Lipid biomarkers of microbial communities involved in carbon dioxide and methane cycling at volcanic CO_2 vents

Dissertation

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Vorwort und Zielsetzung

Die Abtrennung und geologische Speicherung von CO_2 stellt eine Möglichkeit zur Reduktion der anthropogenen CO_2 Emission dar. Eine Speicherung in salinen Aquiferen und ausgebeuteten Gas- und Ölreservoirs ist bereits technisch machbar. Aber vor der Anwendung dieser Technologie ist es notwendig, die Folgen von CO_2-Leckagen aus dem Speicher in die oberflächennahe Umwelt zu bewerten. Natürliche Analoge helfen dabei, die Folgen solcher Leckagen einzuschätzen. So wurden für diese Studie zwei vulkanische CO_2 Austritte als natürliche Labore zur Untersuchung der Auswirkungen von CO_2 induzierten Umweltveränderungen auf das mikrobielle Leben genutzt.

CO_2 ist das wichtigste Treibhausgas des anthropogen bedingten Klimawandels und hat schon bei vorangegangenen Klimaveränderungen eine essentielle Rolle gespielt. Zusätzlich zu den anthropogenen Emissionen haben sowohl die mikrobielle Produktion, als auch der mikrobielle Abbau von CO_2 einen signifikanten Einfluss auf die Konzentration von Treibhausgasen in der Atmosphäre. In früheren geologischen Zeitabschnitten sind mikrobielle Prozesse von wesentlich größerer Bedeutung für die Konzentration von Treibhausgasen in der Atmosphäre gewesen; während des Präkambriums waren sie sogar die dominierenden biologischen Prozesse. Doch es bestehen weiterhin große Wissenslücken bezüglich der Art und Eigenschaften CO_2 verbrauchender und hierbei insbesondere der anaeroben Mikroorganismen. Im Zusammenhang mit der Erforschung des Klimawandels sind die Mikroorganismen des CH_4-Keislaufes von besonderem Interesse. Die autotrophen, methanogenen Mikroorganismen konsumieren CO_2 und produzieren das als Treibhausgas 25-fach effektivere CH_4. Methanotrophe Mikroorganismen wiederum verbrauchen CH_4 und produzieren CO_2. Der CO_2- und der CH_4-Kreislauf sind demzufolge eng miteinander verbunden. Über die beteiligten Mikroorganismen, speziell in anaeroben, terrestrischen Habitaten, ist allerdings wenig bekannt. Deshalb wurden methanogene Organismen die vulkanisches CO_2 verbrauchen und methanotrophe Organismen die den aus dem CO_2 stammenden Kohlenstoff weiter verwerten, besonders intensiv untersucht. Das lückenhafte Verständnis des mikrobiellen Kohlenstoff-Kreislaufs macht eine präzise Vorhersage möglicher Folgen der anthropogenen Emissionen von Treibhausgasen schwierig. Von großem Interesse sind daher die Auswirkungen

von CO_2 induzierten Veränderungen der Umweltbedingungen (z. B. pH-Wert und Nährstoffverfügbarkeit) auf Mikroorganismen.

Physiologie und Aktivität der mikrobiellen Gemeinschaften an diesen natürlichen Gasaustritten wurden mit Hilfe von Lipidbiomarkern ermittelt. Um die Zusammensetzung von mikrobiellen Gemeinschaften mit Hilfe von Lipidbiomarkern bestimmen zu können, ist es erforderlich genaue Kenntnisse über die Verteilung potenzieller Biomarker in Organismen zu haben. Sulfat reduzierende Bakterien profitieren von den Bedingungen an den untersuchten CO_2-Austritten. Sie sind eine heterogene Gruppe, deren Mitglieder u.a. unterschiedlichste Kohlenstoffquellen nutzen können. Um die Rolle der Sulfat reduzierenden Bakterien im Kohlenstoffkreislauf der untersuchten Böden besser beurteilen zu können, wurden verschiedene Spezies der Sulfat reduzierenden Bakterien auf ihre Lipidbiomarker analysiert.

Das vulkanische CO_2 ist im Verhältnis zu den anderen in den Böden vorhandenen Kohlenstoffquellen angereichert an dem Kohlenstoffisotop ^{13}C. Diese Anreicherung stellt eine natürliche Markierung dar, mit deren Hilfe die Aufnahme und Weiterverwendung des CO_2-Kohlenstoffs in diesen komplexen mikrobiellen Systemen nachvollzogen werden konnten. Die Untersuchung der Biomarker wurde daher durch die Analyse ihrer stabilen Isotope ergänzt. Diese Kombination erbrachte detaillierte Auskünfte über vitale mikrobielle Gruppen und den Kohlenstoffkreislauf an den untersuchen CO_2-Austritten.

Preface and Objectives

The capture and geological storage of CO_2 (carbon capture and storage, CCS) presents an option for the reduction of greenhouse gas emissions. The storage in saline aquifers or depleted gas and oil reservoirs is already technically feasible. But before the employment of CCS, the consequences of CO_2 leakages from the reservoir to near surface environment need to be evaluated. Natural analogues are useful to reveal the environmental impacts of such a leakage scenario. In this study, two volcanic CO_2 vents were chosen as natural laboratories to reveal the consequences of CO_2 induced environmental changes on microbial life.

CO_2 is the most important greenhouse gas (GHG) of the anthropogenic climate change, furthermore it played an essential role in past climate changes. Besides anthropogenic emissions, the microbial production and consumption of CO_2 have a significant impact on GHG concentrations in the atmosphere. During earlier geological periods, those microbial processes were of even higher importance for the concentration of GHG in the atmosphere; during the Precambrian they were the dominating biological processes. But gaps in knowledge concerning the nature and characteristics of CO_2 consuming microbial groups, especially in anaerobic environments, still exist. A group of particular interest in the climate change related research are microorganisms connected to the CH_4 cycle. Autotrophic methanogenic microorganisms consume CO_2 and produce CH_4, a 25-times more effective GHG than CO_2. Methanotrophic microorganisms, in turn, consume CH_4 and return it to CO_2. Hence, the CO_2 and CH_4 cycle are closely connected to each other. However, little is known about the responsible microbial groups, especially in anaerobic terrestrial environments. Hence, methanogenic organisms utilising volcanic CO_2 and methanotrophic organisms recycling this CO_2 derived carbon, were studied in great detail. The scarce understanding of the microbial carbon cycle is hindering precise predictions about the consequences of anthropogenic GHG emissions. Additionally, the influences that CO_2-induced changes on environmental parameters (e.g., pH and nutrients availability) have on microbial communities are of special interest.

The physiology and activity of microbial communities at these natural gas vents were studied with the help of lipid biomarker. But to be able to reveal the composition of complex microbial communities with biomarker studies, it is necessary to possess detailed knowledge on the distribution of potential biomarkers in organisms. Sulphate

reducing Bacteria benefit from the conditions at the studied CO_2 vents. They are an heterogenic group, whose members are capable of utilising a divers set of carbon sources. To assess the role of sulphate reducing Bacteria within the carbon cycle of the studied soils different species of the sulphate reducing Bacteria were examined for their lipid biomarkers.

The volcanic CO_2 is enriched in the carbon isotope ^{13}C relative to all other carbon sources within the studied soils. This enrichment presents a natural labelling which helps to trace uptake and recycling of CO_2 carbon in these complex microbial systems. Therefore, the lipid biomarker studies were complemented by the analyses of their stable carbon isotopes. This combination yielded a detailed view on vital microbial groups and carbon cycling in the studied CO_2 vents.

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Introduction

1.1. Carbon cycle and climate change

Earth's carbon cycle is divided into a geochemical and a biochemical component. Within the geochemical carbon cycle 75 x 10^6 Gt of carbon are included, most of it bound in sedimentary rocks. The carbon in the geochemical carbon cycle has a very long residence time, due to the control by slow processes like sedimentation and erosion (Fig. 1.1). Relative to the geochemical cycle, the biochemical cycle is controlled by faster processes. In preindustrial times, the two cycles were mainly linked to each other over the processes of deposition, decomposition and weathering.

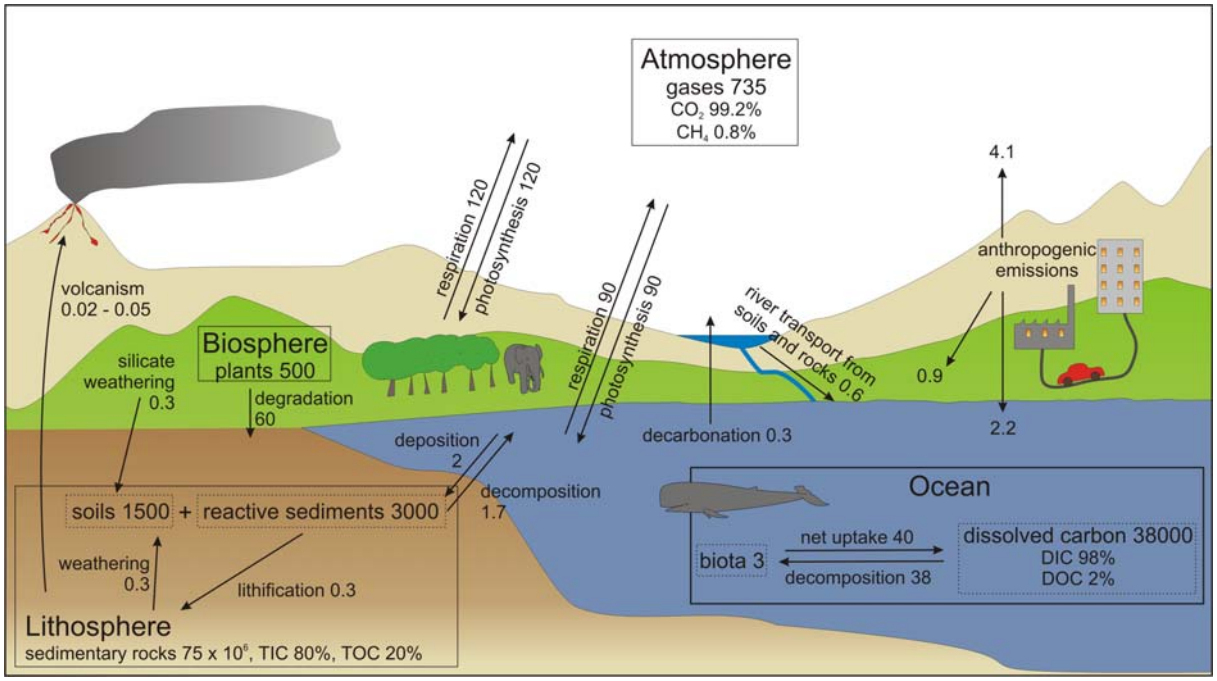

Fig. 1.1. Simplified summary of the global carbon cycle (after several sources (Bolin, 1979; Kempe, 1979; De Vooys, 1979; Bolin *et al.*, 1983; Williams *et al.*, 1992; Siegenthaler & Sarmiento, 1993; Sundquist, 1993; Bickle, 1994; Arthur, 2000; Falkowski *et al.*, 2000; IPCC, 2007a). Amount of carbon in different reservoirs (boxes) is given in Gt C, the exchange rates (associated to arrows) between these reservoirs are given in Gt C yr^{-1}.

But human activity has moved significant amounts of carbon (in form of CO_2 and CH_4) into the biochemical cycle, e.g., by extraction and consumption of fossil biomass. Moreover, anthropogenic activities also lead to enhanced emissions of CO_2 and CH_4 from the biosphere to the atmosphere (e.g., from landfills, paddies and cattle). These emissions have far-reaching consequences as CO_2 and CH_4 are the most important greenhouse gases. Hence, processes controlling the concentrations of CO_2 and CH_4 in the atmosphere are of paramount relevance. Compared to

preindustrial levels the concentration of CO_2 in the atmosphere increased by ca. 28% and that of CH_4 by ca. 250% (Denman *et al.*, 2007). But the consequences of anthropogenic CO_2 emission are insufficiently understood. Only about 55% of this emission is taken up by the atmosphere (Farquhar *et al.*, 2001), while the ocean, soils and the terrestrial biosphere assimilate the remaining part. Hence, human carbon emissions are complete altering the biochemical carbon cycle. To estimate the consequences of such perturbations, detailed knowledge of the size of carbon reservoirs and of processes controlling fluxes between these reservoirs is needed.

Besides changes in the carbon cycle, also CO_2 induced changes in some environmental parameters have to be appraised. Although some of the consequences were identified, e.g., uptake of CO_2 in the oceans and subsequent HCO_3^- formation will lead to decreasing pH values in the oceans, there are still large uncertainties about amplitudes and possible feedback mechanisms. Moreover, the response of most environments to higher temperatures, lower pH and higher CO_2 concentrations are poorly understood yet. For example, reactive sediments and soils are important links between the geochemical and the biochemical carbon cycles. The amounts of carbon being stored in these compartments over longer periods of time are not only determined by the primary production but also by the remineralisation back to CO_2. In both systems, oxygen consumption and subsequent depletion can lead to preservation and accumulation of large quantities of organic carbon. A warmer climate will most likely lead to a decrease in ocean circulation and an increase in density stratification (Denman *et al.*, 2007). The resulting stagnant bottom waters will be cut off from atmospheric O_2 supply, leading to an expansion of oxygen depleted zones in the ocean. Without O_2 the remineralisation of organic matter to CO_2 is slower, possibly enhancing carbon storage in such systems. But this process could also present a positive feedback mechanism, due to the enhanced production of the more effective greenhouse gas CH_4 from organic material under anoxic conditions.

Terrestrial systems are currently a sink for carbon. Major mechanisms controlling the terrestrial carbon budget are known, e.g., climate, stocks of living biomass, chemical and physical properties of litter, stocks of detritus and soil carbon and environmental controls on decomposition. Although these mechanisms are known their, relative contributions are uncertain and factors influencing the strength of the individual mechanisms are insufficiently understood. Hence, a better understanding of these

mechanisms is needed to size CO_2 and climate induced changes in the terrestrial carbon cycle (Farquhar *et al.*, 2001).

1.1.1. Carbon cycle during Earth history

The carbon cycle as it is presented in Figure 1.1 is in equilibrium. It gives reservoir sizes and fluxes as expected before the massive human interventions (ca. 250 BP). But even before human perturbations, massive changes in the carbon cycle have occurred, always having an immense impact on the climate and moreover on the biosphere. Changes in the carbon cycle can be introduced by physical forcing (e.g., the Milankovitch cycles or the solar luminosity), by biological perturbations like the evolution and rise of new organisms (e.g., land plants) or by geological activities emitting large quantities of aerosols, greenhouse gases and water vapour. An understanding of the chronology and effectiveness of different factors and their interaction with each other during past climate changes will help to draw conclusions on present day climate situation and vice versa. In general, transitions from warm periods to cold periods take longer than the other way around. Possibly the melting of gas hydrates contributed to the relative fast shifts to hothouse climates, e.g., during the Proterozoic and Neoproterozoic glaciations (Jacobsen, 2001; Kennedy *et al.*, 2001) and Quaternary ice ages (Dickens *et al.*, 1995; Dickens *et al.*, 1997). Gas hydrates harbour large quantities of greenhouse gases, especially CH_4 (approximately 4000 Gt, Buffett & Archer, (2004)). The dissolution of CH_4 hydrates could explain the rapid acceleration of warming as the melting of some hydrates emits CH_4 into the atmosphere, leading to further climate warming and subsequently more melting of CH_4 hydrates. But the theory of Jacobsen (2001) and Kennedy *et al.* (2001) did not explain how the warming started in the Neoproterozoic. According to Hoffman *et al.* (1998) enhanced volcanic activity and CO_2 emissions caused the climate warming. But it is unknown if autotrophic methanogenic Archaea contributed to the rising CH_4 concentrations. Moreover, it is unsure how the CH_4 was removed from the atmosphere, ether just by abiotic processes or also by biotic processes, which would have caused enhanced CO_2 production.

