Main), the latter with a potential for association with chemosynthetic bacterial symbionts. We did not find adult bivalves at the CO_2 impacted habitats, so it is possible, that their lifespan is reduced by CO_2 effects.

Larger organisms can affect the assemblage structure of smaller organisms by predation, competition for food, or habitat structuring (e.g. bioturbation) (Olafsson 2003). Besides bigger predators, such as echinoderms, also deposit feeders often prey non-selectively on a variety of smaller organisms, and thus not only reduce their densities but also change the dynamics of a

whole ecosystem (Paine 1966). Fig. 2 indicates that meiofauna reached highest densities, where macrofauna abundance was lowest. In addition, camera surveys of Background and the seepage habitats showed an absence of burrowing and sediment-dwelling megafauna at the seepage sites which were abundant at the Background (especially holothurians; data not shown). Epibenthos exclusion experiments in bathyal soft bottom experiments revealed higher meiofauna densities within the exclusion cages (Gallucci et al. 2008) as a consequence of decreased grazing pressure by epibenthic predators (Dayton and Hessler 1972; Bell and Coull 1978; Gee 1987; Smith and Coull 1987; Feller et al. 1992). Furthermore, although direct effects on species richness and diversity could not be observed by Gallucci et al. (2008), the hypothesis of a lower functional diversity of meiofauna in the absence of megafauna was supported based on their results. Especially the apparent sensitivity of echinoderms to low pH and high CO_2, known to be keystone ecosystem engineers as deposit feeders, may have significant consequences for meiofauna communities (Dupont et al. 2010), and potentially also for macrofauna (Kukert and Smith 1992).

Since the Yonaguni Knoll IV area is part of a hydrothermal vent system, CO_2 seepage affecting porewater pH is not the only environmental variable to be considered. Hydrothermal vents are characterized as dynamic habitats with rapidly changing environmental conditions. Differences in the temperature regime between the Background and the CO_2-impacted sites were negligible (range of 4-7°C in the top 5 cm). However, DeBeer et al. (Chapter 2.I) detected a considerable spatial heterogeneity in the porewater CO_2 concentrations as well as in the co-migration of reduced compounds such as sulfide and ammonium. These compounds of the seeping vent fluids may have also affected organism distribution beyond a direct CO_2 effect, but was not further investigated here.

Nematode distribution and diversity

Nematodes have been shown to be relatively insensitive to physical disturbances of the sediment they live in (Sherman and Coull 1980; Austen et al. 1989; Warwick et al. 1990; Gee et al. 1992). At continental margins, bathyal nematodes generally show decreasing densities and biomass with increasing water depth and decreasing surface primary production, as well as increasing distance offshore (Vincx et al. 1994; Soltwedel 2000). Comparing meiobenthos along global continental margins (Soltwedel, 2000), the highest proportions of nematodes (~90% of total metazoan meiofauna) was found in meiobenthic communities from the West-Pacific region off Japan (Shirayama 1994), and food supply from pelagic particle sedimentation was suggested to be the most important factor controlling meiobenthic communities. At deep-sea cold seeps,

however, an increased standing stock in meiofauna and especially nematodes can be expected because of the abundance of chemosynthetically derived food (e.g. bacterial mats) which may lead to elevated nematode densities and/or biomass compared to the background sedimentary habitats (Van Gaever et al. 2006; Vanreusel et al. 2010). At seeps, nematodes can benefit from enhanced bacterial production, occasionally reaching densities of several thousand individuals 10 cm^{-2} , i.e. an order of magnitude higher densities as observed for adjacent deep-sea sediments (Vanreusel et al. 2010). In contrast, most deep-sea hydrothermal vents do not show increased numbers of nematode individuals or biomass. They are characteristically dominated by one single species, which can be as high as 66% on average, ranging from 15-100% (Vanreusel et al. 2010). However, most hydrothermal vent environments sampled today for meiofauna studies consist of relatively coarse debris of rocks and mineral precipitate and show impoverished communities compared to background sedimentary habitats (Shirayama 1992; Flint et al. 2006; Copley et al. 2007; Gollner et al. 2010). Low densities of nematodes were found at bacterial mats with individuals ranging from 1-78 ind. 10 cm^{-2} (Dinet et al. 1988), 1-72 ind. 10 cm^{-2} on bivalves (Flint et al. 2006; Zekely et al. 2006b; Zekely et al. 2006a; Copley et al. 2007), and 1-900 ind 10 cm^{-2} occurring with tubeworms (Gollner et al. 2007). Normally, three different reasons are put forward to explain the low densities of nematodes: (1) unsuitable substrate, since some vents consist of hard substrates such as basalt rocks and sulfide precipitates, (2) bottom-up control, where high energy availability is not facilitating proper food supply for nematodes and its utilization, (3) top-down control, the suppression of nematode numbers due to biotic interactions such as predation, and competition as for instance by macro-invertebrates (Vanreusel et al. 2010). In this study, the densities and diversity of nematodes observed (Fig. 5 and 6) at the Yonaguni Knoll IV hydrothermal systems matches those of cold-seep habitats.

Even at the High CO_2 seepage sites characterized by subsurface liquid CO_2 migration and porewater of pH 5, no substantial reduction in nematode densities was observed compared to the background. However, in a short-term experiment, direct exposure to liquid carbon dioxide caused a high nematode mortality (Barry 2003; Barry et al. 2004). Here, nematode distribution at High CO_2 seepage was confined to 90% to the top 2 cm of seafloor, which was not directly exposed to liquid CO_2, hence the nematode communities may have been adapted to relatively high – but not saturated levels of – CO_2 and low pH. Nevertheless, we observed a change in the composition of nematode genera (e.g. increase of the genus *Terschellingia,* decline of several genera typical for the background zone; Fig. 5)

One of the most prominent nematode taxa of hydrothermal vents is the ubiquitous deep-sea genus *Thalassomonhystera* which was also very numerous in the samples we obtained. Although

little is known about the diet of deep-sea nematodes, non-selective deposit feeders were often shown to be the dominant trophic group in terms of abundance and biomass contribution (Ingels et al. 2009; Ingels et al. 2011). *Thalassomonhystera* can be assigned to this group (Riemann 1995). The reduced sediments at High CO_2 seepage habitat however exhibited a different nematode community with higher occurrence of the nematode genus *Terschellingia*, which is commonly found in organically enriched, oxygen poor shallow water habitats with a widespread geographical range including estuarine sediments of the North Sea (Heip et al. 1985; Vranken et al. 1988; Vranken et al. 1989), mangrove mudflats off northeastern Australia (Alongi 1987; Nicholas et al. 1991; Fisher and Sheaves 2003), the southern coast of India (Chinnadurai and Fernando 2007), off the Atlantic coast of France, in the Black Sea (Sergeeva 1991), and the Gulf of Mexico, off eastern China (Qingdao province, (Zhang 1994)), as well as New Zealand and the Solomon Islands (Burgess 2005). Also, Van Gaever et al. (2010) found the genus occasionally typically associated with reduced sediments along the Nordic margin. The presence of the genus in the High CO_2 seepage site may be associated to the relatively high concentration of sulfides in the vented sediments and illustrates that several nematode genera may be able to adapt to high CO_2 and low pH.

Conclusions

This study focused on the effect of natural high CO_2 leakage on deep-sea benthic meiofauna and macrofauna assemblages. Our results show no significant reduction in total faunal density with increasing seepage intensity, and minor shifts in the distribution at the phylum level (meiofauna), but potentially larger effects to several groups of macro- and megafauna, which needs further investigation. At higher taxonomic resolution, even for the meiofauna a substantial turnover of community composition was observed, supporting previous observations that high CO_2-low pH sensitivity is taxa-specific and may induce substantial ecological effects.