1.2. Carbon capture and storage

Against the background of CO_2-induced changes in the global carbon cycle and the climate and the peril coming along with these changes, a search for adequate methods to reduce GHG emissions, especially CO_2 emissions, has started. One of the options to reduce anthropogenic CO_2 emissions is carbon capture and storage (CCS). The International Panel on Climate Change, which was established by the United Nations Environment Program and the World Meteorological Organization to summarize knowledge on climate change for the public and especially decision makers, considers CCS as one option within a portfolio of mitigation actions. Primary mitigation options for the stabilization of GHG concentrations in the atmosphere include energy efficiency improvements, the switch to less carbon-intensive fuels, renewable energy sources, enhancement of biological sinks, the reduction of non-CO_2-GHG and nuclear power, although the last option is controversially debated in Germany.

But most energy scenarios project that the energy demand will rise and that at least some of this energy will be supplied by fossil fuels (Sims *et al.*, 2007). Therefore, a technique capturing CO_2 after fossil fuel burning and safe storage for longer time period (>100 y) appears to be a promising tool in the fight to mitigate climate change (IPCC, 2005).

In CCS, CO_2 is first separated from other combustion gases. There are three different processes of carbon capture. The first process, the post-combustion, is already widely distributed in the natural gas processing industry. In this process CO_2 is separated from the exhaust after fossil fuel burning. Also the second system to capture CO_2, the pre-combustion, can be adopted from another industry, the production of fertilizer and hydrogen. During pre-combustion fossil fuels are first gasified and combusted with O_2 enriched air. The resulting gas exhaust yields lower concentrations of disturbing gases, making separation of CO_2 easier. Oxyfuel is a CO_2 capture system, still in the demonstration phase of development. It uses highly pure O_2 for combustion and the exhausts contain even higher CO_2 concentration than after the pre-combustion process.

Regardless of the capture system applied, the obliged technical facilities will make CO_2 capture feasible only for large and stationary emission sources. Although agriculture and traffic contribute large amounts to the global anthropogenic CO_2 emission, their small and non-stationary sources are not compatible with the

technical requirements of CO_2 capture. Taking into account only large emission sources, processes producing power contribute by far the largest part to the global CO_2 emissions (Table 1.1). But if the average emission per source is considered, also iron and steel industry and refineries are good locations for CO_2 capture.

Table 1.1. Large stationary CO_2 emission sources worldwide.

Process	Number of sources[1]	Emission [$MtCO_2\ y^{-1}$][1]	Ø-Emissions per source [$MtCO_2\ y^{-1}$]
Power	4942	10539	2.1
Cement production	1175	932	0.8
Refineries	638	798	1.3
Iron and steel industry	269	646	2.4
Petrochemical industry	470	379	0.8
Oil and gas processing	Not available	50	
Other sources	90	33	0.4
Bioethanol and bioenergy	303	91	0.3

[1] after (Sims et al., 2007)

One disadvantage of CCS is the energy consumption during the capture process. The energy need of the capture process will depend on the source type and the capture system applied. Numerous studies in recent years have assessed multiple improvements for the three different capture systems (see Figueroa *et al.*, 2008 for review) and research in this area is continuing. But before industrial scale appliances of this process takes place, the degree of energy consumption can only be appraised (Fig. 1.2). But regardless of the exact technique applied, the capture process will cost additional energy. At a fossil fuel power plant this additional energy will most likely derive from fossil fuel. From this follows that more fossil fuels will be burned in order to provide the same amount of energy, hence the emission factor will increase (Fig. 1.2). Storage can significantly reduce the emission factor, but only 90 to 95% of CO_2 emissions into the atmosphere can be avoided by this. The remaining 5 to 10% arise from CO_2 emissions during fossil fuel extraction, transport, preparation before combustion and incomplete capture after combustion.

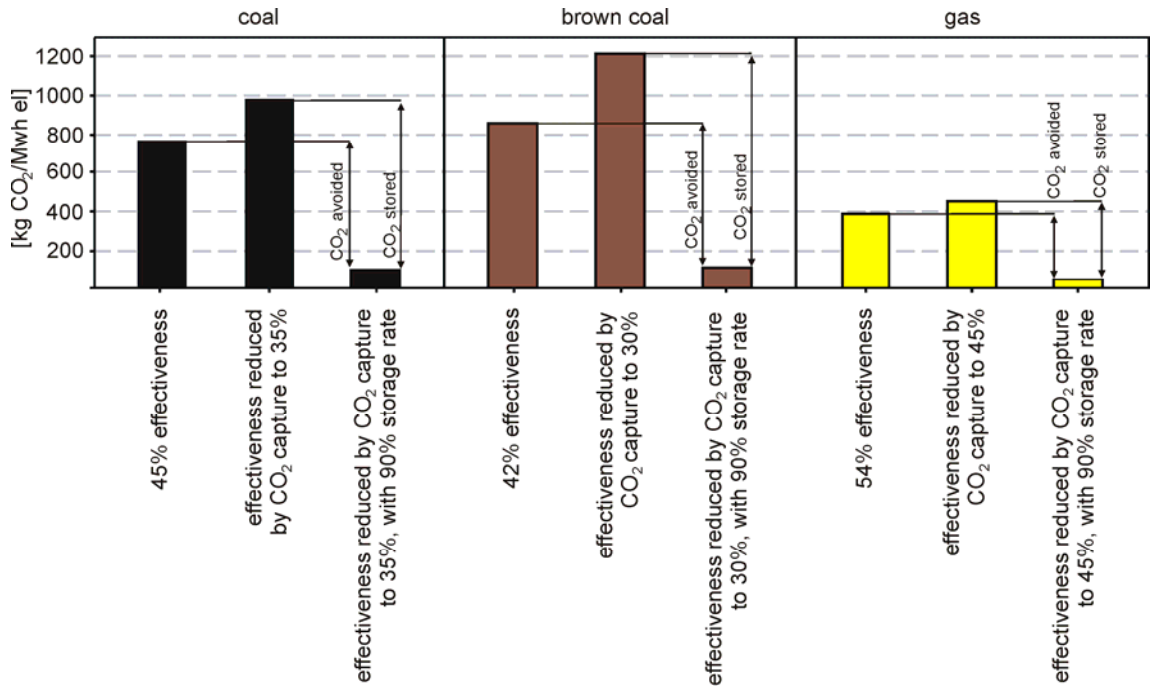

Fig. 1.2. CO_2 emission factors of energy production with different fossil fuels in Mwh el per kg CO_2 emitted to the atmosphere after (Radgen *et al.*, 2005). First bar gives the basic emission factor for each fossil fuel type, the second bar represents the emission factor with CO_2 capture and the last bar gives the emission factor reduced by 90% storage rate.

After the capture, CO_2 is transferred to the supercritical state. Above its triple point (31.1°C and 72.9 atm, Fig. 1.3) CO_2 has the same flow characteristics as in its liquid phase but has got a higher specific density. Since a higher density can reduce the required volume of transport facilities these characteristics are very favourable for transportation. Furthermore, CO_2 keeps its super-critical state if stored in depth of 800 m below earth surface.

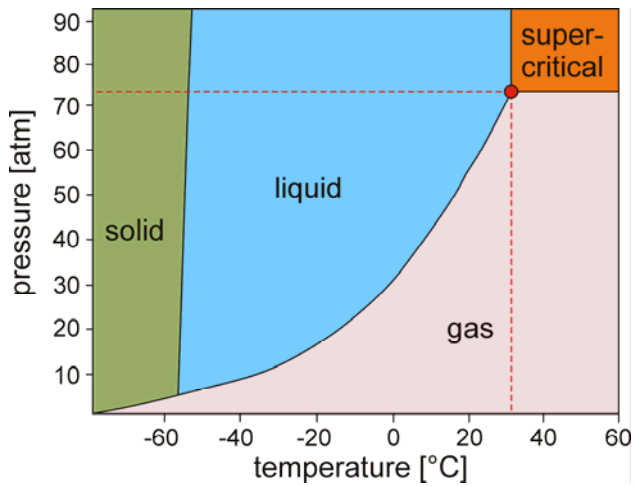

Fig. 1.3. CO_2 pressure-temperature phase diagram.

Four storage types are suggested; storage in depleted oil- or gas reservoirs, in unmineable coal seams, in saline aquifers and in deep ocean basins (Fig. 1.4). Due to the incomplete understanding of deep sea environments, CO_2 storage in the ocean is controversially debated (Shaffer, 2010) and not considered as an option in the EU (The European Parliament and the Council of the European Union, 2009). Storage in depleted oil and gas reservoirs might offer the possibility of enhanced oil or gas recovery. In fact, CO_2 injection has been used for many years to enhance oil or gas

production in almost depleted reservoirs, therefore sufficient experience with this technique exists. Over the past 25 years experiences with the sequestration of CO_2 were gained in the field of enhanced oil and gas recovery. Likewise the storage in unminable coal seams might extract significant amounts of coal bed methane. The extraction of additional fossil fuels would make CCS more competitive but it would also add to the anthropogenic carbon budget. Depleted or unmineable fossil fuel reservoirs are comparatively well explored and usally relatively closely located to emission sources (e.g., coal seams in the Ruhr area/Germany with a high density of coal fire power plants) or already have a pipeline infrastructure which only needs to be adjusted. Moreover, gas and oil reservoirs have proven there retention ability over billions of years. But unfortunately, the storage capacities of depleted oil and gas reservoirs in areas with large emission sources are limited, e.g., May *et al.*, (2002) and Schuppers *et al.* (2003). The injection of CO_2 in unminable coal seams is connected with a number of obstacles, e.g., low permeability, volume increase and fracture after CO_2 injection (Gerling & May, 2001). Deep saline aquifers are a feasible option for storage of CO_2 when depleted oil or gas reservoirs are rare or not availeble, moreover a conflict of interest with usage of geothermal energy has to be considered from region to region. Deep saline aquifers offer the biggest storage capacities, >10-times more than depleted oil and gas reservoirs (IPCC, 2005). But chemical reactions of the brine or the cap rock with supercritical CO_2 are yet unknown and research in this area just started (e.g., Duguid & Scherer, 2010; Michael *et al.* 2010; Ghaderi *et al.,* in press). Nevertheless CCS raised the hope of a feasible compromise between climate protection and economic growth. And nowadays legal and regulatory frameworks are being adjusted to issue the rules of procedure, liability and requirements for CCS (The European

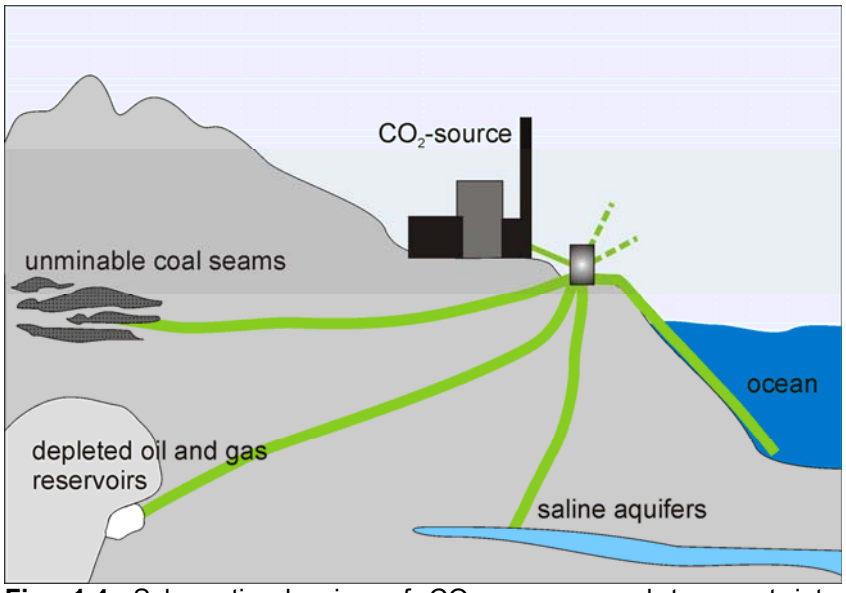

Fig. 1.4. Schematic drawing of CO_2 sources and transport into possible storage types.

Parliament and the Council of the European Union, 2009). But before establishing CCS as a wide spread solution for mitigation of CO_2 emissions we still need to assess risks and consequences. Research in the field of CCS has mainly concentrated on competitive capture techniques, adequate storage sites and prevention of leakage from a storage site. Four main leak mechanisms have been identified, for example a storage formation has contact to atmosphere/ocean, poorly plugged abandoned wells, gas pressure exceeds capillary pressure or the CO_2 rises upward along a fault (Fig. 1.5). Even after thoroughly selection of storage site and careful and skillful injection of CO_2, leakage from the storage formation can never be 100% ruled out.

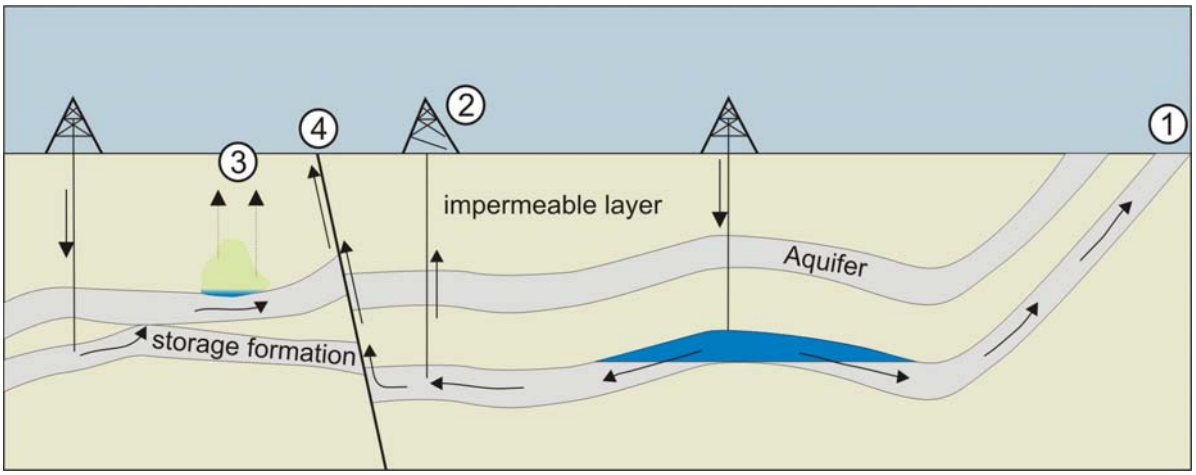

Fig. 1.5. Schematic drawing of different leakage scenarios, modified after (CO2CRC, 2009) 1. formation has contact to atmosphere/ocean, 2. poorly plugged abandoned well, 3. gas pressure exceeds capillary pressure, 4. fault.

Especially the long term stability of CO_2 reservoirs is difficult to predict. Hence, consequences of potential CO_2-leaks and methods to locate these leaks need to be evaluated. Before widespread appliance of CCS legal and regulatory frameworks need to set the maximum value of accepted leakage. One aspect that needs to be considered for an accepted leakage rate is the value at which leakage endangers the positive effect of CCS for GHG mitigation. In this budget also the additional CO_2 production for the capture process has to be considered (Fig. 1.2), as well as the microbial or abiotic production and the extrusion of other GHG during leakage. Another aspect for the determination of maximum leakage rates have to be the direct hazard of CO_2 to humans, the environment and third party property.

In this study the consequences of potential CO_2-leakage into near-surface ecosystems, like changes in community composition of quasi stationary organisms (e.g., plants and microorganisms) and/or soil chemistry are assessed.

Although several studies have been published regarding the effect of increased atmospheric CO_2 concentrations on ecosystems (e.g., Jossi *et al.*, 2006), there are only very few that have examined the effects of increasing CO_2 concentrations in the soil horizon due to upwardly migrating gas. Favourable for such studies are natural, volcanic CO_2 seeps which can serve as natural laboratories, as they exhibit very similar characteristics to leakages from anthropogenic CO_2-storage sites. Reports like these include a detailed study of a terrestrial CO_2 vent at Latera, Italy (Beaubien *et al.* 2008), of Mammoth Mountain (California, USA) regarding the influence of volcanic CO_2 on soil chemistry and mineralogy (Stephens & Hering, 2002; Stephens & Hering, 2004) and of Stavesinci (NE Slovenia) assessing the influence of high soil-gas concentrations of geothermal CO_2 on plants (Macek *et al.*, 2005; Pfanz *et al.*, 2007; Vodnik *et al.*, 2006). The study site was located on pasture land. Pasture land comprises 33% of agricultural used land (about 55 mio. ha) in Europe (Directorate-General for Agriculture and Rural Development, 2006).

1.3. CO_2 and CH_4 in the biochemical carbon cycle

The biochemical carbon cycle was substantially influenced by the evolution of organisms throughout Earth history. Based on molecular comparisons life on earth is divided into three domains, Bacteria, Archaea and Eukarya (Fig.1.6) (Woese *et al.*, 1990).

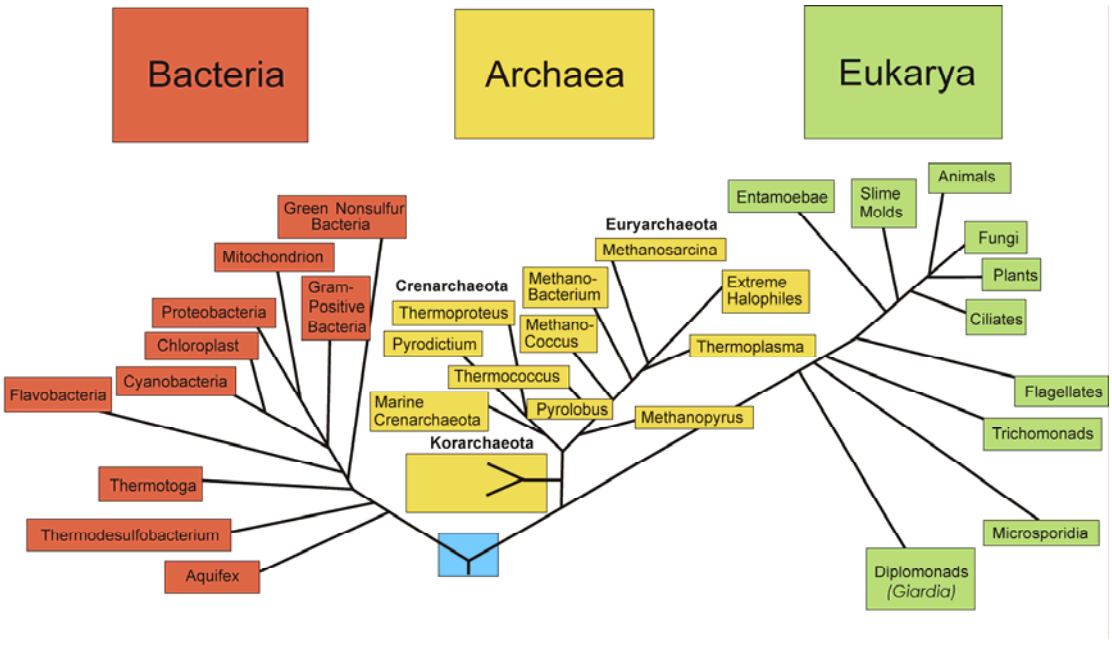

Fig. 1.6. Phylogenetic tree of life based on small subunit 16S-rRNA-sequencing (Madigan *et al.*, 1997).

Eukaryotic cells possess a nuclear envelope (nucleus), carrying the genetic material, while prokaryotic cells (Bacteria and Archaea) lack a cell nucleus and other membrane bound organelles and their DNA is organized in a ring shaped bundle called nucleoid. Prokaryotics cell differ in their membrane lipids, since Archaea produce lipids with isoprenoidic moieties and Bacteria contain lipids with fatty acids. The consumption and production of CO_2 plays a remarkable role in the biochemical carbon cycle. These processes are moving carbon from the geochemical carbon cycle to the biochemical carbon cycle, e.g., fixation of CO_2 by phototrophic organisms and production of CO_2 by methanogenic Archaea. Moreover, the release of CO_2 during remineralisation is of major importance for the recycling of organic carbon. And the uptake of CO_2 by the biosphere mitigates the anthropogenic climate change. Therefore, an understanding of microbial CO_2 consumption, but also of its production, is of fundamental importance to predict effects and possible feedback mechanisms to carbon addition in the atmosphere, the ocean and terrestrial environments.

Production and consumption of CO_2 are strongly linked to CH_4 by a set of biological processes. Autotrophic organisms like plants, phototrophic Bacteria or chemotrophic Bacteria take up CO_2 for biomass build-up. After cell death heterotrophic organisms use this biomass as an energy source, leading to CO_2 and CH_4 production. First organic matter is oxidized with oxygen. After its consumption subsequently nitrate, Mn(IV), Fe(III) and sulphate are used as electron acceptors following the maximum energy yields of these oxidation reactions. The last step in organic matter degradation is methanogenesis by methanogenic Archaea. Two major groups of methanogenic Archaea are known, heterotrophic methanogens using acetate to produce CO_2 and CH_4 and autotrophic methanogens using $CO_2 + 4H_2$ producing CH_4 and $2H_2O$ (Conrad, 1996).

Both groups live in diverse habitats and are responsible for the methane production in marine sediments, wetland, paddies, forests, landfills and guts of termites. Therefore, they are responsible for natural as well as human induced CH_4 emissions. Biological sources account for 70% of the global CH_4 emissions. Since anthropogenic CO_2 emissions result in higher concentrations of substrates for both autotrophic (CO_2) and heterotrophic (biomass; Farquhar *et al.*, 2001) methanogenesis, they might strengthen microbial CH_4 production. CH_4 has a global warming potential 25-times higher than that of CO_2 (Forster *et al.*, 2007). Therefore enhanced CH_4 production presents a positive feedback mechanism to CO_2 emissions.

The major sink of atmospheric CH_4 is the abiotic oxidation with OH-radicals in the troposphere, followed by stratospheric losses. Without the biological oxidation, however, atmospheric concentrations of methane would be much higher. Reeburgh (2007) found that more than 88% of CH_4 in the ocean is oxidised before it reaches the atmosphere.

Similarly to the oxidation of organic matter, the oxidation of CH_4 can be most likely conducted by a set of oxidants, with oxygen being the energetically most favourable. But as described before, methanogenesis and therefore major CH_4 sources are located in areas depleted of oxidants. From the zones of its formation CH_4 migrates upwards and first passes through the zone of sulphate reduction. In marine environments were sulphate is prevalent, the anaerobic oxidation of methane (AOM) coupled to sulphate reduction is the dominating process in CH_4 consumption (Hoehler *et al.*, 1994; Boetius *et al.*, 2000; Michaelis *et al.*, 2002b). Only recently, Ettwig *et al.* (2008), Beal *et al.* (2009) and Ettwig *et al.* (2010) showed that also Mn(IV), Fe(III) and NO_3^- could serve as oxidants for the AOM. In terrestrial environments were sulphate concentrations are usually low, these additional electron acceptors theoretically could play an important role in the AOM. But the relevance of Mn(IV), Fe(III) and NO_3^- coupled AOM under natural conditions is yet uncertain. Until now there are only enrichment cultures available, being capable of sulphate independent AOM, the denitrifying '*Candidatus* Methylomirabilis oxyfera' (Ettwig *et al.*, 2008) and a manganese and iron reducing culture (Beal *et al.*, 2009). Due to the lack of isolates these uncertainties can only be approached by environmental surveys. Aerobic methanotrophic Bacteria were studied in much more detail (Summons, *et al.*, 1994; Hanson & Hanson, 1996; Jahnke *et al.*, 1999).

Moreover, considerable gaps in knowledge concerning the global rates of the different AOM pathways and their sensitivity to different parameters, e.g., pH and temperature, exist. Due to the lack of isolates of AOM performing microorganisms these gaps can be most efficently closed by culture independent methods.

1.3.1. Assessment of microbial communities

Several approaches are available to study the microbial diversity and ecology in complex biological systems. Each approach offers particular information but also drawbacks. In order to study complex biological systems, methods should be chosen conscientiously, considering the goal of the research and the limits of the selected methods.

Culture depended methods, for example, offer the potential to characterise the physiology of isolated microorganisms in detail. Unfortunately, today only a small fraction of known microorganisms has been isolated and cultured, approximately 10% (Amann *et al.*, 1995), leaving the majority of microorganisms undescribed. Moreover, abilities or characteristics of organisms in culture experiments cannot simply be transferred to complex biological systems. Environmental factors and other microorganisms, which are not engaged in culture depended studies, usually have a large effect on microbial physiology. Hence, it is hardly possible to appraise the role of individual microorganisms in certain environments by culture depended methods. Likewise, growth experiments, studying certain microbial activities within environmental samples, only yield biosynthetic capabilities under the conditions applied. Moreover, already perturbation during the sampling process alters the samples.

Molecular microbiological methods can be used to assess microbial communities by culture independent methods. The most commonly used culture independent method to evaluate the phylogeny of active microorganisms in environmental samples is the 16S rRNA sequence analysis (Amann *et al.*, 1998). 16S rRNA allows a rapid description of phylogenetic variety of microbial communities. Another possibility to analyse the *in-situ* population is based on DNA. But DNA can be physically protected from degradation by adsorption to sediment particles (Romanowski *et al.*, 1991; Demaneche *et al.*, 2001) thus containing the risk of inclusion of dead material. RNA is less stable than DNA and it is believed to present a signal of living cells (Fritsche, 1999). Additionally, 16S rRNA probes can be equipped with fluorescent labels. With the fluorescence *in situ* hybridisation and sufficient microscopes, the arrangement and structure of cells can be studied (Amann *et al.*, 1998). But 16S rRNA probes are selective, targeting only matching rRNA. Therefore, it is likely that not the entire microbial variety is displayed with this method. In addition, 16S rRNA probing does not reveal the physiology of the studied microorganisms.

Besides molecular microbiological methods, lipid biomarkers can be used to study microbial communities. Lipid biomarkers are specific organic molecules, assignable to particular groups of organisms, but they are often not as specific as rRNA. So knowledge of the biomarker distribution in different organisms is needed to assign them to possible source organisms. Nevertheless, they are non-selective and yield information on the relative importance of distinct prokaryotic groups. Lipid biomarkers

are relatively resistant against chemical or biological degradation and thus they can survive geological timescales within sediments, rocks and oils. Hence, they are the only tools to assess fossil microbial communities. Moreover, combined with stable isotope analyses they can yield information on the biosynthetic pathways applied by organisms *in situ*.

1.3.2. Lipid Biomarker

Most biomarkers are cell membrane lipids. Cell membranes consist of a selectively permeable, phospholipid bilayer (Fig. 1.7). Phospholipids consist of a hydrophobic group, containing a phosphate moiety and possibly other polar groups (e.g., choline, ethanolamine), a hydrophobic group containing straight chain, branched or isoprenoic acids and a glycerol moiety over which the two parts are linked. In Archaea the phosphate group is bound to the glycerol moiety at the *sn*-1 position, while in Eukarya and Bacteria it is bound at the *sn*-3 position. Further differences between the phospholipids of Bacteria, Eukarya and Archaea are the type of the hydrophobic groups and the type of bonding between the glycerol backbone and the hydrophobic group. Archaea produce only ether bound lipids with isoprenoic hydrophobic groups, while in Eukarya the hydrophilic groups consist of fatty acids which are linked to the glycerol back bone via ester bounds.

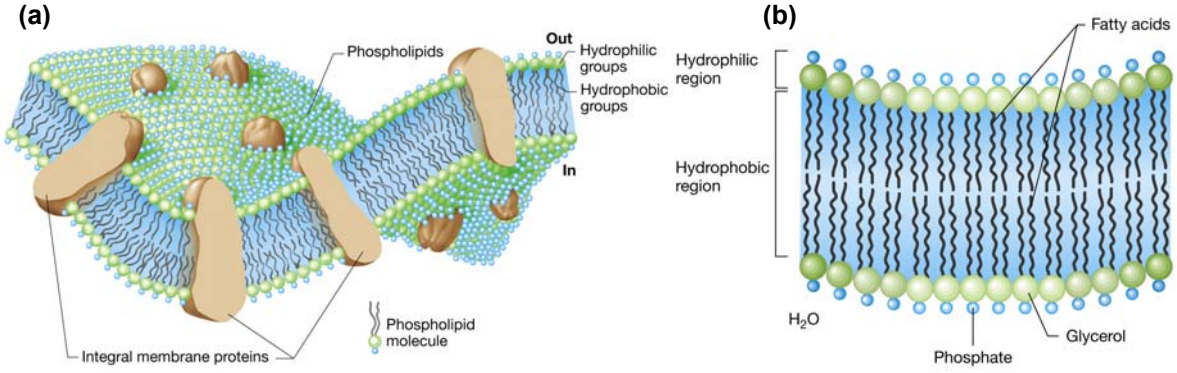

Fig. 1.7. Diagram of the structure of a bacterial cytoplasmic membrane composed of a phospholipid bilayer after (Madigan *et al.*, 2000). The hydrophobic groups face each other while the hydrophilic groups either point into the cytoplasm or to the exterior of the cell. (a) Hydrophobic proteins are embedded in the membrane. (b) Phospholipids consist of a phosphate, a glycerol and a fatty acid moiety.

Bacteria produce fatty acids as hydrophilic moieties, which might be ester- or ether-bound to the glycerol backbone. Bacterial ethers were first found in Bacteria from extreme environments, e.g., thermophilic Bacteria (Huber *et al.*, 1992; Huber *et al.*, 1996) or in environmental samples from extreme environments, e.g., from the shales

of the euxinic lake Messel (Chappe *et al.*, 1979) or hydrothermal vents (Blumenberg *et al.*, 2007a).