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Figures and Tables

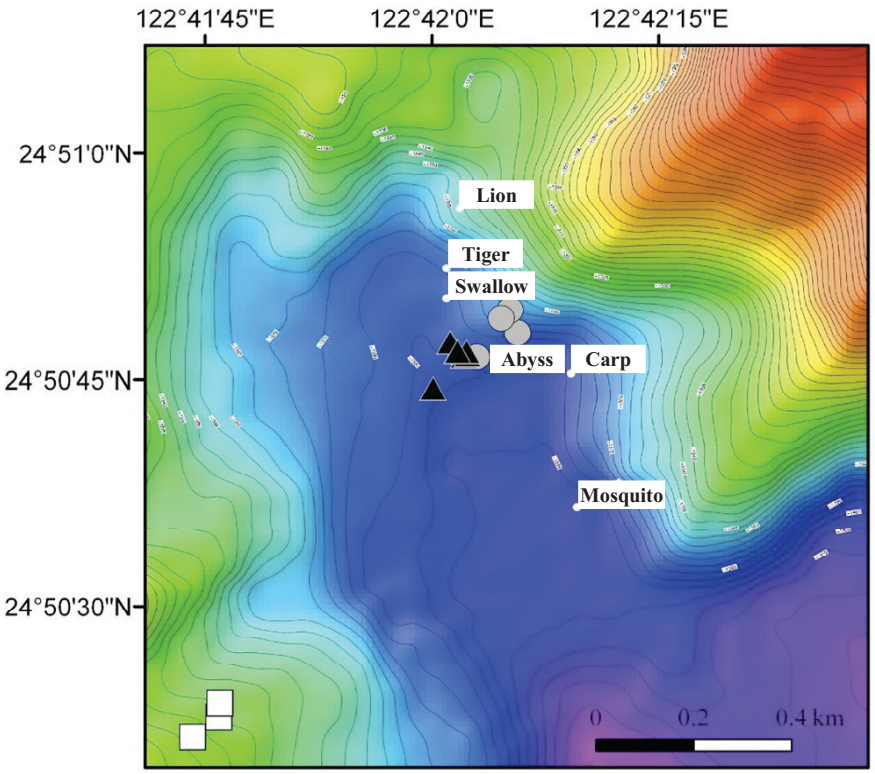

Figure 1 Overview of the Yonaguni Knoll IV hydrothermal vent system. Symbols for sediment samples for the Background are white squares, for High CO_2 seepage black triangles, and for Low CO_2 seepage grey circles, respectively.

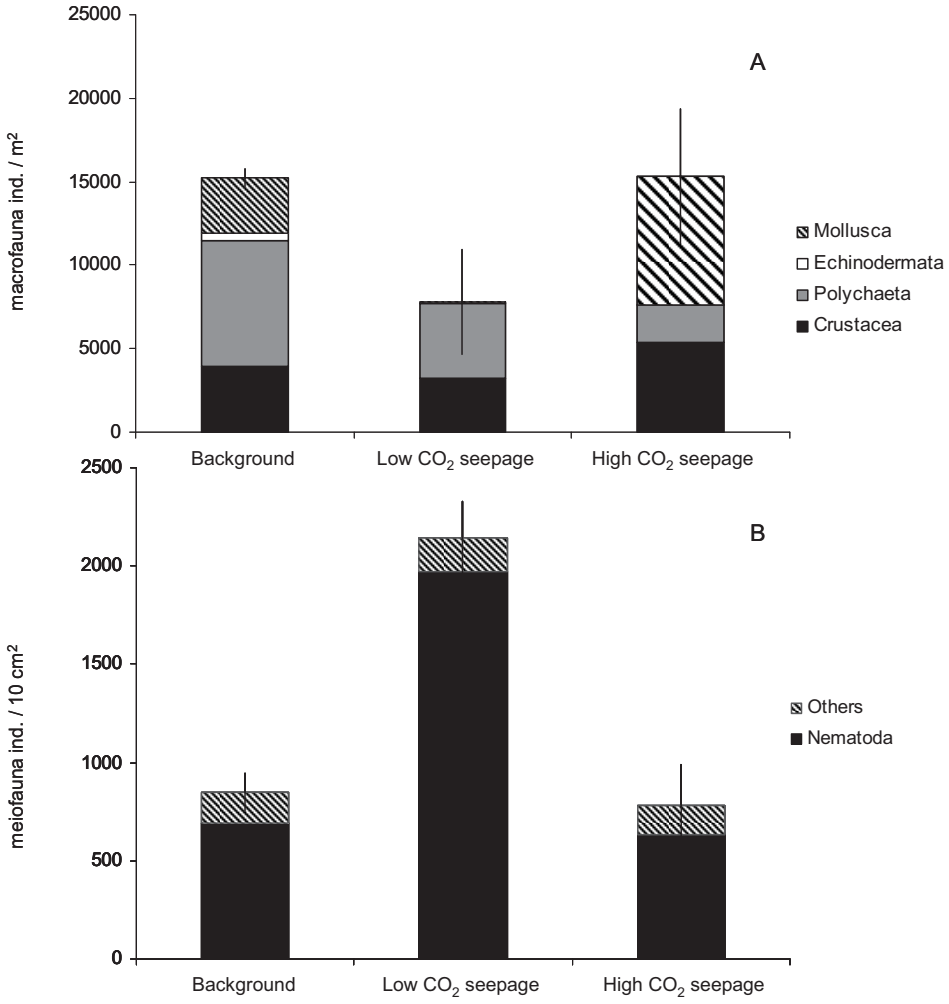

Figure 2 (A) Macrofauna densities, (B) Meiofauna densities at the different habitats Background, Low CO_2 seepage, and High CO_2 seepage. Color coding for macrofauna mollusks at High CO_2 seepage site indicates the predominance of mainly juveniles. Columns show mean ± SE.

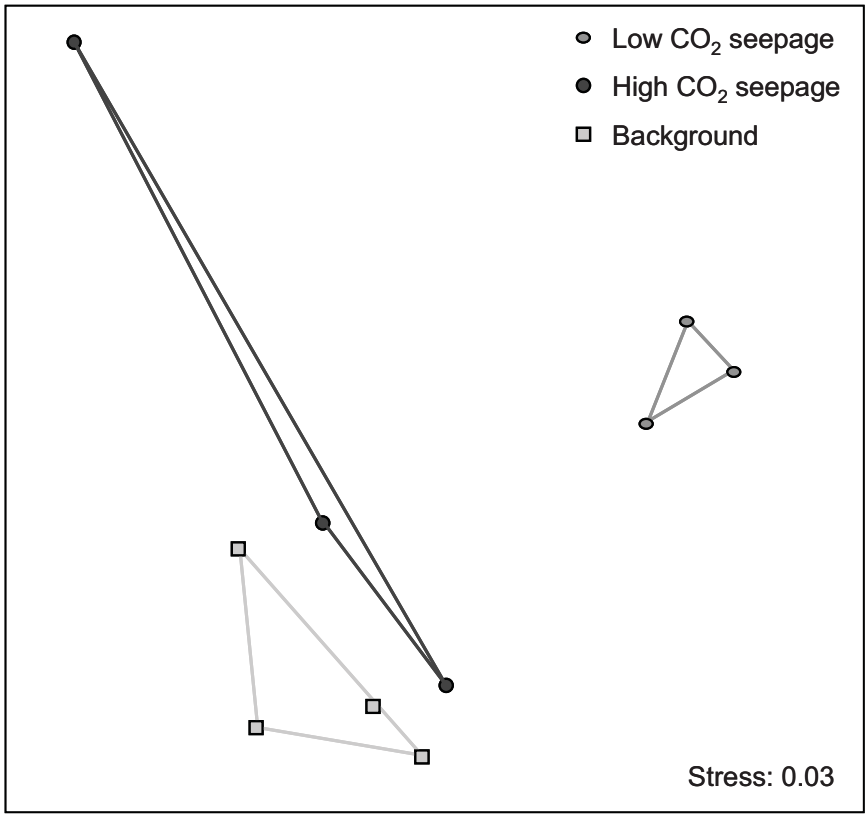

Figure 3 Non-metric multi-dimensional scaling based on meiofauna taxa densities. ANOSIM confirmed significant differences for the overall model ($p = 0.01$), as well as between Background and Low CO_2 Seepage ($p = 0.03$). R values illustrate higher variation within the groups of High CO_2 seepage than for the other habitats. Overall R value = 0.61.

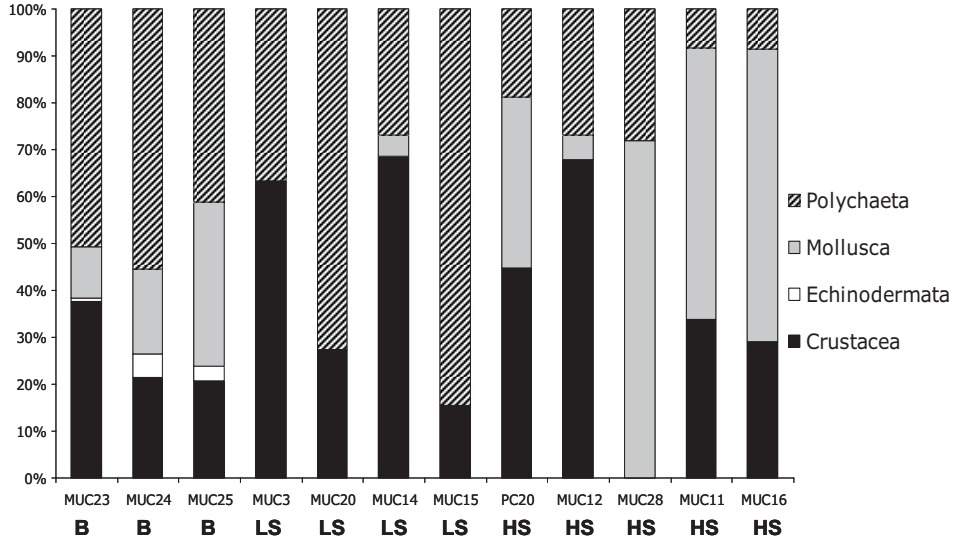

Figure 4 Proportional abundance of the identified macrofaunal groups of all samples for the Background (B), Low CO_2 seepage (LS), and High CO_2 seepage (HS) habitats.