But recent findings illustrate the occurrence of bacterial ethers also in mesophilic environments (Schouten *et al.* 2000; Rütters *et al.*, 2002). Three groups of bacterial ethers are known, as there are monoalkyl glycerol monoethers (MAGEs), dialkyl glycerol diethers (DAGEs) and glycerol dialkyl glycerol tetraethers (GDGTs) (Fig. 1.8). Bacterial ethers appear to be particular widespread in anaerobic environments. For example, in AOM performing microbial mats DAGE *ai*-15:0 (Fig. 1.8.b) is found (Hinrichs *et al.*, 2000, Pancost *et al.*, 2001; Michaelis *et al.* 2002). According to Michaelis *et al.* (2002) this DAGE derives from SRB associated to the anaerobic oxidation of methane. Also SRB from other environment are known to produce MAGE and DAGE (Langworthy *et al.*, 1983; Rütters *et al.*, 2001, Sturt *et al.,* 2004). Besides SRB also species of the obligate anaerobic *Clostridium* (Langworthy & Pond, 1986), the facultative anaerobic *Streptococcus mutans* (Brissette *et al.*, 1986), *Aquifex pyrophilus* (Huber *et al.*, 1992), *Ammonifex degensii* (Huber *et al.*, 1996), *Mycoplasma fermentans* (Wagner *et al.*, 2000) and *Propionibacterium propionicum* (Pasciak *et al.*, 2003) possess bacterial ethers. The only aerobic Bacteria known to contain ether lipids are *Myxobacteria* (Caillon *et al.*, 1983; Ring *et al.*, 2006), which produce ether lipids in the process of spore formation under unfavourable conditions.

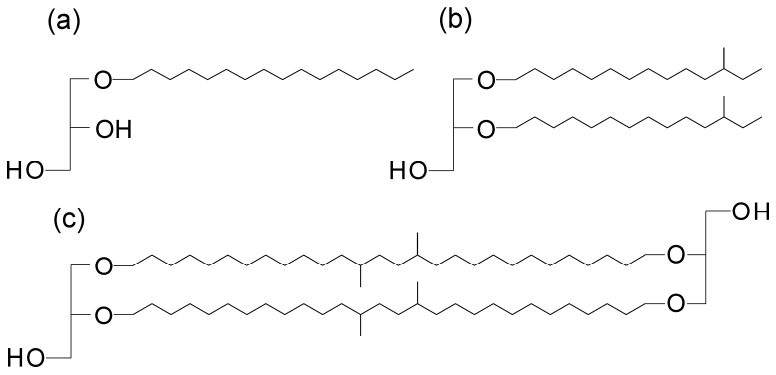

Fig. 1.8. Examples for the chemical structures of MAGE, DAGE and GDGT (a) MAGE containing a $C_{16:0}$-moeity, (b) DAGE containing two *anteiso*-methyl-branched $C_{15:0}$-moeities and (c) GDGT containing two mid chain methyl branched C30:0 moeities.

The source-organisms of the third group of bacterial ethers, the GDGTs, are unknown. But based on the predominance of branched GDGTs in anoxic compartments of soils and peat bogs, Pancost and Sinninghe Damsté (2003) and Weijers *et al.* (2007) proposed these compounds to derive from yet unknown anaerobic soil Bacteria. GDGTs are, so far, only found in terrestrial environments. In soils they even can be the dominating group of bacterial lipids (Weijers *et al.*, 2006a).

Hence, they are used to estimate terrestrial input within marine sediments (Hopmans *et al.*, 2004).

GDGTs were first found in Archaea, *Thermoplasma acidophilum* (Langworthy, 1977). Instead of straight chain and branched acids, archaeal GDGTs contain isoprenoic core moieties (Fig. 1.9). Shortly after the first finding (Michaelis and Albrecht, 1979) found GDGT derived biphytanes containing up to one pentacyclic moiety in the fossil sediments of the Lake Messel. Interestingly, the Lake Messel is located within a volcanic caldera very similar to the study sites of this study (Jackoby *et al.*, 2000).

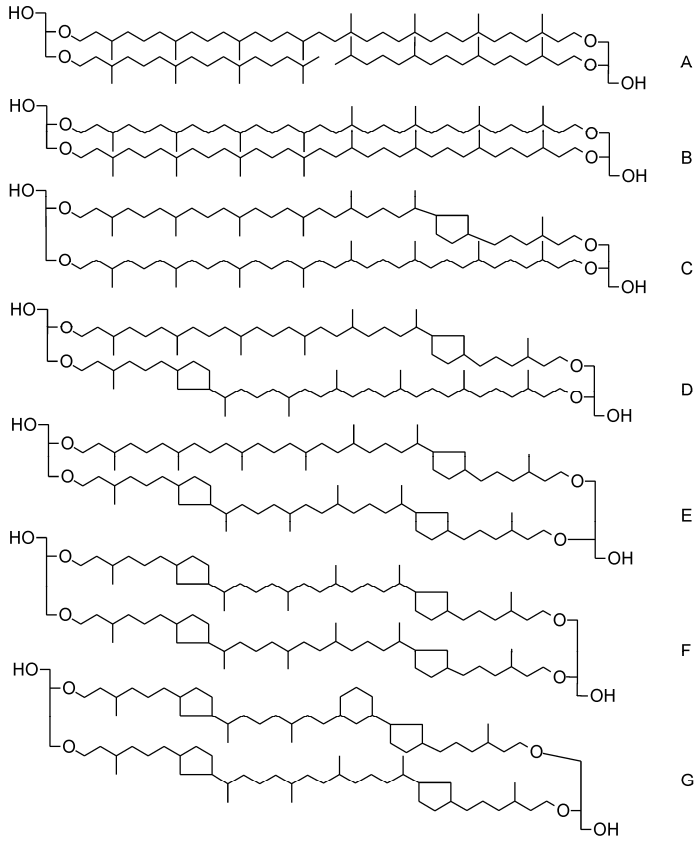

Fig. 1.9. Chemical structures of archaeal tetraethers.

The acyclic, archaeal GDGT (Fig. 1.9B) is wide spread in methanogenic Archaea (De Rosa and Gambacorta, 1988; Koga *et al.* 2006 and ref. therein) and the biphytane core lipids were first used as biomarkers for methanogenic Archaea in mesophilic environments (Michaelis and Albrecht, 1979; Chappe *et al.*, 1980; Chappe *et al.*, 1982; Hoefs *et al.*, 1997). But additionally GDGTs were lately found in many mesophilic environments originating from mesophilic Crenarchaeota (DeLong *et al.*, 1998; Schouten *et al.*, 2000; Weijers *et al.*, 2006a; Escala *et al.*, 2007). Creanarcheaote produce a highly diagnostic lipid biomarker, Crenarchaeol (Fig. 1.9G) (Sinninghe Damsté *et al.*, 2002).

In addition to phospholipids, terpenoids can be used as lipid biomarkers. Terpenoids consist of isoprene units and display a great diversity of structures and functions. One group of terpenoids used as biomarker are hopanoids. They occur in plants, ferns, mosses, fungi, protists and Bacteria. But bacteriohopanepolyols (C_{35}) are only found in Bacteria. They are components of cell membranes in Bacteria and the precursors of the ubiquitous geohopanoids (Ourisson & Albrecht, 1992). Kannenberg

& Porella (1999) and Ourisson & Rohmer (1992) proposed that bacteriohopane-polyols (BHP) rigidify the cell membrane comparable to sterols in Eukaryotes. However, according to Pearson *et al.* (2007) only 10% of Bacteria are capable of producing hopanoids, suggesting a different less elementary role in the bacterial cells. Nevertheless, hopanoids are believed to be the most abundant organic molecules on Earth (Ourisson & Albrecht, 1992). Some BHPs are very diagnostic biomarkers, e.g., 35-aminobacteriohopentriol, which is only produced by purple non-sulphur α-proteobacteria.

Only few anaerobic Bacteria are known to synthesise BHP, Planctomycetes (Sinninghe Damsté *et al.*, 2004) and some *Geobacter* species (Fischer *et al.*, 2005).

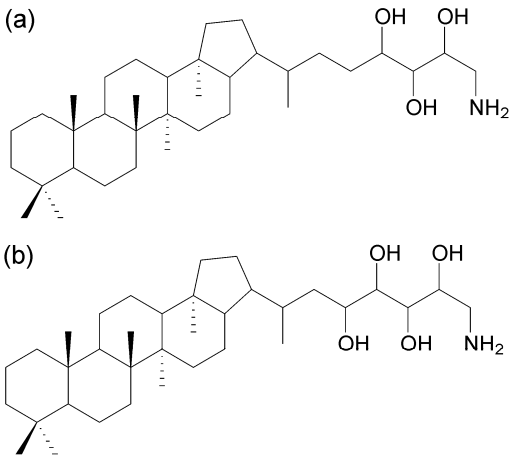

Fig. 1.10. Examples for the chemical structures of BHPs (a) aminotriol and (b) aminotetrol.

But the recent discovery of BHP in microbial mats performing AOM (Thiel *et al.* 2003) hints to additional source organisms, possibly SRB. Aerobic methanotrophic Bacteria also contain BHPs, which are used to distinguish between the two main groups of methanotrophic Bacteria. 3β-methylbacteriohopanetetrol and 35-amino-bacteriohopanepentol (aminopentol) occur in type I methanotrophs (Neunlist & Rohmer, 1985a; Zundel & Rohmer, 1985) whereas 35-aminobacteriohopanetriol (aminotriol; Fig. 1.10) and 35-amino-bacteriohopanetetrol (aminotetrol; Fig. 1.10) are found in type II methanotrophs (Neunlist & Rohmer, 1985b; Talbot *et al.*, 2001), but some of these BHPs are present also in other bacterial groups, e.g., 35-aminobacteriohopanetriol is contained in cyanobacteria (Talbot *et al.*, 2008).

In such cases, lipid biomarkers need to be evaluated by taking the studied environment and their $\delta^{13}C$-values into account. The $\delta^{13}C$-values of biomarkers contain information regarding the carbon source and the utilized carbon assimilation pathway of source organism.

1.3.3. Stable carbon isotopes and biomarkers

Carbon has two stable isotopes, ^{12}C and ^{13}C. The isotope ^{13}C is usually less reactive than the isotope ^{12}C. Hence, ^{13}C is discriminated against ^{12}C in biological processes. In non-equilibrium processes this leads to a relative enrichment of ^{12}C in the products of biological processes and an enrichment of ^{13}C in the remaining educt. This effect can be used to track carbon cycling in biological systems.

The isotopic composition of all natural compounds is governed by the $\delta^{13}C$-value of the carbon source, the isotopic fractionation during carbon uptake, the isotopic fractionation during biosynthesis and metabolism, as well as the cellular carbon budget (Craig, 1954; Sirevag *et al.*, 1977; Hayes, 1993; Summons *et al.,* 1994; Sakata *et al.,* 1997). The isotopic composition is expressed as the deviation relative to a standard of known composition ($\delta^{13}C$). The $\delta^{13}C$-value is calculated according to the following formula:

$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)_{Probe}}{(^{13}C/^{12}C)_{Standard}} - 1 \right] \times 1000 \qquad (1)$$

The two stable isotopes of carbon ^{12}C and ^{13}C are contained in the Vienna Pee Dee Belemnite in a relative abundance of 98.890% and 1.110%, respectively. The $\delta^{13}C$ notation gives the changes in composition in promille (‰), e.g., 1‰ equals a shift in relative abundance of 0.001%.

Carbon sources have a broad range of $\delta^{13}C$-values depending on their origin (Fig. 1.11 and Table 1.1).

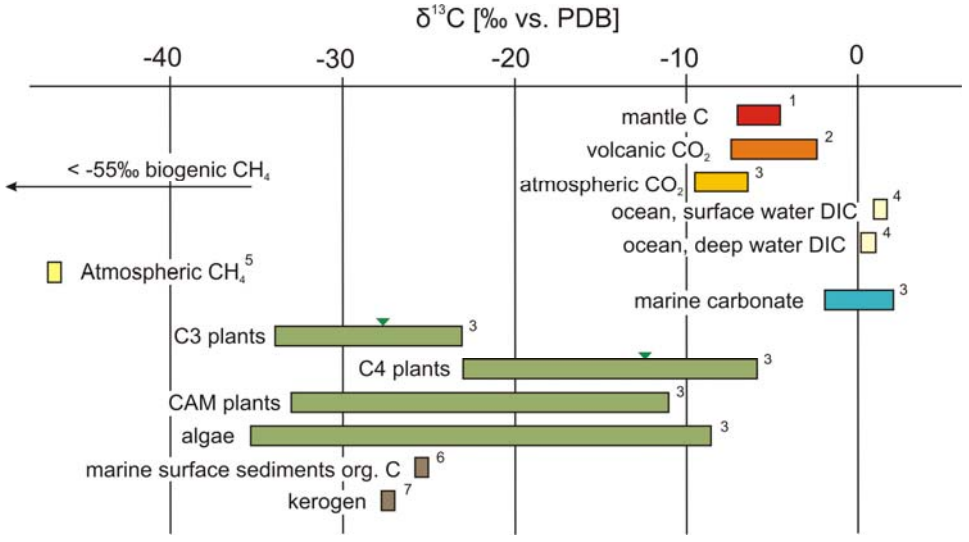

Fig. 1.11. The $\delta^{13}C$-values of selected large carbon reservoirs. Numbers indicate references for given $\delta^{13}C$-values: [1] (Craig, 1953; Deines & Gold, 1973; Deines *et al.*, 1989); [2] (Gerlach & Taylor, 1990; Javoy & Pineau, 1991; Poorter *et al.*, 1991; Poreda *et al.*, 1992), [3] (Schidlowski, 1988), [4] (Deuser & Hunt, 1969; Kroopnick, 1985), [5] (Craig *et al.*, 1988; Stevens, 1988), [6] (Deines, 1980), [7] (Hayes, 1983). Green triangles indicate the mean.

Atmospheric CO_2 has $\delta^{13}C$-values ranging from -6‰ to -10‰ becoming progressively enriched in ^{12}C with increasing emissions of CO_2 from fossil fuel burning. Dissolved inorganic carbon (DIC) displays $\delta^{13}C$-values from +1‰ to -1‰. Depending on the carbon fixation pathway utilized by the plants the $\delta^{13}C$-values of their biomass ranges between -8‰ and -34‰.

The $\delta^{13}C$- and δD values of methane vary over a relatively large range depending on whether the methane is thermogenic or biogenic (Fig. 1.12). Moreover, biogenic methane can show broad variance depending on the carbon source and its $\delta^{13}C$-value and on the mode of methanogenesis (autotrophic or heterotrophic). Combining information on stable carbon and hydrogen isotope Whiticar (1999) developed a model that helps to identify the origin of CH_4 (Fig. 1.12).

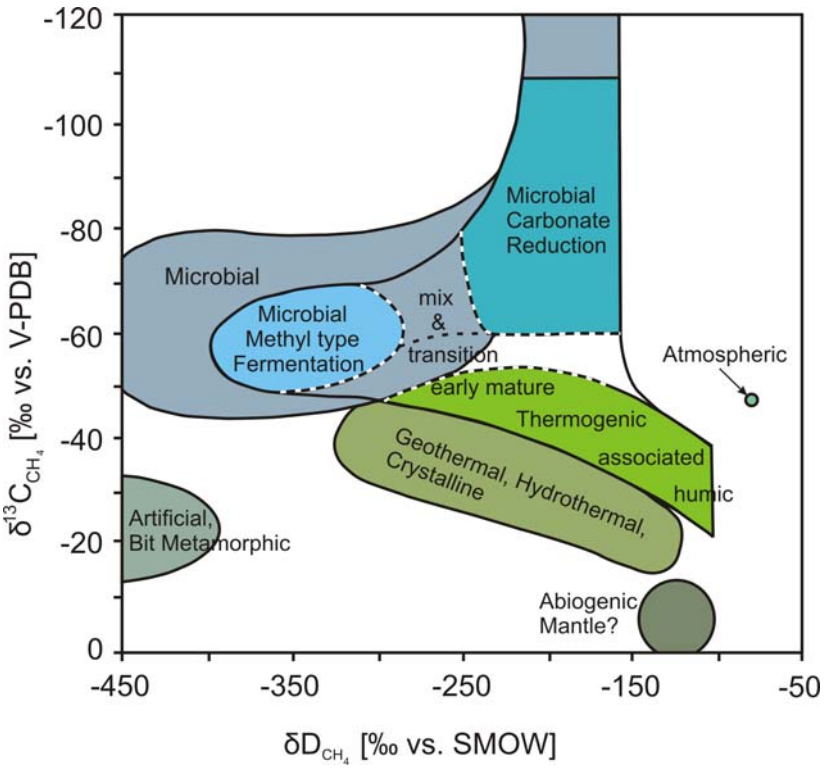

Fig. 1.12. Classification of microbial and thermogenic CH_4 by stable C- and H-isotopes, after Whiticar (1999).

The assimilation of CO_2 and CH_4 can lead to significant kinetic isotope effects. Although fractionation factors also depend on environmental conditions like temperature, pressure, salinity etc., the mode of carbon fixation sets the limits of this fractionation (Goericke & Fry, 1994). During the calvin cycle, utilised by many chemo-autotrophic microorganisms, ^{12}C can be further enriched in organic carbon, yielding isotopic shifts of up to -27‰ (Goericke & Fry, 1994). The reverse tricarboxylic acid cycle and the 3-hydroxypropionate pathway have lower fractionation factors (Table 1.2).

Table 1.2. Selected fractionation between inorganic carbon and biomass after O'Leary (1981), Belyaev *et al.* (1983), Preuss *et al.* (1989), Guy *et al.* (1993), Goericke & Fry (1994), Jahnke *et al.* (1999), Hayes (2001) and Zhang *et al.* (2003).

Pathway	Organisms	ε (‰)
Autotrophic fixation of inorganic carbon		
C3	tropical phytoplankton	≈ 18
	land plants	≈ 30
C4	land plants	≈ 10
CAM	land plants	≈ 23
Acetyl-CoA	Eukarya and Prokarya	15 to 36
Reductive or reverse TCA cycle	Bacteria and Archaea	4 to 13
Heterotrophic carbon fixation		
Iron reducing	Geobacter	4.5 to 15.5
Aerobic methane oxidation		
RuMP pathway	Type I-methanotrophs	5.3 to 24.8
Serine pathway	Type II-methanotrophs	-3.6 to 14.1

Substantial variance was also detected concerning the carbon fixation of methanothrophs, depending on the pathway used for CH_4-oxidation. Aerobic methanotrophic Bacteria are phylogenetically a divers groups. Most of the methanotrophic bacteria belong to the Proteobacteria, either the Gammaproteo-bacteria or the Alphaproteobacteria (Hanson & Hanson, 1996, Op den Camp *et al.*, 2009). The methanotrophic Bacteria of the Gammaproteobacteria, also called the type I methanotrophs, all belong to the *Methyloccoccaceae*. For carbon fixation they typically utilise the ribulose monophosphate pathway (RuMP-pathway), additional they can apply the calvin cycle. The two methanotrophic groups of the Alphaproteobacteria use the serine pathway (*Methylocystaceae* or type II methanotrophs) or the serine pathway and the calvin cycle (*Beijerinckiaceae* or type X methanotrophs) for carbon fixation (Op den Camp *et al.*, 2009 and references therein). Outside the Proteobacteria only Bacteria of the Verrucomicrobia are known to oxidise CH_4. The newly discovered methanotrophic Bacteria of the Verrucomicrobia are, by far, the most acidophilic methanotrophs known. For carbon fixation they apply the the serine-pathway and the calvin cycle.

The fractionation between CH_4 and biomass within the RuMP pathway can reach -24.8‰, while the serine pathway yields in general a lower fractionation maximum of -14.1‰ (Jahnke *et al.*, 1999). Due to the lack of isolates, there is a high uncertainty concerning anaerobic methanotrophs. To include microbial groups lacking pure culture isolates in isotope analyses, the $\delta^{13}C$-values of their biomarkers can be used.

The isotopic composition of lipids is not only controlled by the isotopic composition of the carbon source and the fractionation during assimilation, but also by isotope effects associated with metabolism and biosynthesis. This leads to different $\delta^{13}C$-values among compound classes even within one organism (e.g., DeNiro & Epstein, 1977; Monson & Hayes, 1982). Since the substrate pool within the cell is smaller than outside the cell, the carbon fractionation during biosyntheses is antagonised by subsequent ^{13}C enrichment of the residual substrate pool within the cell. If a substrate is consumed by more than one reaction, e.g., for acetate by the energy-, the carbohydrate- and the lipid metabolism, isotopic effects of each of the reactions influence the final isotopic composition of all possible products (Hayes, 1993). If carbon sources are limited, this can even lead to ^{12}C depletion of some compounds as reported for example by Jahnke *et al.* (1999). However, lipids seem to have a very strong fractionating metabolism, being still enriched in ^{12}C under low substrate conditions as described by Jahnke *et al.* (1999).

In general, fatty acids from microorganisms grown under aerobic conditions are enriched in ^{12}C relative to the biomass by 2‰ to 3‰. But multiple exceptions to this general trend exist. Especially anaerobic and autotrophic microorganisms show a wider range of $\Delta\delta^{13}C_{biomass\text{-}lipid}$-values (Tab. 1.3).

Table 1.3. $\Delta\delta^{13}C_{substrate\text{-}lipid}$-values and $\Delta\delta^{13}C_{biomass\text{-}lipid}$-values of selected microbial groups

Metabolism	$\Delta\delta^{13}C_{substrate\text{-}lipid}$-value (‰)	$\Delta\delta^{13}C_{biomass\text{-}lipid}$-value (‰)	Reference
Aerobic heterotrophic		-2 to -3	Monson & Hayes (1982), Blair *et al.* (1985) and Teece *et al.* (1999)
Iron reducing heterotrophic		-7.1 to -7.2	Zhang *et al.* (2003)
Denitrifing heterotrophic		-7.3 to -10	Teece *et al.* (1999)
Aerobic methanotrophic			
Type I (RuMP pathway)		-2 to -5	Jahnke *et al.* (1999) and Summons *et al.* (1994)
Type II (serine pathway)		-12	Jahnke *et al.* (1999)
Methanogenic			
autotrophic		-13	Takigiku (1987)
heterotrophic		-18 to -28	Summons *et al.* (1998)
AOM coupled to SRB			
SRB	-2 to -34		Niemann & Elvert (2008) and references therein
Archaea	-13 to -74		
AOM coupled to denitrification	unknown		
AOM coupled to metal reduction	unknown		

Consequently, the carbon isotopic composition of lipids also provides insights into the carbon metabolism. Analyses from natural habitats revealed that the microorganisms involved in sulphate coupled AOM display relatively high carbon isotope fractionation compared to other known biosynthetic pathways (Tab. 1.3) (Elvert, 1999; Michaelis *et al.*, 2002; Blumenberg *et al.*, 2004; Niemann & Elvert, 2008). Moreover, by means of characteristic ether lipids and their $\delta^{13}C$-values it is possible to distinguish between different ANME consortia, even in fossil sediments (Michaelis *et al.*, 2002; Thiel *et al.*, 2002). Less is known about the microorganisms conducting AOM coupled to denitrification. Raghoebarsing *et al.* (2006) determined the $\delta^{13}C$-value of lipids in an enrichment culture performing nitrate coupled AOM.

But additionally to CH_4 they also fed bicarbonate. Most of the observed lipids were enriched in ^{13}C relative to the CH_4, indicating uptake of the ^{13}C enriched bicarbonate. Nevertheless, it is unlikely that fractionation during nitrate dependent AOM is as large as during sulphate dependent AOM. Otherwise, the studied lipids should have shown stronger ^{13}C depletion. So far, no study has assessed the lipids of microorganisms involved in AOM coupled to metal reduction.

Combined with their $\delta^{13}C$-values, biomarkers are a powerful tool to assess microbial live and carbon cycling in recent environments, as for palaeo environments they are often the only tools available.

In this thesis, microbial communities at natural CO_2 vents were studied with the help of lipid biomarker and stable carbon isotope analyses. Lipid biomarker studies combined with stable carbon isotope analyses are a powerful tool to assess vital microbial groups and carbon cycling in complex environments. My research was focused on microorganisms involved in the production and consumption of CO_2 and CH_4 in anaerobic environments.

The following chapters give the results of the conducted research summarised in articles for scientific journals.

Chapter III: Oppermann, B. I., Michaelis, W., Blumenberg, M., Frerichs, J., Schulz, H.M., Schippers, A., Beaubien, S. E. and Krüger, M. (2010) Soil microbial community changes as a result of long-term exposure to a natural CO_2 vent. *Geochimica et Cosmochimica Acta* **74**, 2697-2716

Chapter IV: Oppermann, B.I., Knoblauch, C., Krüger, M., Blumenberg, M., Seifert, R., Frerichs, J. and W. Michaelis. Anaerobic microbial methane consumption in a soil overlying a natural CO_2 vent. Submitted to *Organic Geochemistry*.

Chapter V: Krüger, M., Jones, D., Frerichs, J., Oppermann, B.I., West, J., Coombs, P., Green, K., Barlow, T., Lister, B. and Möller, I. Effects of elevated CO_2 concentrations on the vegetation and microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. Submitted to *International Journal of Greenhouse Gas Control.*

Contributions to chapter V:

- sample recovery, determination of soil pH and TOC, lipid biomarker analyses and interpretation
- co-working in manuscript preparation

Chapter VI: Blumenberg, M., Krüger, M., Nauhaus, K., Talbot, H.M., Oppermann, B.I., Seifert, R., Pape, T. and Michaelis, W. (2006) Biosynthesis of hopanoids by sulfate-reducing bacteria (genus *Desulfovibrio*). *Environmental Microbiology* **8**(7), 1220–1227

Contributions to chapter VI:

- lipid biomarker analyses and interpretation
- co-working in manuscript preparation

Chapter VII: Blumenberg M., Oppermann B.I., Guyoneaud R. and Michaelis W. (2009) Hopanoid-production by *Desulfovibrio bastinii* isolated from oilfield formation water. *FEMS Microbial Letters* **293**, 73-78

Contributions to chapter IV:

- selection and cultivation of Bacteria
- co-working in biomarker interpretation and manuscript preparation

CO_2 dominated environments

To study the adaptation and composition of microbial communities living under high CO_2 conditions (>60%), natural environments dominated by this gas species can be used. Laboratory based studies assessing complex microbial communities come with the risk of being biased. Natural laboratories offer the possibility to study microbial key players and important environmental factors on their activities in relative undisturbed samples.

To assess the environmental impact of high CO_2 concentrations on the abundance and functional diversity of microorganisms, two terrestrial, geothermal CO_2 vents (>90% CO_2 in the soil gas) were studied. Moreover, these environments are an excellent natural analogue to study the consequences of a leakage from a CO_2 storage site. Such leakages could occur during CCS, but field test of this technique are rare and no leakages are reported from them yet. Therefore, CO_2 vents are an elegant way to appraise the impact of such CO_2 leakages onto microbial communities.

In chapter 3 and 4 the microbial soil community at a volcanic CO_2 vent in the Caldera di Latera/Italy was examined.

Already during the first survey (Chapter 3), a shift in the microbial community towards anaerobic and acidophilic microorganisms, as a consequence of the long-term exposure to high CO_2 concentrations, was detected. Moreover, substantial amounts of geothermal CO_2 were incorporated into the microbial, plant and soil carbon pools. The $\delta^{13}C$-values of diagnostic biomarkers demonstrated that methanogenic Archaea and sulphate reducing Bacteria were the dominating autotrophic organisms utilizing the volcanic CO_2 for assimilatory biosynthesis in this environment.

As autotrophic methanogenesis is enhanced under high CO_2 conditions the fate of CH_4 was assessed in the second survey (Chapter 4). Sulphate reducing Bacteria were not found to contribute significantly in the consumption of CH_4 and were rather associated with the consumption of CO_2. Recent studies by Ettwig *et al.* (2008), Beal *et al.* (2009) and Ettwig *et al.* (2010) suggested that also Mn(IV), Fe(III) and NO_3^- could theoretically serve as oxidants for the AOM. And biomarker distributions and $\delta^{13}C$-values in the studied samples indicated, that bacterial groups connected to nitrogen and/or to the metal cycle were involved in the degradation of CH_4 and the

mitigation of its emissions. These findings illustrated a more diverse methanotrophic community in terrestrial systems than previously assumed.

In Chapter 5, a CO_2 vent at the Laacher See/Germany was studied. In an interdisciplinary survey it was possible to show strong correlations to the previously studied CO_2 vent in the Caldera di Latera/Italy. Shifts towards anaerobic and acidophilic species appeared to be the general consequences of high CO_2 conditions. In particular, a significant shift towards microorganisms producing more stable membrane lipids, probably as an adaptation to the CO_2-induced low pH values at the CO_2 vent, was confirmed.

2.1. Sampling sites

2.1.1. Caldera di Latera/Italy

The first study area is located in central Italy, in the Vulsini Vulcanic District within the Caldera di Latera (Fig. 2.1). The Vulsini Vulcanic District contains besides the Caldera di Latera also other volcanic structures like the Bolsena Lake and some smaller structures like the Mezzano Lake and the craters near Montefiascone. The Latera volcano became active during the Quaternary 232 ka BP, further eruptions followed at 206, 195 and 156 ka BP. The last eruption caused the formation of the caldera (Turbeville, 1993). In the beginning, the eruptions yield tephritic to phonolithic and trachytic to phonolithic lava towards the end of the activity (Varekamp, 1980). These alkali volcanites erupted mostly as pyroclastic flows. After the development of the caldera, smaller volcanoes formed within the caldera and at its rim. These smaller volcanoes provided increasingly mafic lava. Together with younger alluvial sediments these mafic volcanites present the dominating near surface deposits within the caldera (Varekamp, 1980). Although the Latera Volcano is inactive for more than 150ka, the area within the Caldera and its surrounding is characterised by a high geothermal gradient (Frondini *et al.*, 2008). This high heat flow does not affect the soils within the

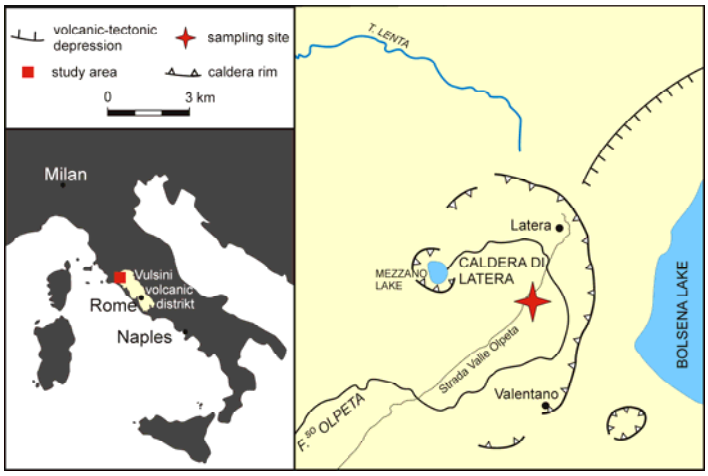

Fig. 2.1. Map of the sampling area modified after Palladino and Agosta, (1997), Beaubien *et al*. (2008) and Oppermann *et al*. (2010).

caldera directly. But in the underlying carbonate rocks this heat causes thermo-metamorphic reaction, CO_2 build up and subsequent CO_2 seepage throughout the caldera (Barberi *et al.*, 1984).