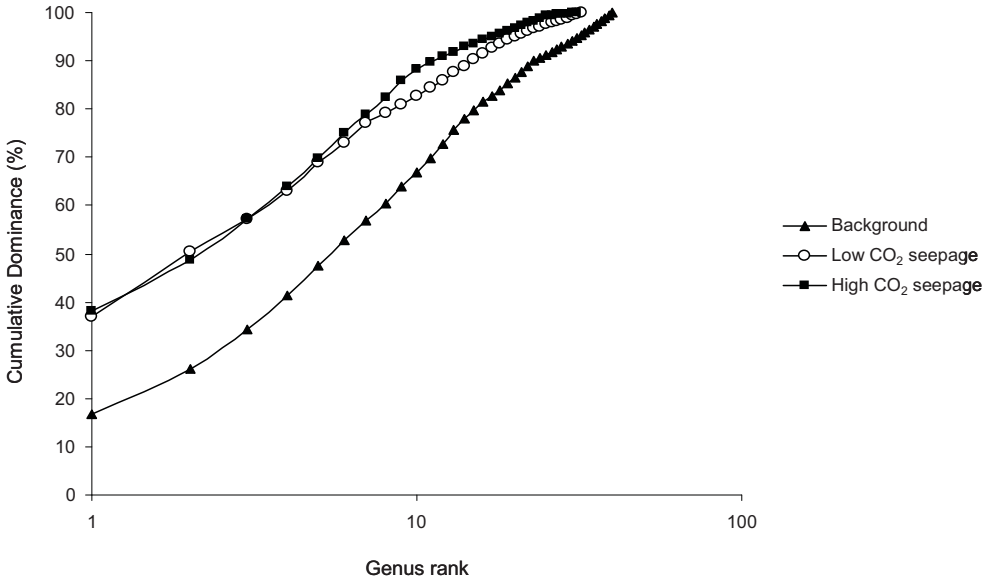

Figure 5 K-dominance curve of all nematode genera identified within the different replicates and pooled for the habitats Background, Low CO_2 seepage, and High CO_2 seepage. The x-axis shows on a logarithmic scale the different genera ranked according to their relative abundance. The y-axis shows the cumulative percentage (that is the most prominent genus plus all other genera).

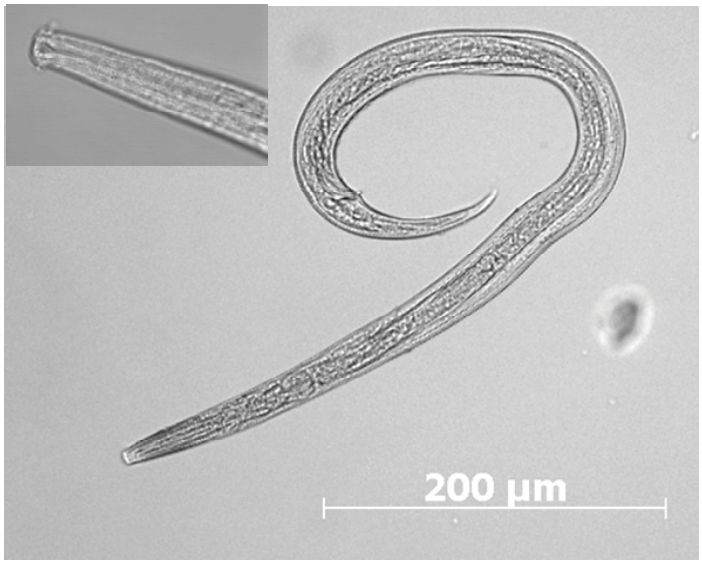

Figure 6 Micrograph of the most abundant nematode genus: *Thalassomonhystera*, inset figure showing the buccal cavity.

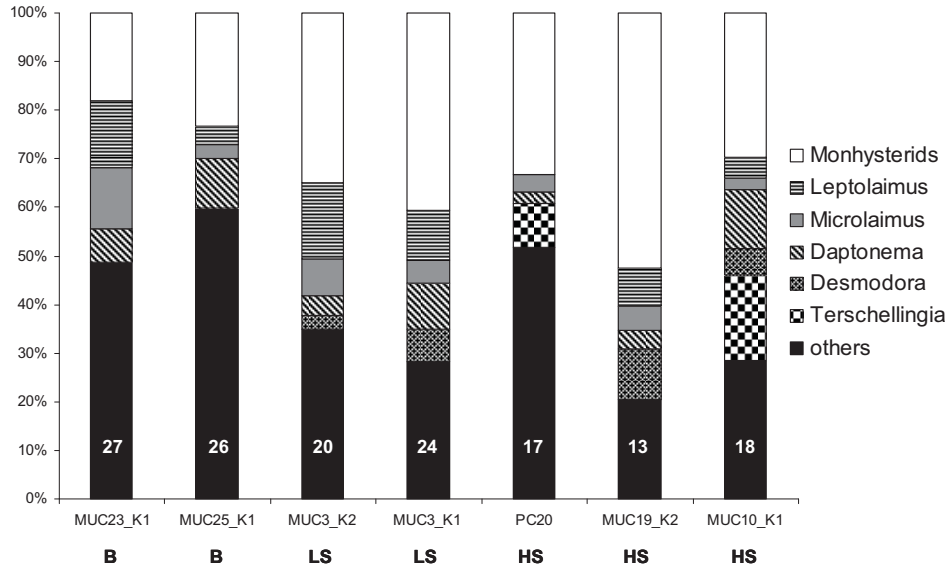

Figure 7 Relative abundance of the most prominent nematode genera present at the three different habitats Background (B) Low CO_2 seepage (LS), and High CO_2 seepage (HS). Numbers in white represent the total of all genera identified at each site. *Thalassomonhystera* and *Halomonhystera* combined in Monhysterids.

Table 1 Sampling sites of the three investigated habitats. Meiofauna samples are labeled [A], macrofauna samples are labeled [B]. The Pangaea event label is linked to further metadata in the Word Data Center PANGAEA (http://www.pangaea.de/ddi?retr=events/Sonne/SO196.retr&conf=events/CruiseReportHTML.conf&title=Station+list+of+cruise+SO196&format=html).

Site	Station	Date	Latitude	Longitude	Depth [m]	Pangaea_event
Background	MUC23 [A,B]	3/18/08	24.83925	122.69560	1324	SO196_65
Background	MUC24 [A,B]	3/18/08	24.83963	122.69608	1323	SO196_66
Background	MUC25 [A,B]	3/18/08	24.83990	122.69610	1318	SO196_67
Low CO_2 seepage	MUC3 [A,B]	3/09/08	24.84712	122.70143	1372	SO196_15
Low CO_2 seepage	MUC20 [B]	3/17/08	24.84697	122.70127	1318	SO196_55
Low CO_2 seepage	MUC14 [B]	3/14/08	24.84625	122.70082	1385	SO196_39
Low CO_2 seepage	MUC15 [B]	3/14/08	24.84670	122.70157	1382	SO196_40
High CO_2 seepage	PC20_Dive 201 [A,B]	3/13/08	24.84635	122.70046	1382	SO196_30_PUC20
High CO_2 seepage	MUC10 [A]	3/08/08	24.84652	122.70033	1392	SO196_25
High CO_2 seepage	MUC11 [B]	3/14/08	24.84568	122.70002	1389	SO196_36
High CO_2 seepage	MUC12 [B]	3/14/08	24.84625	122.70058	1385	SO196_37
High CO_2 seepage	MUC16 [B]	3/17/08	24.84628	122.70065	1365	SO196_49
High CO_2 seepage	MUC19 [A]	3/08/08	24.84635	122.70063	1387	SO196_54
High CO_2 seepage	MUC28 [B]	3/22/08	24.84635	122.70047	1394	SO196_95

Table 2 A-C Densities, relative abundance of all meiofaunal taxa, and taxa richness found in 0-2 cm sediment at (A) Background, (B) Low CO_2 Seepage, and (C) High CO_2 Seepage normalized to 10 cm^{-2}; Shannon Wiener and Hill's index are shown. Significance was tested using ANOVA; * significant values < 0.001).