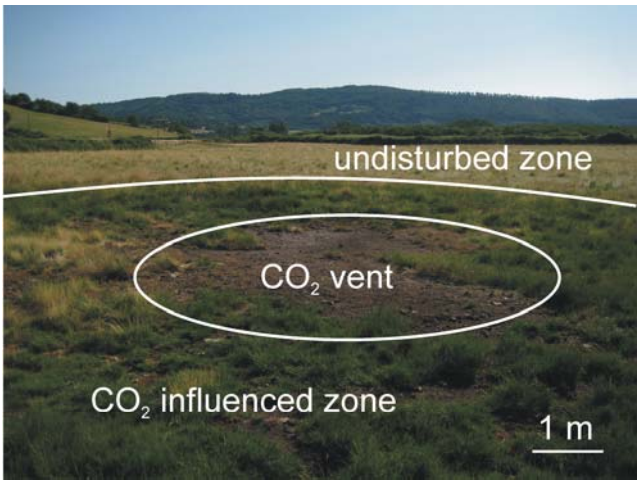

Fig. 2.2. Picture of the sampling site with zones of different CO_2 impact modified after Oppermann et al. (2010).

The actual sampling site is located on pastureland. The CO_2 vent can easily be identified by an elliptic, 5 to 6 m wide and almost vegetation-free area (Fig. 2.2). Beaubien *et al.* (2008) have detected a CO_2 flow of 2000 to 3000 g $m^{-2}d^{-1}$ in this area. According to their measurements the entire vent emits approximately 48 kg CO_2 d^{-1}. Due to this high flow rate CO_2 is the dominating soil gas (>95%) in the studied soil profile at the CO_2 vent (Beaubien *et al.*, 2008). Besides CO_2, also CH_4 (>1000 ppm), H_2S (>200 ppm) and He (>9 ppm) are contained in the soil gas. Since the CO_2 migrates upward along fault zones the CO_2 vents are narrow and closely tide to faults (Annunziatellis *et al.*, 2008). Towards the margin of the CO_2 vent the concentrations of these gases decrease rapidly. Already at 1 m away from the CO_2 vent, within the CO_2 influenced zone, air derived gases like O_2 and N_2 dominate and the CO_2 concentrations decreased to 20% of total soil gas (Beaubien *et al.*, 2008). The soil around the CO_2 vent in the approximately 2.5 m wide CO_2 influenced zone is covered almost exclusively by the acidophilic grass, *Agrostis capillaris* (Beaubien *et al.*, 2008). Outside of this area, in the undisturbed zone, ordinary vegetation consisting of grasses, mosses and clover, with 80% *Trifolium campestre*, a C3-plant, was found.

2.1.2. Laacher See/Germany

The second sampling area is located in western Germany in the East Eifel in the Laacher See Caldera (Fig. 2.3). The Eifel displays a part of the Rhenish Massif, which was formed during the variscan orogeny. Towards the east the Eifel borders on the Rhine and the Moselle, in the north on Bonn, Cologne and Aachen and in the south on Treves. Under the name of High Fens and Ardennes these mountain ranges stretch over Belgium and Luxembourg into France.

The volcanic activity in the Eifel started during the Tertiary, reaching the East Eifel in the Quaternary. The largest eruption in the Eifel mountain range was the eruption of

the Laacher See volcano which took place 13.1 ka BP (Nowell *et al.*, 2006). Maars like the Laacher See and the Caldera di Latera are always the result of phreatomagmatic eruption. During phreatomagmatic eruptions the hot ascending magma gets in contact with ground water. The high temperatures of the lava rapidly heats this water leading to a volume increase and a violent explosion. During the Laacher See eruption enormous amounts of tephra (16 km^3) were produced, which is 6-times more than during the eruption of Mount St. Helens in 1980 (Wörner & Schmincke, 1984b). According to (Worner & Schmincke, 1984) the beginning of the eruption was characterised by gas rich phonolitic magma, becoming increasingly mafic as the eruption continued. The eruption phase during which most of the pyroclastica were produced, lasted only a week (Worner & Schmincke, 1984). The wind transported these pyroclastica over large distances and tephra of the Laacher See eruption is found between Turin (Italy) and Gotland (Sweden) (Bogaard & Schmincke, 1985). Due to their widespread occurrence the Laacher See tephra is used as an isochrone.

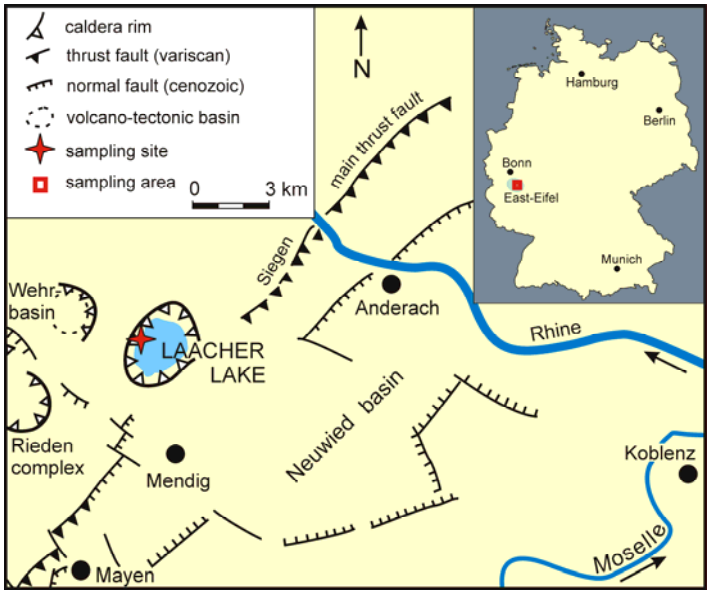

Fig. 2.3. Map of the sampling area in the Laacher See Caldera/Germany modified after Wörner & Schmincke (1984a).

The variscan orogenesis started in the Upper Carboniferous in the Eifel. After this variscan uplift the Eifel was an area of erosion until the Tertiary, when small depressions, like the Neuwiedener Basin formed in the East Eifel. Despite the localised formation of depressions, the entire East Eifel is still being uplifted.

The exposed rocks in the study area are dominated by tephritic layers of the Laacher See eruption, e.g., layers of lappili, ignimbrites and pyroclastic surge deposits. A 90 to 240 m high ring wall, consisting of pyroclastica, surrounds the Laacher See Caldera. Within the caldera a 3.3 km^2 large lake is located. The area around this lake is used for agricultural and silvicultural purposes. The actual sampling site is located on pastureland.

Soil microbial community changes as a result of long-term exposure to a natural CO_2 vent

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Abstract

The capture and geological storage of CO_2 can be used to reduce anthropogenic greenhouse gas emissions. To assess the environmental impact of potential CO_2 leakage from deep storage reservoirs on the abundance and functional diversity of microorganisms in near-surface terrestrial environments, a natural CO_2 vent (>90% CO_2 in the soil gas) was studied as an analogue. The microbial communities were investigated using lipid biomarkers combined with compound-specific stable carbon isotope analyses, the determination of microbial activities, and the use of quantitative polymerase chain reactions (Q-PCR). With this complementary set of methods, significant differences between the CO_2-rich vent and a reference site with a normal CO_2 concentration were detected. The $\delta^{13}C$-values of the plant and microbial lipids within the CO_2 vent demonstrate that substantial amounts of geothermal CO_2 were incorporated into the microbial, plant, and soil carbon pools. Moreover, the numbers of Archaea and Bacteria were highest at the reference site and substantially lower at the CO_2 vent. Lipid biomarker analyses, Q-PCR, and the determination of microbial activities showed the presence of CO_2-utilising methanogenic Archaea, *Geobacteraceae*, and sulphate-reducing Bacteria (SRB) mainly at the CO_2 vent, only minor quantities were found at the reference site. Stable carbon isotopic analyses revealed that the methanogenic Archaea and SRB utilised the vent-derived CO_2 for assimilatory biosynthesis. Our results show a shift in the microbial community towards anaerobic and acidophilic microorganisms as a consequence of the long-term exposure of the soil environment to high CO_2 concentrations.

3.1. Introduction

According to the International Panel of Climate Change (IPCC), the capture of CO_2 from large emission sources and its subsequent storage in geological structures (CCS) is one option that can be used to reduce anthropogenic CO_2 emissions (IPCC, 2005). Before establishing CCS as a widespread solution for the mitigation of CO_2 emissions, it is necessary to evaluate the possible consequences of CO_2 leakage into near-surface ecosystems, including changes in the community composition of quasi-stationary organisms (e.g., plants and microorganisms) and in the soil chemistry.

Although several studies have been published regarding the effect of higher atmospheric CO_2 concentrations on ecosystems (e. g., Jossi *et al.*, 2006), there are very few studies that have examined the effects of increasing CO_2 concentrations in the soil due to upwardly migrating gas. Naturally occurring, mostly volcanic CO_2 vents are suitable for such studies as they provide natural laboratories that exhibit characteristic that are similar to the conditions that would occur with potential leaks from anthropogenic CO_2 storage sites. Currently, there is a lack of detailed investigations that report on changes in the biological carbon cycle and the community structure of soil microorganisms, yet there are some reports and studies on the other characteristics of natural, terrestrial CO_2 vents. These include the influence of volcanic CO_2 on soil chemistry and mineralogy at Mammoth Mountain (USA) (Stephens and Hering, 2002, 2004) and on plants in NE Slovenia (Macek *et al.*, 2005; Vodnik *et al.*, 2006; Pfanz *et al.*, 2007). No studies have addressed the issue of CO_2 induced changes in soil microbial communities.

By combining biogeochemical, microbiological and molecular biological methods in this study, we show profound changes in the soil microbial community that are caused by these relatively high CO_2 concentrations. In addition, the key microbial players that benefited from the environmental changes induced by the high CO_2 levels were identified by tracing the uptake of the ^{13}C-enriched geothermal CO_2.

3.2. Materials and methods

3.2.1. Site description and sampling

A detailed description of the study site, including the exact location and a general introduction on gas composition and fluxes, soil chemistry, vegetation, and soil organisms can be found in Beaubien *et al.* (2008). The study area is located within the Latera Caldera in the Vulsinian volcanic in central Italy (Fig. 3.1a) on pasture land (Fig. 3.1b). In addition to the Latera Caldera, this area also comprises various other volcanic complexes. The study area possesses a high geothermal gradient, although it has been inactive for over 0.16 Ma (Vezzoli *et al.*, 1987). This high geothermal gradient does not directly influence the soil in the caldera but causes thermo-metamorphic reactions in the underlying carbonate-rocks, leading to CO_2 formation and localised release throughout the caldera (Barberi *et al.*, 1984). The fertile soils in the caldera are formed from the alkali-potassic unit of the Latera volcanic complex and are underlain by alluvial sediments (Beaubien *et al.*, 2008). Significant environmental changes were found that were due to high CO_2 fluxes (2000-3000 g m^{-2} d^{-1}, Beaubien *et al.*, 2008) within the vent-impacted area, such as low pH, anoxia, reduced microbial activities, lower concentrations of oxides in the soil, and a lack of vegetation.

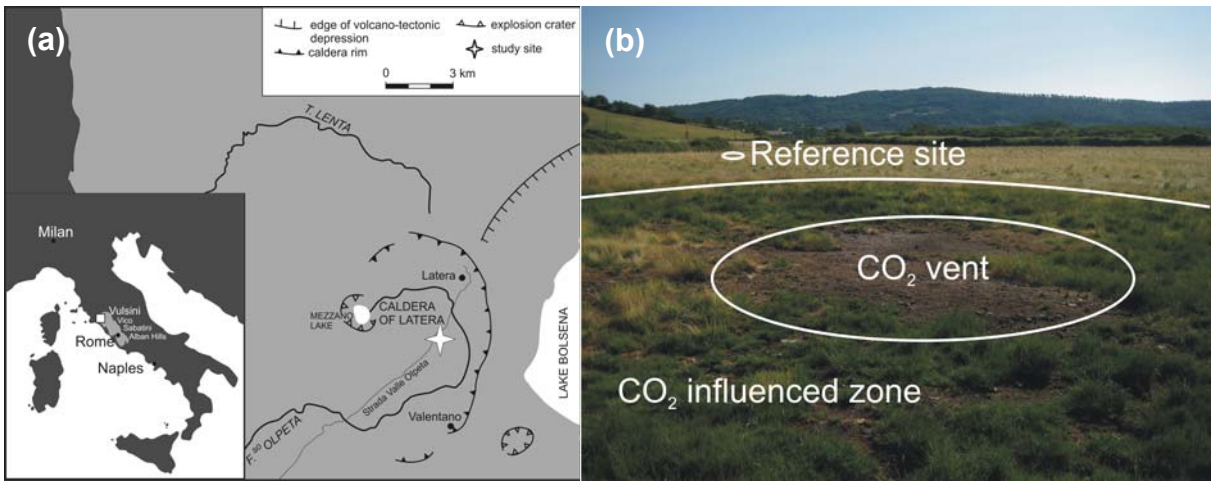


Fig. 3.1. (a) Map of the study area, modified after Beaubien *et al.* (2008) and Palladino and Agosta (1997). (b) Picture of the sampling site, showing the 6-m wide CO_2 vent area with reduced vegetation, the CO_2 influenced zone with acidophilic grasses and the non CO_2 influenced reference site.

The samples were taken along a 50-m transect from the centre of the gas vent to an area that was not notably affected by the gas venting (Fig. 3.1b). Soil, plant, and gas samples were taken during a field campaign in June 2006. One soil sample was

taken from the 6-m wide vegetation-free vent zone. In the area around the vegetation-free vent zone, the acid-tolerant grass *Agrostis capillaris* dominated; eight plant samples were taken from this area. The overall zone was adjacent to an area with a more diverse plant community of grasses and clover; two plant samples were taken from this plant community. Patches of moss were observed in an interval from 5.5 to 8.5 m. The reference soil sample was taken 50 m from the centre of the CO_2 vent. At the CO_2 vent site, a thick (20 cm) A-horizon underlain by a silt loam was detected. At the reference site, the A-horizon was only 5 cm thick. Below the A-horizon, a poorly sorted granular soil was detected. This horizon was also found below 50 cm at the CO_2 vent site. Therefore, both of the soil samples were taken below the root zone (50–70 cm depth) to minimise the soil-horizon heterogeneity as well as the seasonal temperature variation, the plant, and the atmospheric influences. All of the samples were collected using a hand-held auger. The soil samples that were to be used for molecular biological and biogeochemical analyses were stored at –20°C and those for activity measurements were stored at 5°C. To assess the interannual and seasonal variability, additional gas samples were taken in March and September 2006. Similar soil gas concentration measurements were taken for the top 20 cm in June 2009 (data not shown). Furthermore, the pH values were determined in a sampling campaign that occurred in June 2009 and were compared with values determined by Beaubien *et al.* (2008).

3.2.2. Gas sampling and analysis

The gas samples were taken according to Ciotoli *et al.* (1998) and Ciotoli *et al.* (1999) using a modified thick-walled, stainless steel tube (6.4-mm diameter) that was pounded to the desired sampling depth (20 cm or 80 cm). A rubber-septum sampling port was securely attached to the tube to allow it to be purged of atmospheric air and subsequently sampled using an air-tight plastic syringe fitted with a Teflon valve. The sampled gas was rapidly transferred into either air-tight glass containers (for stable isotopes) or 25 ml stainless-steel containers (for quantifications). The stable carbon isotopes of the hydrocarbons and CO_2 were analysed according to Vandre *et al.* (2007). The precision, as determined by a repeated analysis of the air, was about 0.6‰. Methane was quantified using a Fison 8000-series bench gas chromatography (GC) equipped with a flame ionisation detector (FID) and a 2-m PoraPlot packed column. CO_2, N_2, and O_2 were quantified with the same type of gas chromatograph

but with a 2-m column packed with a molecular sieve of 5 Å and a thermal conductivity detector (TCD). Based on >30 replicate measurements of the certified standards during the sample analysis period, the analytical precision for each gas was: 1.97 ppm CH_4 standard = 2.01 ±0.1 ppm; 1.10% CO_2 = 1.08 ±0.05%; 19.5% O_2 = 19.9 ±0.5%; and 80.5% N_2 = 80.93 ±1.1%. Replicate measurements in the field showed a similar range of precision.

The field analyses were conducted using an infrared gas analyser (Draeger Multiwarn®) that was equipped with electrochemical detector for H_2S and H_2. Since the concentrations for H_2S and H_2 were off of the scale for direct measurements, the samples were diluted using Tedlar bags and atmospheric air. The precision for these dilutions and field analyses was on the order of ±10% based on the field trials.

3.2.3. Bulk carbon, nitrogen, and sulphur analyses

The soil and plant samples were lyophilised and homogenised prior to analysis. The total carbon and nitrogen (TC and TN in wt. %) content were determined from either soil or plant samples. An aliquot of the soil sample (2–3 mg) was used to determine the organic carbon content. For this analysis, the carbonate was removed with 50 µL 1N HCl followed by drying the sample on a 40°C heating plate (repeated three times). The total carbon, total nitrogen, and organic carbon (TOC) were measured by combustion to CO_2 and N_2 using a Carlo Erba Science 1500 CNS Analyser (Erba Science, Italy). The carbonate carbon was calculated as the difference between the total and the organic carbon. The sulphur content of the soil samples was measured on a Laser Sampler 320 (Perkin–Elmer, USA). Duplicate analyses yielded a standard deviation of 0.08% for the total sulphur, of 0.01% for the total nitrogen, and of 0.05% for the carbon content.

3.2.4. Bulk stable carbon isotope analyses

The samples taken for the isotopic analyses of organic carbon were prepared as described above for TC and TOC. The $\delta^{13}C$ values of TOC were determined using a Finnigan MAT 252 mass spectrometer after high-temperature combustion to CO_2 in a Carlo Erba NA-2500 elemental analyser (Erba Science, Italy) at 1020°C. To remove the sulphur after the combustion of the soil samples, an adjusted version of the method described by Carlson *et al.* (1998) was used. In brief, the column (quartz, 49 x 1.3 cm) was packed from the bottom to the zone where the samples are combusted

with 4 cm quartz wool, 1 cm Sulfix (850–2360 µm; 8–20 mesh; WAKO Chemicals, Japan), 6 cm silver coated cobalt oxide, and 12 cm chromium oxide. All of the reagents were separated by a thin layer of quartz wool. Based on replicate measurements of the reference standard, the analytical precision was better than 0.12‰. Duplicate measurements showed a mean deviation of 0.18‰.

3.2.5. Biomarker analyses

3.2.5.1. Extraction and isolation

Prior to the analysis, the soil samples were lyophilised and homogenised. Ester-bound lipids were released using alkaline hydrolysis with 6% KOH in methanol (MeOH) in excess for 2 h at 80°C with additional ultrasonication. Neutral lipids (hydrocarbons, ketones, and alcohols) were extracted from the alkaline reaction solution with *n*-hexane, at least five times or until the supernatant was colourless. The residual KOH was removed from the extract by extraction with H_2O (cleaned by Milli-Q and extraction against dichloromethane (DCM)). The remaining aquatic solution was acidified to a pH of 1–2. The acid solution was extracted with *n*-hexane once and then five times with DCM to yield the carboxylic acids. Hydrocarbons in the neutral lipid fraction were separated from the alcohols and ketones using column chromatography (Merck silica gel 60) and elution with organic solvents of increasing polarity (*n*-hexane, DCM, MeOH).

An aliquot of the neutral lipid fraction was separated according to the method of Michaelis *et al.* (2002) by thin layer chromatography (TLC) using silica gel 60, 0.25 mm plates (Merck, Germany). The compounds were eluted with DCM and yielded a di- and polyol fraction that included monoalkyl glycerol monoethers (MAGEs) and glycerol dialkyl glycerol tetraethers (GDGTs) (Rf 0–0.1) as well as a mono-alcohol fraction that included dialkyl glycerol diethers (DAGEs) (Rf 0.1–0.35). To analyse the alkyl moiety, the ether bond lipids were subjected to ether cleavage with HI treatment (HI 57% and CH_3COOH 1:1, v:v; 4 h at 110°C) and a further reduction of the resulting iodides by using $LiAlH_4$ in dry tetrahydrofuran under an argon atmosphere (modified after Kohnen *et al.*, 1992). The GDGTs were extracted with DCM/MeOH (3:1, v/v) via ultrasonification of the freeze-dried soil samples. The extracts and the remaining soil samples were hydrolysed with KOH in MeOH according to the method described above. The resulting fractions were combined. A schematic diagram of the extraction and the isolation of all of the fractions can be found in Appendix, Fig. 3.12.

3.2.5.2. Derivatisation

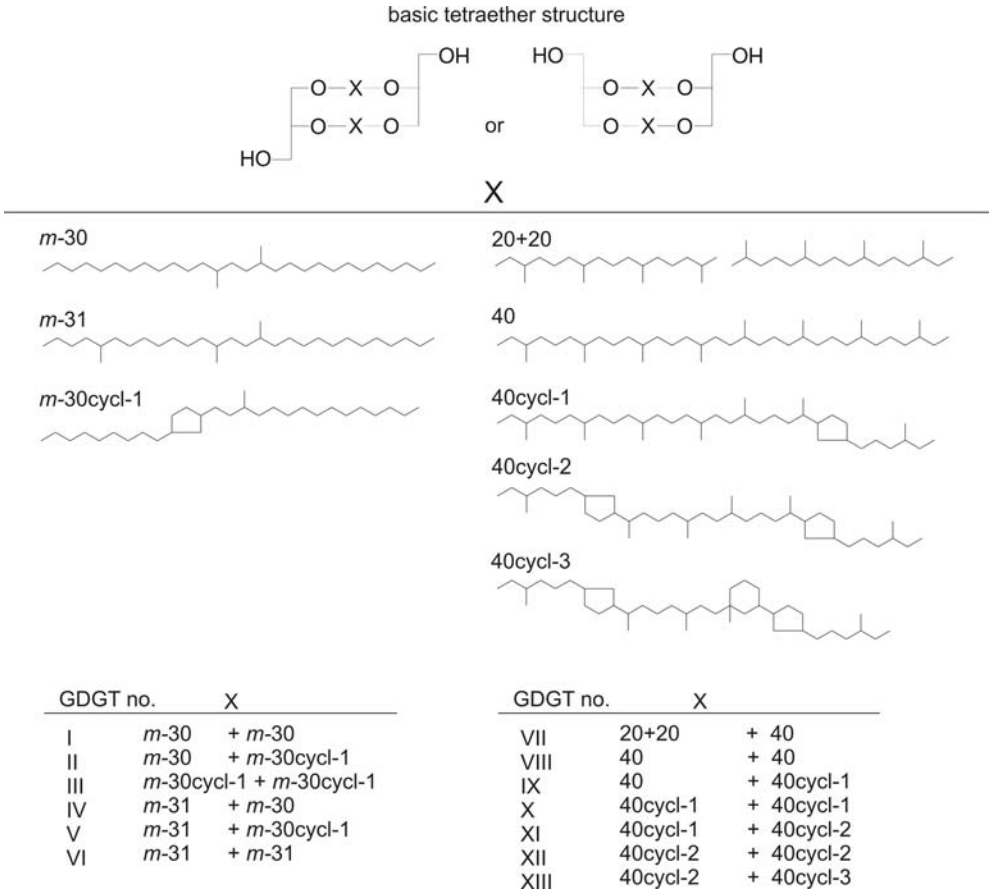
Prior to the analysis, the alcohols were silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 2 h at 80°C. Carboxylic acids were converted to their methyl esters before analysis (trimethylchlorosilane in MeOH 1:9; v:v; 2 h, 80°C). The double bond positions of the carboxylic acid methyl esters were determined using the method described by Buser *et al.* (1983). In short, dimethyl disulfide (DMDS) was used to derivatise the double bonds. The resulting analytes yielded easy to recognise molecular ions and fragments.

3.2.5.3. Instrumental setup

The hydrocarbon-, silylated alcohol-, and methylated carboxylic acid-fractions (abbreviation of these lipids, see Appendix, Fig. 3.13), were analysed by coupled gas chromatography–mass spectrometry (GC–MS) using a Fisons MD 800 spectrometer (EI, 70 eV) interfaced with a Fisons 8060 GC with a 30-m fused silica capillary column (DB5-MS, 0.32 mm i.d. 0.25 µm film thickness). The carrier gas was helium, and the temperature program was: 3 min at 80°C; from 80°C to 310°C at 6°C min^{-1}; 20 min at 310°C. The lipids were tentatively identified by a comparison of the GC-retention times, the published mass spectra, and the mass spectra from the NIST Mass Spectral Search Program 2.0.0.26 (US Secretary of Commerce, USA). If this was not sufficient, the lipids were checked with reference compounds. The software packages used for these analyses were MassLynx NT v3.2 (Micromass, UK) and Varian MS Workstation, Star Toolbar v6.5 (Varian, USA). The concentrations of the lipids were calculated relative to an authentic, internal standard (Squalane, Fluka, Switzerland) using the GC-FID signals processed in ChromStar 6.3 (Chromatography Software, Germany).

The filtered GDGT-fractions were analysed using a Prostar Dynamax HPLC coupled to a 1200L triple-quad mass spectrometer (Varian, USA). The LC–MS-parameters were adapted from Hopmans *et al.* (2000) and Hopmans *et al.* (2004). In short, the compounds were separated using a Nucleodur 100-3 CN column (2 x 150 mm, Macherey-Nagel, Germany), equipped with a pre-column of the same material maintained at 30°C. The mobile phase was 99% *n*-hexane and 1% propanol for 5 min, followed by a gradient to 1.8% propanol in 45 min, with a 0.2 mL min^{-1} flow rate. At the end of each run, the column was washed with 95% *n*-hexane and 5% propanol for 10 min. The compounds were ionised with an atmospheric pressure

chemical ionisation source. Detection was achieved in the positive ion mode under the following conditions: Corona current 5 µA, vaporising gas 400°C (17 psi), drying gas (N_2) 200°C (12 psi), nebulising pressure 58 psi, capillary voltage 90 V. Masses with *m/z* 950–1400 were scanned (for abbreviation of GDGTs see Fig. 3.2). The GDGTs were quantified using the relative intensity of their $[M+H]^+$ ion compared to a standard of known concentration, (acyclic biphytane GDGT extracted from *Sulfolobus solfataricus*; kindly provided by P. Adam and P. Schaeffer).



GDGT no.	X	
I	*m*-30	+ *m*-30
II	*m*-30	+ *m*-30cycl-1
III	*m*-30cycl-1	+ *m*-30cycl-1
IV	*m*-31	+ *m*-30
V	*m*-31	+ *m*-30cycl-1
VI	*m*-31	+ *m*-31

GDGT no.	X	
VII	20+20	+ 40
VIII	40	+ 40
IX	40	+ 40cycl-1
X	40cycl-1	+ 40cycl-1
XI	40cycl-1	+ 40cycl-2
XII	40cycl-2	+ 40cycl-2
XIII	40cycl-2	+ 40cycl-3

Fig. 3.2. The basic structures and the different alkyl moieties of the detected GDGTs. The archaeal derived C_{40} (biphytans) were detected with up to three cyclic moieties. The bacterial derived C_{30}–C_{31} alkyl-moieties contained up to two cyclic moieties. The number of cyclic moieties is given by the number behind the hyphen (e.g., –1 for one cyclic moiety). The position of the methyl branches and the cyclic moieties were tentatively assigned after De Rosa *et al.* (1980) and references therein, Sinninghe Damsté *et al.* (2000), and Weijers *et al.* (2006b).

3.2.6. Compound specific stable carbon isotope analyses

The stable carbon isotope compositions are given in the delta notation ($\delta^{13}C$) versus the Vienna Pee Dee Belemnite (V-PDB). The coupled gas chromatography-combustion-isotope ratio mass spectrometry (GC-*C*-IRMS) was conducted using a Finnigan DeltaPlus XL mass spectrometer coupled to a HP 6890 GC via a CuO/Ni/Pt combustion furnace operated at 940°C. Samples were applied using splitless injected

(1 min). The GC was equipped with a 30-m fused silica column (DB5-MS, 0.32 mm i.d., 0.25 µm film thickness); the same temperature programme was applied as for the GC–MS. The precision was checked daily using a standard alkane mix (C_{15}–C_{29}) with a known isotopic composition. Based on these measurements, the analytical precision was determined to be generally better than 0.9‰ (three replicates). For a few of the compounds, the deviation was higher than 0.9‰. Where this was the case, the higher deviation is denoted. To be able to calculate the $\delta^{13}C$ values of the underivatised compounds, the $\delta^{13}C$ values of the carbon atoms introduced into the molecule by the derivatisation reagent were determined. For this measure, about 0.1 mg of BSTFA was applied on 1.0–1.5 mg silica gel in a silver-capsule and was measured using a Finnigan MAT 252 mass spectrometer after high-temperature flash combustion in a Carlo Erba NA-2500 elemental analyser (Erba Science, Italy) at 1020°C. The $\delta^{13}C$-value of the MeOH, from which the carbon atom of the methyl group of the carboxylic acid methyl esters (CAMEs) derived, was determined by the injection of 0.05–0.1 µL of MeOH into the GC-*C*-IRMS system described above. The $\delta^{13}C$-values of derivatised components were corrected by a formula modified after Goñi and Eglinton (1996).