(A) Background								
Taxon	**MUC23_1**	**MUC23_2**	**MUC24_1**	**MUC25_1**	**Sum**	**mean**	**SD**	**rel. abundance**
Density (ind. 10 cm^{-2})								
Aelosomatidae								
Amphipoda	5.66		0.71	0.35	6.72	1.68	2.67	0.20
Bivalve	0.35	2.83	0.35	0.71	4.24	1.06	1.19	0.13
Cladocera	6.01	0.35	1.41		7.78	1.95	2.78	0.23
Cnidaria								
Copepoda	75.33	92.31	35.72	34.66	238.03	59.51	28.92	7.18
Nauplii	24.76	81.70	10.26	30.42	147.13	36.78	31.12	4.44
Cumacea								
Echinodermata			0.35		0.35	0.09	0.18	0.01
Gastrotricha	1.41				1.41	0.35	0.71	0.04
Gastropoda		0.35		0.35	0.71	0.18	0.20	0.02
Gnathostomulida	1.41				1.41	0.35	0.71	0.04
Halacaroida			0.71		0.71	0.18	0.35	0.02
Isopoda		0.35			0.35	0.09	0.18	0.01
Kinorhyncha	5.31	7.78	2.83	1.77	17.68	4.42	2.69	0.53
Nematoda	587.46	860.85	537.94	774.20	2,760.45	690.11	152.66	83.27
Nemertina								
Oligocheata	2.12			7.78	9.90	2.48	3.68	0.30
Ostracoda	7.43	1.41	1.41	2.48	12.73	3.18	2.87	0.38
Polychaetes	57.65	11.67	8.84	3.54	81.70	20.42	25.04	2.46
Priapulida	7.07	0.35			7.43	1.86	3.48	0.22
Rotifera	1.06	1.06			2.12	0.53	0.61	0.06
Solenogaster	0.71	1.41		3.18	5.31	1.33	1.37	0.16
Syncardia		0.35			0.35	0.09	0.18	0.01
Tanadiacea	0.35	0.71		1.77	2.83	0.71	0.76	0.09
Tunicata								
Turbellaria	4.95		0.71		5.66	1.41	2.38	0.17
Total	789.1	1,063.5	601.3	861.2	3,315.0	828.8	191.1	
No. of taxa	17	15	12	12	22			
Shannon Wiener index					0.32			
Hill's index					1.37			

Table 2B

(B) Low CO_2 seepage Taxon	MUC3_1	MUC3_2	MUC3_3	Sum	mean	SD	rel. abundance
Density (ind. 10 cm^{-2})							
Aelosomatidae							
Amphipoda							
Bivalve	0.71	1.41	0.35	2.48	0.83	0.54	0.04
Cladocera			1.77	1.77	0.59	1.02	0.03
Cnidaria							
Copepoda	56.94	68.26	60.13	185.33	61.78	5.84	2.88
Nauplii	30.77	71.44	23.34	125.56	41.85	25.89	1.95
Cumacea							
Echinodermata							
Gastrotricha		9.20	12.38	21.57	7.19	6.43	0.34
Gastropoda							
Gnathostomulida							
Halacaroida			0.35	0.35	0.12	0.20	0.01
Isopoda	1.41	0.35		1.77	0.59	0.74	0.03
Kinorhyncha	34.31	24.76	22.99	82.05	27.35	6.09	1.27
Nematoda	1,915.87	2,288.29	1,701.90	5,906.06	1,968.69*	296.75	91.73
Nemertina							
Oligocheata		0.71		0.71	0.24	0.41	0.01
Ostracoda	1.06	1.77		2.83	0.94	0.89	0.04
Polychaetes	5.31	8.13	16.27	29.71	9.90	5.69	0.46
Priapulida							
Rotifera	2.48			2.48	0.83	1.43	0.04
Solenogaster	59.06	1.06	1.06	61.19	20.40	33.49	0.95
Syncardia			1.06	1.06	0.35	0.61	0.02
Tanadiacea	1.77	4.24	4.95	10.96	3.65	1.67	0.17
Tunicata		0.35	1.77	2.12	0.71	0.94	0.03
Turbelaria	0.35		0.35	0.71	0.24	0.20	0.01
Total	2,110.0	2,480.0	1,848.7	6,438.7	2,146.2*	317.2	
No. of taxa	12	13	14	18			
Shannon Wiener index				0.19			
Hill's index				1.21			

Table 2C

(C) High CO_2 seepage Taxon	PC20	MUC19_2	MUC10_1	Sum	mean	SD	rel. abundance
Density (ind. 10 cm^{-2})							
Aelosomatidae			0.35	0.35	0.12	0.20	0.02
Amphipoda	0.40	0.35		0.75	0.25	0.22	0.03
Bivalve	5.77	4.24	1.77	11.78	3.93	2.02	0.50
Cladocera		0.35	0.71	1.06	0.35	0.35	0.05
Cnidaria	0.40			0.40	0.13	0.23	0.02
Copepoda	56.50	62.60	52.34	171.45	57.15	5.16	7.28
Nauplii	21.68	44.92	54.11	120.71	40.24	16.71	5.13
Cumacea	0.20		0.35	0.55	0.18	0.18	0.02
Echinodermata							
Gastrotricha		16.98	6.01	22.99	7.66	8.61	0.98
Gastropoda		0.35	0.71	1.06	0.35	0.35	0.05
Gnathostomulida							
Halacaroida							
Isopoda			1.06	1.06	0.35	0.61	0.05
Kinorhyncha	0.60	52.34	7.07	60.01	20.00	28.19	2.55
Nematoda	294.04	652.89	952.10	1,899.03	633.01	329.48	80.66
Nemertina			0.35	0.35	0.12	0.20	0.02
Oligocheata	0.20			0.20	0.07	0.11	0.01
Ostracoda		1.06	1.06	2.12	0.71	0.61	0.09
Polychaetes	17.51	5.66	9.90	33.07	11.02	6.00	1.40
Priapulida	0.20		1.06	1.26	0.42	0.56	0.05
Rotifera			0.35	0.35	0.12	0.20	0.02
Solenogaster		6.37	0.71	7.07	2.36	3.49	0.30
Syncardia							
Tanadiacea	0.40		3.89	4.29	1.43	2.14	0.18
Tunicata							
Turbelaria	6.37	8.13		14.50	4.83	4.28	0.62
Total	404.25	856.25	1,093.92	2,354.43	784.81	350.34	
No. of taxa	13	13	18	22			
Shannon Wiener index				0.36			
Hill's index				1.44			

Table 3 Density of all nematode genera (0-1 cm), nematodes which were only identified to family level are in parentheses.

	MUC23_1	MUC25_1	MUC3_2	MUC3_1	PC20	MUC19_2	MUC10_1
Density (ind. 10 cm^{-2})							
Acantholaimus	19.42	19.12	39.32	11.25			
Actinonema	6.47	12.75	39.32			22.76	7.45
Aegialoalaimus	19.42	25.49					7.45
Camacolaimus	6.47						
Comesoma	12.95						
Ammotheristhus	6.47						
Amphimonhystera	6.47						
Amphimonhystrella	12.95	19.12					
Anticyathus	6.47						
Antomicron				11.25			
Calomicrolaimus	6.47					5.69	
Campylaimus						5.69	7.45
Cervonema		38.24	39.32	22.49			
Chromodora		12.75	19.66	22.49	12.04	11.38	22.36
Chromodorina					3.28	5.69	
Coninckia			19.66				
(Cyatholaimidae)			19.66				
Daptonema	32.37	70.11	78.63	112.47	2.19	17.07	82.00
Desmodora			58.97	78.73		45.52	37.27
Desmocolex	6.47	25.49		44.99		5.69	
Desmolaimus							14.91
Dichromadora				11.25			
Diplopeltula	6.47	6.37					
Dorylaimopsis	6.47			11.25			
Echinodesmodora					1.09		
Halalaimus	6.47	25.49	19.66	11.25	3.28	22.76	44.73
Halichoanolaimus	19.42	38.24	137.61	44.99			7.45
Haliplectus	6.47						
Halomonhystera		63.73		11.25	1.09		
Innocuonema		12.75					
Ledovitia					1.09		
Leptolaimoides	6.47		58.97		3.28		

	MUC23_1	MUC25_1	MUC3_2	MUC3_1	PC20	MUC19_2	MUC10_1
Leptolaimus	64.74	25.49	314.53	123.72		34.14	29.82
Linhystera			19.66				
Marylinnia		12.75					
Megadosmolaimus					1.09		
Metadasynemella	6.47						
Metadesmolaimus			58.97				
Metalinhomeus					1.09		
Microlaimus	58.27	19.12	157.27	56.23	3.28	22.76	14.91
Molgolaimus				11.25	5.47		
Monhystrella				22.49			
Neochromadora	6.47						
Neotonchus							7.45
Odontanticoma		25.49	19.66				7.45
Oxystomina		6.37		11.25			
Paracyatholaimus		6.37			5.47		
Paralongicyatholaimus		6.37					
Paramesonchium				11.25			
Pomponema			39.32	11.25			
Prochromadorella				11.25			44.73
Pselionema		12.75					
Quadricoma	19.42	12.75	39.32				
Richtersia	6.47						
Sabatieria							7.45
Setoplectus		6.37					
Sphaerolaimus					1.09	11.38	
Spilophorella	6.47	12.75	117.95	11.25			
Spirinia	6.47			11.25	1.09		14.91
Terschellingia					8.75		119.27
Thalasssomonhystera	84.17	95.60	707.70	472.37	30.64	233.31	201.27
Viscosia		6.37	19.66	33.74			
(Xyalidae)	12.95						

3.

Discussion & Perspectives

Organisms adapted to the deep-sea environment are confronted with a number of challenges such as high hydrostatic pressure, cold temperatures, constant darkness, low food availability, and hypoxia. Accordingly it is assumed that metabolic rates are slow and environmentally constrained, although this might be negligible concerning predator-prey interactions as recently reported by (Seibel and Drazen 2007). However, benthic deep-sea organisms have adapted to these environmental parameters and human-induced changes in terms of ocean acidification and CO_2 leakage from CCS may have substantial effects. The injection of CO_2 into deep layers of sediments in the deep sea are advocated for CCS projects because at high hydrostatic pressure and low temperature CO_2 is in liquid phase and might even form hydrates and thus serves as a cap sealing the liquid CO_2 below. Reactions with carbonate and silicate minerals in the deep-sea sediment may further add to the stability of the introduced CO_2. However, leakage from CCS sites cannot be ruled out completely. To analyze the influence of naturally high CO_2 concentrations on the benthic deep-sea community, the Yonaguni Knoll CO_2 hydrothermal vent system was investigated during this PhD study. Such "natural laboratories" appear to be the most suitable tool in order to improve predictions on environmental long-term impacts of possible CO_2 leakage that comes as a risk of CCS projects. On the one hand, this "natural laboratories" might differ from CCS sites due to different magnitude and duration of CO_2 seepage at these sites in contrast to the gradient that is established by CCS leakage. On the other hand, all benthic size classes are subject to the conditions that evolve from high CO_2 concentrations due to possible leakage.

The biogeochemical processes in CO_2-vented deep-sea sediments of the Yonaguni Knoll IV hydrothermal system were studied with *in situ* and *ex situ* analysis techniques and indicated microbial activity, albeit only at relatively low rates. The analysis of bacterial community composition by means of the fingerprinting method ARISA and next-generation-sequencing, as well as evaluation of cell numbers in combination with contextual parameters, allowed for interpretation of community responses at the base of the ecosystem. Additional investigation of the meiofauna and macrofauna using morphological analysis and taxonomic identification added to the general view on ecosystem response to low pH.

The results presented in the chapters of this thesis demonstrate that deep-sea benthic communities may fundamentally change in response to the release of CO_2 in the context of CCS. Combined effects of the release of other, maybe toxic, fluids may further alter community composition and also functioning at the seafloor. Organisms were identified

during this work that were highly susceptible to low pH and thus could function as indicator organisms to monitor the state of benthic ecosystem at specific sites.

3.1 Naturally CO_2-influenced sediments may provide information on the impact of CCS leakage on benthic communities

Hydrothermally-induced liquid and supercritical CO_2 venting in the deep-sea realm of the Yonaguni Knoll IV CO_2 hydrothermal system was investigated with respect to the impact on the biogeochemistry of the deep-sea sediments (Chapter I). The area disclosed a high heterogeneity in terms of porewater geochemistry, transport processes, and microbial rates. CO_2 was transported to the sediment surface in supercritical form and condensed at the surface to liquid and CO_2 hydrates (Konno et al. 2006). However, CO_2 concentrations reached values one order of magnitude higher as indicated by the measured low pH values (< 6). In proximity to the vent sites pH values were approximately 4.5 at 6 cm below seafloor (bsf). Measured rates of sulfate reduction (SR) and anaerobic oxidation of methane (AOM) showed that microbial activity was restricted to the upper 15 cm sediment depth, although methane, sulfate and numerous other potential electron donors were present in the deep sediments (Chapter I). At the vent sites, SR and AOM rates were low compared to methane-rich hydrothermal vents and seeps lacking CO_2 (Felden et al. 2010; Biddle et al. 2012). Neither temperature nor low pH, seemed to restrict microbial activity to these depths as recently reported from *ex situ* experiments, showing potential for sulfate reduction up to temperatures of 60°C (Yanagawa et al. submitted). This lethal temperature was only reached at 0.6 m bsf at 0.5 m distance to the vent, far below the upper 15 cm horizon. The experiments of Yanagawa et al. (submitted) further disclosed that SR was not inhibited at pH 4.5, or even pH 3. However, main differences in the *ex situ* experiments of Yanagawa et al. (submitted) and the *in situ* conditions was the level of CO_2. Considering the *in situ* ambient low pH, dissolved inorganic carbon (DIC) is almost completely present in the form of CO_2. Important to note is that at *in situ* pressures exceeding 130 atmospheres (at ca. 1380 m depth), the concentration of dissolved CO_2 is in equilibrium with CO_2 hydrate, liquid or supercritical CO_2 and concentrations will be as high as 1000-1700 mM compared to < 0.02 mM in seawater at atmospheric pressure (Chapter I). The apolar compound of liquid CO_2 is known to be a powerful solvent that affects microbes by dissolving the cytoplasmic membrane. Furthermore, the protonated form of CO_2 (H_2CO_3) may as well be toxic as it functions as uncoupler of the membrane potential.

Although microbes have evolved many different cellular properties to maintain pH homeostasis, such as cell membranes that are highly impermeable to protons or reduced pore size of membrane channels (see BOX 4) (Baker-Austin and Dopson 2007), these and other mechanisms do not constrain H_2CO_3 to pass through the membrane thus dissipating the proton motive force. These uncoupling processes consequently interrupt the basic energy supply of the cells and leave no possibility for adaptation. Therefore, in the context of CCS and potential CO_2 leakage, microbes might be able to survive low pH values but most likely will suffer mortality as a consequence of the dissolving and uncoupling characteristics of liquid and supercritical CO_2.

3.2 Bacterial communities influenced by high CO_2 and low pH

Based on the results obtained from the biogeochemical study of the Yonaguni Knoll CO_2 hydrothermal system, it was expected that abundance and diversity of bacteria in samples obtained from CO_2-vented sediments may decrease compared to samples taken at a background site. Inagaki and colleagues (2006) identified a decrease in bacterial cell numbers of two orders of magnitude at the CO_2/CO_2-hydrate interphase at the Yonaguni Knoll IV CO_2 hydrothermal system. However, bacteria showed diverging responses, depending on the sites and thus differences in CO_2 concentrations and according pH values. In contrast to the expected decrease of bacteria, cell numbers increased from the background to the High CO_2 seepage site and were surprisingly high at this site (Chapter II). This increase in cell number correlates with a decrease in diversity on the level of ARISA phylotypes ($OTUs_A$). We could identify some sequences assigned to bacterial phyla, classes, and genera that decreased with low pH, while others increased, suggesting that they may profit from the energy supply of hydrothermal fluids (Chapter II), which is consistent with other recent studies (Fig. 11) (e.g. (Nunoura et al. 2010). The presence of vent fluids as energy sources probably enhanced cell numbers at the most CO_2-impacted site, e.g. sulfate reduction was observed at high CO_2 seepage in close proximity to the vents. The changes in bacterial community structure, however, were related to changes in the relative abundance of ARISA OTUs (OTU_A) and not necessarily to a loss of bacterial types. Further analysis of MPTS OTUs (OTU_{454}) revealed that bacterial community composition changed. However, again the changes were unexpected. The total abundance of bacterial sequences and sequences that appeared only once in the 454 data set (total singletons, Chapter I) were highest at the

low CO_2 seepage site. At the intermediate CO_2-impacted site a substantial decrease in pH was observed (pH < 6.4), however concentrations of CO_2 were lower compared to the high-impacted site. The shift in community structure was caused by a replacement of some ubiquitous phyla and classes with bacterial types known to be at least acid-tolerant (Chapter II). Some of the sequences affiliated to bacterial phyla and classes typical for deep-sea sediments (*Acidobacteria*, *Actinobacteria, Planctomycetes and Alphaproteobateria*) (Dang et al. 2009) decreased in relative abundances, while sequences affiliated to acidophilic members of the *Deltaproteobacteria* increased. Numbers of sequences affiliated with *Epsilonproteobateria*, a dominant bacterial group at hydrothermal vents (together with *Gammaproteobacteria*) (Nakagawa and Takai 2008), increased one order of magnitude at the High CO_2 seepage site. This bacterial group, known to follow and quickly adapt to changes in geochemical properties (Huber et al. 2003), was pronounced in the hydrothermal environment of the high CO_2 seepage site where members may benefit from hydrothermal fluids (Chapter II). Investigations on pure cultures of mesophilic to moderately thermophilic deep-sea vent *Epsilonproteobacteria* from endosymbiotic or episymbiotic associations with various vent fauna, confirmed that the majority were flexible chemoautotrophs capable of oxidizing H_2 and sulfur compounds coupled with a reduction of oxygen, nitrate and sulfur compounds (Takai et al. 2003; Campbell et al. 2006; Nakagawa et al. 2007). *Epsilonproteobacteria* account for a significant fraction of the deep-sea vent chemoautotrophs (Takai et al. 2004b; Nakagawa et al. 2005b; Nakagawa et al. 2005a; Takai et al. 2006; Huber et al. 2010) and are major components of the free-living benthic bacteria, e.g. at a Mid-Atlantic Ridge hydrothermal vent site (Polz and Cavanaugh 1995).

The link between geological and geochemical settings of (CO_2) hydrothermal systems and associated habitats and the chemoautotrophic microbial community structures, have become increasingly evident (Takai et al. 2004a; Nakagawa et al. 2005b; Nakagawa et al. 2005a; Nakagawa and Takai 2008; Huber et al. 2010). Consistent with this study, Nunoura and colleagues (2010) found an increase in *Epsilonproteobacteria* and a loss in *Alphaproteobacteria* in sedimentary bacterial communities at the Yonaguni Knoll CO_2 hydrothermal vent system when compared to bacterial communities found in deep-sea waters (Fig. 11) (Nunoura et al. 2010). Also the increase in the family *Thermodesulfobacteriaceae*, reported by Nunoura and colleagues, especially in the deeper sediments, is in accordance with the results based on the 454 MPTS of this study (Chapter II). Although changes in the bacterial community structure were present, the

highly CO_2-vented sediments with consequently low pH did not repress bacterial abundance and diversity as assumed, responses were observed in a shift in community structure towards acidophilic and hydrothermal vent associated bacteria.

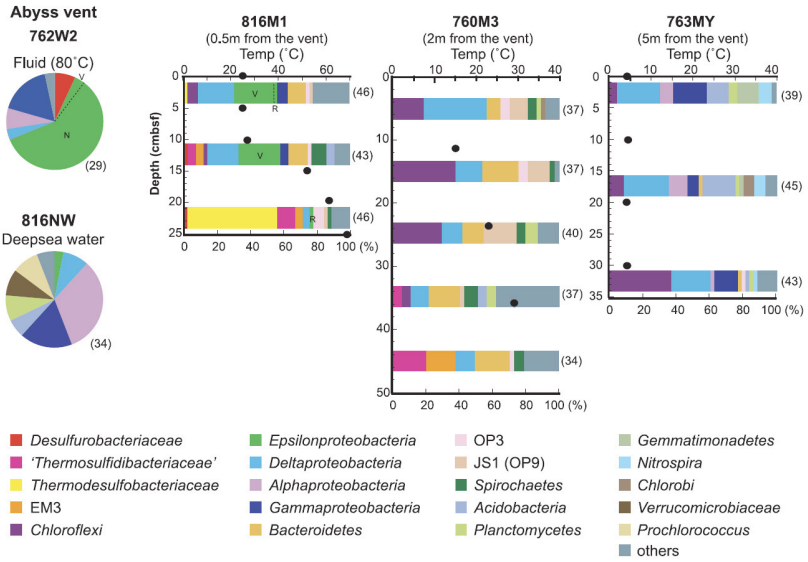

Figure 11 Distribution profiles of *Bacteria* based on gene clone analysis of the small subunit (SSU) rRNA of hydrothermal sediments, *in situ* colonization system (ISCS), vent emission, and deep-seawater from the Yonaguni Knoll IV hydrothermal field. Numbers of sequenced clones in each clone library are shown in parantheses. Black dots indicate *in situ* temperature of the sediment. Abbreviations: V, N, and R in *Epsilonproteobacteria* indicate the families *Thiovulgaceae*, *Nautiliaceae*, and *Thioreductoraceae*, respectively (Nunoura et al. 2010).

3.3 Impact of high CO_2 and low pH on the benthic faunal assemblage

CO_2 can directly impair physiological functions of animals, for instance by respiratory distress (Tamburri et al. 2000), but it can also affect organisms indirectly due to the resulting decrease in pH of seawater (Fleeger et al. 2006). This study did not focus on physiological responses but evaluated the subsequent distribution patterns. However, this may already give insights into adaptation capabilities of the different taxa of megafauna, macrofauna and meiofauna.

The benthic megafauna survey along the CO_2 gradient revealed a loss of typical deep-sea megafauna with decreasing pH. Both, echinoderms and anthozoa were excluded from areas with pH < 7.5. Echinoderms are especially sensitive to high CO_2 and low pH, as dissolution of their internal skeleton impairs organism functions. At the chimney structures of the vents, dense accumulations of vent associated fauna were observed

comprising the mussel *Bathymodiolus platifrons,* the shrimp *Alvinocaris longirostris,* and the crab *Shinkaia crosnieri*.

Metazoan macro- and meiofauna showed opposing responses to high CO_2 and low pH. The macrofauna appeared to be higher impacted than the meiofauna. The macrofauna community shifted between the background and the High CO_2 seepage with regard to three of the four investigated taxa (echinoderms, molluscs, polychaetes), as echinoderms disappeared from the background to the high CO_2 and polychaetes decreased considerably (Chapter III). Molluscs, mainly bivalves, had highest densities at the high CO_2 site, due to an increase in juvenile representatives. Some of the genera identified are known to host symbiotic bacteria (e.g. *Thyasira*). Sequences affiliated to the genus *Thiobacillus*, that are known to be associated to some *Thyasira* species, were found in all samples of the 454 data set; however, abundances were lowest in samples obtained near the vent site. Bacterial symbiotic affiliates of the bivalves are dependent on the energy flow of hydrothermal fluids emanating from subsurface reservoirs. Enhanced abundance in juvenile bivalves may point towards colonization attempts attracted by the release of the hydrothermal fluid flow. Eventually, it has not been clarified if bivalves will survive at theses high CO_2 concentrations. The decrease in sequences of *Thiobacillus* presumably indicates a low survival rate.

In contrast to the macrofauna, total faunal densities of deep-sea benthic meiofauna showed no significant reduction in abundance and no significant loss in diversity. The meiofauna community was predominated by nematodes and shifts in community on phylum level were primarily caused by increased numbers of nematodes at the low CO_2 impacted site. These findings are novel, as the gross of studies concerned with CO_2 and low pH did either not investigate all benthic size classes, or did not incorporate long-term effects on community level. Other studies investigated the response of deep-sea meiofauna benthic assemblage to direct exposure of liquid CO_2 and found a high rate of mortality for, e.g. nematodes and harpacticoid copepods (Barry 2003; Barry et al. 2004; Thistle et al. 2005), while in other studies no effect on the abundance of the meiofauna could be observed (Carman et al. 2004). Recent studies by Fleeger et al. (2006, 2010) revealed that impact of high CO_2 may rather show on the morphology of meiofauna, in particular on nematodes. Investigated effects of CO_2 disposal experiments on foraminiferal survival clearly indicated a loss in abundance and species richness (Ricketts et al. 2009), as well as a lower survival rate of calcareous

foraminifera in contrast to agglutinated and thecate foraminifera (Bernhard et al. 2009). However, foraminifera were not subject to this study.

The meiofaunal assemblage investigated in this study was dominated by nematodes, which contributed relative proportions of up to 90% to total meiofauna abundance. Due to their high abundance, short turnover time, and important ecological role, nematodes were investigated in more detail. The shift in the community composition of the meiofauna was mainly due to an increase in nematode abundances. Nematode densities changed, as some nematode genera could thrive in highly acidified areas (*Thalassomonhystera* and *Teschellingia*) and reached numbers that doubled those found at the reference site, while other genera disappeared, e.g. *Amphymonhystrella* (Chapter III). However, since it was not distinguishable whether nematodes were alive at the time of sampling, it cannot be clarified if nematode abundance was biased because corpses had not decayed (Fleeger et al. 2006). Based on experimental disposal of liquid CO_2, Fleeger et al. (2006) report several ways in which CO_2 may possibly impair nematodes without causing a change in total abundance. The recruitment of nematodes after exposure to CO_2 could offset the reduction in abundance that is caused by lethal effects (Fleeger et al. 2006). This would cause a shift in community composition without changing the abundance. Nematodes could colonize sites exposed to CO_2 either by migration from surrounding sediments (by errant burrowing) or by settlement through the water column (Fleeger et al. 2006). In their approach, Fleeger and colleagues tested the hypothesis, that body dimensions (length:width ratio) and biovolume may be appropriate measures to estimate changes in nematode community and thus evade abundance. Their observations suggested that nematode body dimension and biovolume increased post-mortem after exposure to CO_2. Thus, they postulate that nematodes community suffered high mortality rates after the exposure to CO_2, as individual biovolume significantly differed relative to the concentrations of CO_2 and pH values. Further they concluded that "moderate" CO_2 (pH 7) may also cause substantial mortality to infaunal nematodes, which is consistent with the conclusions of Thistle et al. (2005) (Thistle et al. 2005; Fleeger et al. 2006).

Another study suggested that large nematodes seem to respond differently than small nematodes to increased levels of CO_2, probably because large nematodes suffer less mortality (Fleeger et al. 2010). Fleeger et al. (2010) suggested that reduced mortality was due to differences in diffusion rates as diffusion varies with body size (Powell 1989; Brown et al. 2004). Thus, CO_2 and proton uptake may have been reduced by a slower

diffusion rate or by a less permeable cuticle of the larger nematodes (Fleeger et al. 2010). Furthermore, acid-base regulation is supposed to moderate mortality. Studies on a terrestrial nematode indicated that external pH influenced the transcriptional profile of carbonic anhydrases most likely enhancing protection against pH changes (Hall et al. 2008). Differences in responses exist also in terms of attraction or avoidance behaviour observable for some nematode species (Riemann and Schrage 1988; Bretscher et al. 2008). Size-related higher mobility of larger nematodes might as well help to avoid harmful conditions and thus reduce mortality through a vertical emigration (Soetaert et al. 2002). This emphasizes that not all nematodes will respond equally when exposed to high CO_2 concentrations. Further studies are needed to investigate these specific response patterns in nematode communities of different CO_2-impacted habitats.

3.4 Responses on ecosystem level

This study focused on the effects of naturally high CO_2 vented deep-sea sediments and responses on the benthic community therein. At broad taxonomic resolution (phylum level), neither bacteria nor the benthic faunal assemblage disclosed severe negative impacts, as for example a total collapse of the community. Only minor shifts in the distribution were observable. Certain groups of mega- and macrofauna, however, exhibited high sensitivity to acidification, especially echinoderms which were excluded from the highly CO_2 saturated sediments near the venting site. However, the reduction of these organisms may already be enough to change the dynamics of the ecosystem. Besides bigger predators, such as echinoderms, also deposit feeders often prey non-selectively on a variety of smaller organisms, and thus reduce their densities (Paine 1966). Larger organisms, as for example polychaetes, are known to affect the assemblage structure of smaller organisms by predation, competition for food, or habitat structuring (e.g bioturbation) (Olafsson 2003). Changes on ecosystem level may be even more evident at higher taxonomic resolution. The impact of high-CO_2/low-pH on biodiversity as measured by the reduction of species abundance and richness may not be sufficient. Besides biodiversity measures, studies on ecological interactions of the deep-sea benthic community are essential to unravel negative impacts that may appear on the level of community ecology by modifying, e.g. predator/prey interactions or competition. McCauley and colleagues (2012) stress, that elimination of ecological interactions as a consequence of anthropogenic disturbances has long been neglected. Disruption of these complex interactions may have long-lasting effects in a way that the ecological interaction

chain length will shorten thus simplifying and isolating ecosystems (McCauley et al. 2012). Further research is required to investigate taxon-specific responses to high CO_2-low pH, while taking interconnections and interactions on community level into consideration. The following paragraphs are suggestions for additional future studies and experiments used as monitoring approaches, based on what was learned from this study.

3.5 Perspectives for monitoring

The results of this work suggest that some organisms can adapt to highly acidified conditions, while others are severely impaired leading to decreased abundances or disappearance. Evaluation at phylum level may not be sufficient to comprehend the full extent of the effects on ecosystem functioning. Hence, the shift in community composition on ecosystem level may be criticized, because feedback mechanisms are not yet completely and satisfactorily investigated. Studies are missing on the impact on ecological interactions and consequently the simplification and isolation of the otherwise well balanced deep-sea ecosystems.

The current state of literature illustrates that the majority of organisms exposed to high CO_2 and low pH will suffer metabolic depression (disruption in acid-base balance) (Widdicombe and Spicer 2008), inhibited growth and reproduction (Pörtner 2008), reduced hatching success of eggs, impacts on cleavage, larval development, and settlement (Mayor et al. 2007; Kurihara 2008) and at the most extreme mortality (Barry et al. 2004), especially if time for adaptation is missing. Exact specifications of thresholds might be at some point of matured research given on species level, but demand a closer look at interconnections and interaction on community level and must look beyond mortality of adults but integrate responses of juveniles. Fleeger et al. (2010) also accentuated that body size discriminates for mortality rates. There is also evidence that factors related to an organism's lifestyle and activity are closely linked to potential species extinction as due to interactions of infauna vs. epifauna, fauna of the deep vs. shallow, or deposit feeders vs. suspension feeders (Widdicombe and Spicer 2008).

Acidification and concomitant low pH might induce survival limitations and shifts in community composition, which will depend on the magnitude and duration of acidification and additional stressors such as temperature increase due to global warming and hypoxia (Pörtner et al. 2005). Beyond biomass and diversity, pertinent parameters to evaluate overall responses to ocean acidification on ecosystem level are metabolic rates and activity. Further research should aim at combining theses response parameters and

evaluate ecosystem responses on species level. Temporal scales of many ecosystem studies are short-term, excluding the understanding of ecological processes and community dynamics that only show on a long-term. Long-term time series are lacking, which certainly would be important to evaluate natural versus human-induced changes. Natural laboratories constitute sites where species have already adapted to changes in acidity and temperature over longer time scales. These natural laboratories thus present a kind of long-term projection opposing short time experiments in the laboratory. These ends, short term experiments and long term adaptation in nature, need to be connected and the data combined. Quantification of the short- and long-term effects may add to an overall assessment of the risk deriving from CO_2 leakage.

Why is monitoring required?

Ecosystem assessments and monitoring have been neglected during the last decades, but are required in order to better predict future responses of marine ecosystems to climate change and associated risks from mitigation strategies. Previously, winners and losers in climate change and ocean acidification have been identified foremost in calcifying organisms, since changes occur on short term. Further insights are needed to try and link different fields of studies that describe geological, physical, chemical, and biological (ecology, physiology) conditions to gain an overall picture of what marine biota might face. There is an urgent need for further data collection and data linkage to provide politics with appropriate scientific expertise to enable informed decision-making.

There is a need to establish monitoring programs in order to estimate possible impacts and damages to CCS adjacent ecosystems. Currently, four large-scale commercial CCS projects with varying degree of maturity are operating. In 1996, the international energy company Statoil marked the beginning of CCS at Sleipner Vest, 800 m below seabed in an aquifer of porous sandstone and saline waters. By own account, Statoil accomplished seismic testing in 2008 to investigate safety of the storage location and no leakage was observed. At the beginning of 2011, 12 million tonnes of CO_2 had been stored with a CO_2 concentration of 2.5% (www.statoil.com, status on 30.5.2012). The yearly reduction of 1 million tonnes of CO_2 emissions accounted for nearly 3% of the Norwegian CO_2 emission in 1990 but in comparison to the extensive global emissions of 31.6 gigatonnes in 2011 (according to preliminary estimates of the International Energy Agency), this fraction is negligible.

Where monitoring should be implemented

Monitoring might not be easily undertaken, due to the remoteness of the deep sea and because we still lack important information on the deep-sea ecosystem. Thus, it is critical to focus particularly on the deep sea, as it is the largest coherent habitat on earth and changes in this ecosystem will back-loop to associated environments, e.g. the pelagic. Additionally, many CCS projects are operated or are planned in geological formations of the deep sea.

Environmental monitoring is used to assess the condition of the specific environment investigated. When undertaking a monitoring and assessment of deep-sea organisms and the consequences of CO_2 leakage events, it is crucial to pinpoint the areas of interest and surveillance. These defined areas need to be exemplary for different areas of the deep sea and should be appropriate in their dimensions. For accurate and precise sampling clearly defined aims of the monitoring program are critical. The first approach to monitoring thus would be the observation and evaluation of the environment in the vicinity of running CCS sites. Another possible approach may be a comparative study of normal deep-sea sediments and sediments highly enriched with CO_2 as described for the area of the Yonaguni Knoll IV hydrothermal system.

BOX 5 | Monitoring approaches:

How:

- Multidisciplinary research
- Long-term experiments and observations
- Small to large spatial scale observations

What:

- Model organisms
- Establishment of different, experimentally designed small scale CO_2 leakage scenarios
- Gradient-surveys
- *In situ* camera surveys
- All benthic size classes
- Key species and functional groups

How monitoring should be implemented

Based on this survey it has been illustrated that there will be winners and losers associated to scenarios of CO_2 leakage at CCS sites. If it would come to an accident during CCS with leakage of CO_2, organisms will likely not be able to adapt as fast and effective as circumstances demand. And previous studies have shown that some organisms would suffer mortality. However, organisms that are able to at least cope with the increased CO_2 concentrations could exhibit resistance to a short-term exposure. Nevertheless, depending on geology and geography, as well as currents, the leaked CO_2 could remain for a long time and thus organisms would need to adapt on the long run. Echinoderms, such as sea urchins and starfish have been demonstrated to be absent in acidic environments. This study shows that at least for some organisms (besides echinoderms, some nematode genera, gnathostomulids, and polychaetes) it would either result in migration if possible or perhaps mortality. Studies at broad taxonomic resolution may not be sufficient to comprehend the consequences of the aftermath of such an accident and monitoring strategies should aim at long-term experiments that include high resolution taxonomy on species level. Continuative recommendations are

summarized and discussed in the following and may be used for future approaches (BOX 5).

There is a need for **multidisciplinary research** that includes not only ecology and physiology, but also population dynamics, community and evolutionary biology. Sampling strategies should therefore include contextual parameters such as geochemical (e.g. pH, CO_2, oxygen, temperature), oceanographic (e.g. direction and velocity of currents), geological (e.g. grain size, porosity), and geographic properties, in order to not overlook important variables that would in retrospect bias interpretation and results. Changes on the deep-sea biota have to date been monitored on a short-temporal scale and are lacking **long-term observations**. Thus, a future aim is to establish long-term observation on the abundance, distribution and diversity of species, as well as ecological interactions which will allow for detection of changes on long temporal scale. Monitoring programs need to further implement spatial observations that range from **micro-scale** (µm – several cm) **to larger spatial scale** (100s – 1000s m) in order to comprehend gradual changes that may be a consequence of the movement and dispersion of CO_2. Biogeochemical processes (e.g. AOM, SR) based on microbial activity may be evaluated *in situ* e.g. with microprofilers and benthic chambers which elucidate direct and short-term responses of the microbenthos. The question, how the microbial community adapts to gradients of high CO_2/low-pH may be answered with these approaches. *In situ* measurements are likewise important for measurements of pH and CO_2 concentration gradients as these parameters are determined by pressure and temperature. Gradients of pH and CO_2 and their spatial and temporal variability could, hence, determine the dimension of the monitored area. In combination with porewater chemistry, these chemical gradients are important to reveal changes occurring in the biogeochemistry of the area exposed to high CO_2.

It is indispensable to determine the variations in geochemistry and link these data to corresponding variables of interest, which in this case could be indicator organisms or assemblages that respond to high CO_2 concentrations and low pH. In this study it was shown that echinoderms and also polychaetes might be possible **indicator organisms**.
The following section shortly summarizes the changes and effects that are suggested by this study and proposes possible aims of a monitoring program. The site investigated by this study has previously been described in terms of biogeochemistry and ecology and it has been demonstrated that venting of CO_2 is highly variable, leading to environmental heterogeneity. Thus, different leakage speed and amounts as supposed for CCS leakage

may have different effects. It can not be predicted at what magnitude and extent leakage will occur, whether it may release liquid CO_2 slowly but continuously, thereby declining pH at a relatively constant manner, or if the release might be sudden and will result in an outburst of compressed CO_2 gas. Therefore, **gradient-surveys** testing for the effect of different values of CO_2 concentrations and low pH are essential.

Due to the fact that the deep sea is a relatively stable and slowly changing environment, short-term effects of acidification on sensitive species (reduction in echinoderm abundances) will begin to show quickly but responses that occur slowly might be overlooked. Despite the remoteness of the deep sea, variations in the megabenthos can easily be observed through, e.g. Remotely Operated Vehicles (ROV) with attached cameras. The planarity of most parts of the deep sea allow for **camera-surveys** of megafauna assemblages, as we accomplished in our study of the Yonaguni Knoll IV CO_2 hydrothermal vent system. This method has proven to be quite efficient in identifying loss of megafauna along a transect from no/low to high CO_2 concentrations. However, to distinguish between human–induced effects of low pH and natural variations in benthic communities, it is necessary to extract and to understand changes that might occur naturally. When observing changes in the benthic faunal composition, it will be crucial to focus on the **species level**, as changes might not be obvious at lower taxonomic resolution. Monitoring should further evaluate the dependence of reproduction and recruitment of key species with a wide distribution (e.g. the typical deep-sea holothurian *Pannychia moseleyi* found in deep waters off Japan, Hawaii, and New Zealand; c.f. World Register of Marine Species (WoRMS)) to increased CO_2 concentrations which would give insight into the resilience characteristics of the ecosystem.

Monitoring approaches at ecosystem level must comprise **all benthic size classes**, i.e. microbes, meiofauna, macrofauna and megafauna. In order to assess alterations on significant functions of microbes, e.g. organic matter respiration, the recycling of nutrients, monitoring microbial activity is essential. **Biomass estimations, rate measurements, and cell counts** for microbes could indicate functional loss. Respiration of microbes could be assessed on short-term by using *in situ* techniques, which would also enable an evaluation of changes occurring on micro-scale. Meiofauna (especially nematodes) are likewise important as they link energy to higher trophic levels in terms of nutrient recycling. The turnover of meiofauna is short and thus short-term monitoring could be accomplished. However, sampling strategies must be exactly

defined as for the mesh size that is used for sampling when surveying different size classes and estimation of smaller benthic fauna in sediment samples. Different studies on meiofauna have used a variety of different mesh sizes making direct comparisons difficult and biasing subsequent statistical analyzes (Soltwedel 2000).

Macrofauna exhibit a short lifespan and responses to high CO_2 show quickly. They function as predators and bioturbators and shape the structure of the meiofauna community. Short-term approaches should be appropriate. Surveying megafauna however, may be accomplished via camera surveys on short-term. Besides the monitoring of benthic communities exposed to high CO_2 concentrations, it might also be helpful to further incorporate pelagic abyssal animals, as some are bottom dwellers and act as scavengers on deadfalls (Barry and Drazen 2007). Hence, they might as well be directly exposed to high CO_2 concentrations, if liquid CO_2 disperses through the sediment and reaches the water column. It is likely that through the leakage and circulation of liquid CO_2 changes may persist for extended periods of time. Thus, **invasion of new species** is possible and therefore manipulative experiments could give insights into dynamics of recruitment and may elucidate interactions between invasive and resident species under newly established environmental conditions, e.g. in colonization experiments. However, dispersal and retention time of liquid CO_2 on the seabed can not be accurately predicted due to currents, topography, or sediment properties, and porosity and knowledge on the regeneration time of the ecosystem hence is not straightforward.

BOX 6 | Following predictions on the response of deep-sea organisms should be assessed:

- Megafauna (especially echinoderms) abundance will decline at worst towards a total collapse of the population; repressed reproduction success and juvenile development might drive these variations.
    - Megafauna surveys could give further insights

- A loss of key species will modulate biological interactions of predation and competition with consequences on the assemblage structure of the ecosystem.
    - Colonization experiments along CO_2-gradients

- Biodiversity of all benthic size classes will be reduced.
    - Conducting taxonomic studies on species level, including biovolume measurements

- Biomass will decline.
    - Measures of biomass might be helpful in assessing the health of organisms

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Posters and Oral Presentations

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Ufkes J, Ramette A, de Beer D, Haeckel M, Inagaki F, Vanreusel A, Boetius A (2009). CO_2 vents – natural analogues for potential leakage from CO_2 storage in the seabed. MARUM/GLOMAR PhD student day, Bremen. Poster presentation.

Ufkes J, Bienhold C, Schulz I, Wang T, Hubert A-M, Assmy P (2010). Geo-engineering the climate - from science to governance. GLOMAR/MARUM PhD Days, Bremen. Oral group presentation.

Ufkes J, Ramette A, Haeckel M, de Beer D, Inagaki F, Boetius A (2010). Effect of seawater acidification on microbial community diversity – lessons from natural CO_2 leaks. GfOe conference, Giessen. Oral presentation.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Effect of high CO_2 and low pH on benthic communities of the deep sea

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, das es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

.....
(Unterschrift)