3.2.7. Determination of the microbial activities

As an important indicator for the overall microbial activity in the soil, the CO_2 production rates were determined, both with and without oxygen (gross mineralisation). More specifically, the aerobic methane oxidation, the anaerobic methane production, and the sulphate reduction were measured.

These experiments were carried out in glass tubes (20 ml) sealed with butyl-rubber stoppers and screw caps. The soil samples were mixed 1:1 with artificial mineral medium after Widdel and Bak (1992) to obtain homogenous slurries. Subsequently, 9 ml of medium was added to 3 ml of soil slurry. All of the manipulations were performed under an atmosphere of nitrogen in a glove box. The headspace of the incubation tubes consisted either of methane (100%) or N_2/CO_2 (90/10; v:v) in the control and in the methane-production experiments. The potential aerobic methane oxidation rates in the soil samples were determined *in vitro* as described in Krüger *et al.* (2002). The slurries were prepared under oxic conditions as noted above. Aliquots (20 ml) were transferred into sterile glass bottles (175 ml) and supplemented with 5% methane. Methane depletion was quantified by sampling the headspace after the thorough shaking of the bottles for subsequent GC-FID analysis. Triplicate tubes

were incubated horizontally at 20°C and gently shaken once per day to ensure an even distribution of methane and sulphate within the medium. The rates were calculated per gram of dry weight (g_{dw}). The dry weight was determined after drying at 80°C for 48 h.

The sulphide content was determined using the formation of copper sulphide (Cord-Ruwisch, 1985). Methane and CO_2 were determined using a GC 14B gas chromatograph (Shimadzu) as described in Nauhaus *et al.* (2002), which was additionally equipped with a methaniser to quantify the CO_2.

3.2.8. Quantification of microbial populations

For the Q-PCR, high-molecular-weight DNA was extracted from 450 mg of the frozen soil samples. Cell lysis was performed with 10% SDS and horizontal shaking for 45 s at 5.5 m/s after the addition of zirconium-silica beads. The DNA was purified using NH_4-acetate and isopropanol precipitations as described in detail by Henckel *et al.* (1999). To determine the 16S rRNA copy numbers of Archaea (Takai *et al.*, 2000) and Bacteria (Nadkarni *et al.*, 2002), Q-PCR (ABI Prism 7000, Applied Biosystems) was used. The eukaryotic 18S rDNA was quantified using a commercially available Q-PCR assay from Applied Biosystems (TaqMan® ribosomal RNA control reagents, VIC™ probe). Iron-reducing Bacteria (*Geobacteraceae*), SRB and methanogenic Archaea were quantified after Holmes *et al.* (2005), Schippers and Neretin (2006) or Wilms *et al.* (2006) using either the 16S rRNA (*Geobacter*) or the functional genes in cases of SRB and methanogens. The detection limit for the Q-PCR analyses was at 10^3 DNA copies per g of sample or lower, depending on the specific assay. The purified PCR products obtained from the DNA of the pure culture cell extracts were used as DNA standards for the Q-PCR assays. The amplification efficiency of the Q-PCR reactions was between 85% and 100% depending on the particular Q-PCR assay, with average standard deviations between 5% and 15%.

3.2.9. Molecular community analysis via DGGE

The primer set 533f/907r, which is a phylogenetic marker encoding the small ribosomal subunit of Eubacteria (Henkel *et al.*, 1999), was used to amplify the 16S rRNA gene. For the Denaturing Gradient Gel Electrophoresis (DGGE), the backward primer was labelled at the 5'-end with a GC-clamp (Muyzer *et al.*, 1993). The 16S rRNA of Archaea was amplified with the primer set 340F and 915R (Ovreas *et al.*,

1997; Coolen *et al.*, 2002). All of the approaches were combined in the subsequent DGGE analysis that was carried out in an Ingeny phorU-2 system (Ingeny, Netherlands) for 20 h at 60°C, with denaturing gradients at 30–80% and acrylamide concentrations of 6–8%. The selected bands were subsequently sequenced (for details see Eller *et al.*, 2005). These bands were checked for their next relatives using the BLAST search in the Genbank Data Library (http://www.ncbi.nlm.nih.gov/BLAST/). Additionally, the sequences were aligned and uploaded into the SILVA database to generate a tree alignment using the ARB software package (www.arb-home.de). The tree was calculated with the SILVA alignment based upon the maximum likelihood algorithm after Pruesse *et al.* (2007).

3.2.10. Nucleotide accession numbers

All of the sequence data have been submitted to the EMBL database (http//:www.ebi.ac.uk) under accession numbers GU566176 to GU566187 for the sequences of DGGE bands.

3.3. Results

3.3.1. Gas composition

The soil gas in the CO_2 vent consisted mainly of CO_2, and the contents were measured at 93.1% at the 20 cm depth (Beaubien *et al.*, 2008) and 95.6% at the 80 cm depth (Appendix, Fig. 3.2). Methane followed this trend in increasing concentration with depth, with a maximum concentration of 1015 ppm at 80 cm. In contrast, the concentrations of oxygen (1.0% at 20 cm and 0.1% at 80 cm) and nitrogen (5.8% at 20 cm and 4.4% at 80 cm) decreased with depth. At the reference site, the concentrations of oxygen and nitrogen were much higher (20.6% and 76.3% at 80 cm, respectively).

3.3.2. Bulk carbon, nitrogen and sulphur content

The total organic carbon (TOC) content of the soil samples (Fig. 3.3) showed no significant differences between the CO_2 vent and the reference site (1.3% and 1.4%, respectively). The organic carbon accounted for 100% of the total carbon at the CO_2 vent. In addition to organic carbon, also carbonate carbon was detected (0.4%) at the

reference site. The soil at the CO_2 vent contained 0.9% sulphur, while a sulphur content of 1.1% was determined at the reference site.

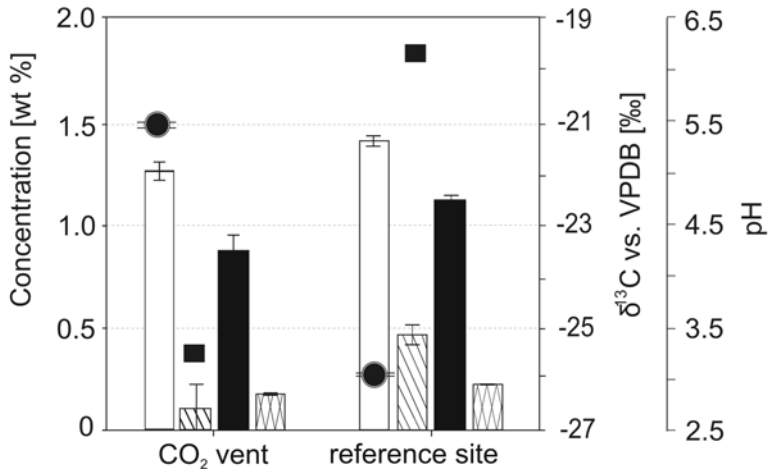


Fig. 3.3. The concentrations of TOC (white), carbonate (striped), sulphur (black), and nitrogen (checked pattern) in the dried soil samples, as well as the $\delta^{13}C$ value of TOC (dots) and the pH (rectangles).

3.3.3. Stable carbon isotope signatures of bulk carbon

The $\delta^{13}CO_2$ in the soil gas samples from the CO_2 vent was found to vary between +1.8 and +3.3‰, with no detectable trend over depth. The total organic carbon in the CO_2 vent was enriched in ^{13}C (-21.1‰) relative to the reference site (-25.7‰). Although all of the important plants at the sampling site are C3-plants, the isotopic values of the organic carbon in the plant samples changes significantly along the profile from the reference site to the CO_2 vent. We observed a trend of ^{13}C enrichment toward the CO_2 vent, with samples collected 6 m from the CO_2 vent having more ^{13}C-enriched compositions (-22.8‰ leaves and stalks, -23.1‰ roots) compared to those collected 16 m from the vent (-27.0‰ leaves and stalks, -27.6‰ roots).

3.3.4. N-alkyl lipids and steroids

The prevalent lipids in the analysed soils were putative plant-derived lipids, including *n*-alkanes dominated by odd numbers of carbon atoms, steroids (not shown) and *n*-alcohols dominated by even numbers of carbon atoms (Appendix, Fig. 3.3). Together they accounted for more than 600 µg g^{-1} of the TOC at the CO_2 vent and more than 900 µg g^{-1} of the TOC at the reference site. The plant waxes were also enriched in ^{13}C at the CO_2 vent compared to the reference site, e.g., HC 31:0 -24.3‰ versus -33.9‰, respectively (Appendix, Fig. 3.3).

3.3.5. Glycerolipids

3.3.5.1. Carboxylic acids

The $C_{14–19}$ straight chain or mono methyl branched (*i*-, *ai*-, *m*-) carboxylic acids were found in significant quantities in both of the soil samples (Fig. 3.4). Within the CO_2 vent, these lipids were strongly enriched in ^{13}C compared to the reference site soil.

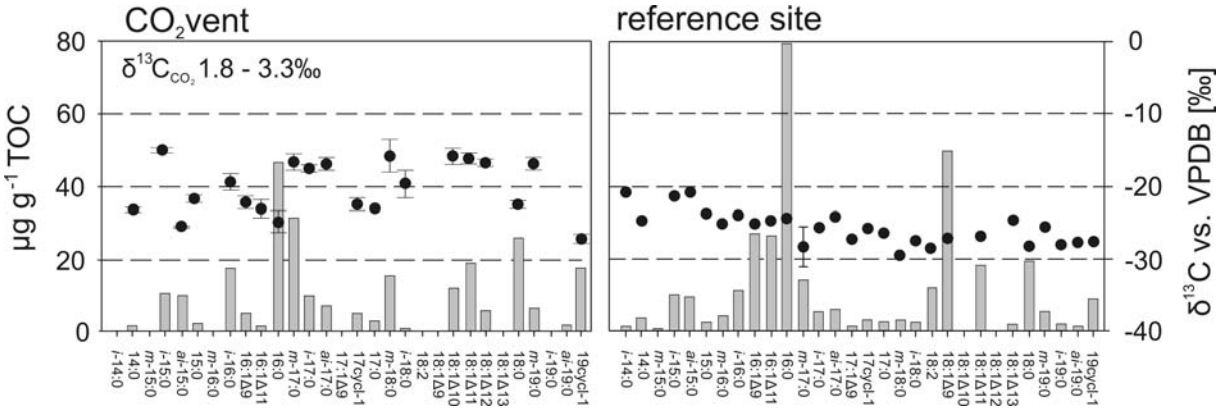


Fig. 3.4. The carboxylic acids extracted from the studied soils. The dots indicate the $\delta^{13}C$ values of individual compounds versus V-PDB. The grey bars give the µg of compounds per g TOC. *m*- are mid chain branched compounds.

3.3.5.1.1. Saturated carboxylic acids

The main carboxylic acid detected in the two samples was hexadecanoic acid (16:0), although its relative concentration in the CO_2 vent compared to the other lipids was lower than at the reference site (Fig. 3.4). A number of mid-chain branched heptadecanoic acids (three different acids added together, represented as *m*-17:0) and octadecanoic acids (three different acids added together, represented as *m*-18:0) were observed in high concentrations in the CO_2 vent soil. These lipids are also strongly enriched in ^{13}C compared with the reference site.

3.3.5.1.2. Unsaturated carboxylic acids. The unsaturated carboxylic acids 16:1 and 18:1 were detected at both of the sampling sites, although at the reference site the concentrations of the unsaturated carboxylic acids were particularly high (Fig. 3.4). The 9-heptadecenoic acid (17:1Δ9), the only odd-numbered unsaturated carboxylic acid found in the studied samples, was only detected at the reference site. Various octadecenoic acids were also detected, with 18:1Δ9, 18:1Δ13 and 18:2 only present at the reference site, while 18:1Δ10 and 18:1Δ12 were only found in the CO_2 vent soil. Octadecenoic acids within the CO_2 vent were relative enriched in ^{13}C, as compared to most other lipids at this site.

3.3.5.2. MAGEs and DAGEs

In addition to the free or ester bound alcohols, MAGEs and DAGEs were also detected (Figs. 3.5 and 3.6). These ether lipids were retrieved in higher concentrations from the CO_2 vent. Only DAGE *i*-15:0/*i*-15:0 was identified at a higher concentration at the reference site compared to the CO_2 vent; all of the other MAGEs and DAGEs were less abundant (or even absent).

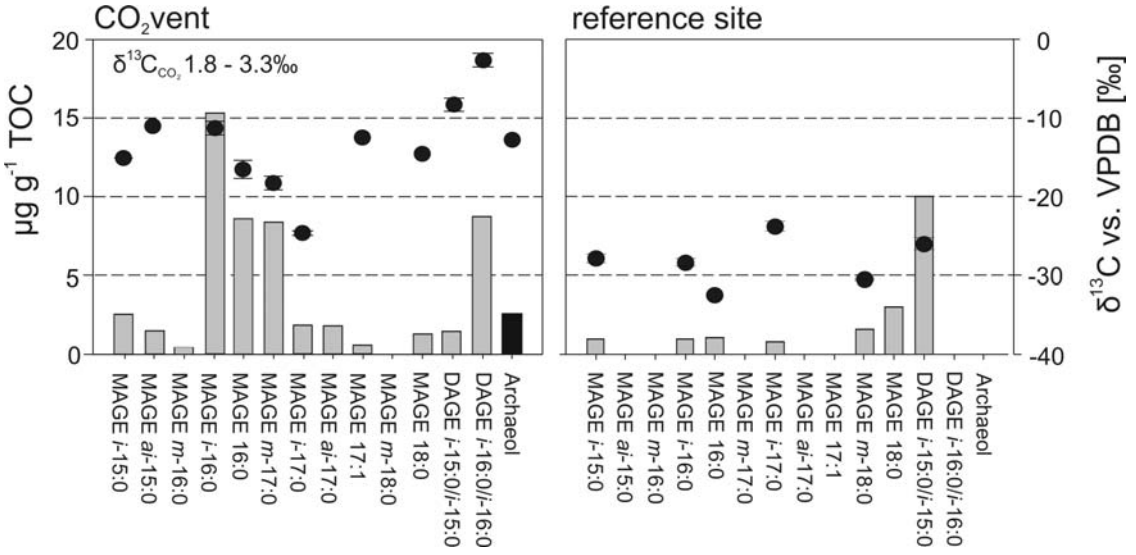


Fig. 3.5. The alcohols extracted from the studied soils samples. For MAGEs and DAGEs, the numbers of carbon atoms in the alkyl moiety are given before the colon; behind the colon the number of undersaturations is given. The dots indicate the $\delta^{13}C$ values of individual compounds versus the V-PDB-Standard. The heights of the bars give the µg of compounds per gram TOC. Gray bars – bacterial biomarkers; black bars – archaeal biomarkers.

Bacterial ethers containing C_{16} moieties, MAGE *i*-16:0, MAGE 16:0 and DAGE *i*-16:0/*i*-16:0 were found in particularly high concentrations in the CO_2 vent soil. These ethers also displayed a strong enrichment in ^{13}C, in particular the DAGE *i*-16:0/*i*-16:0 (-2.6‰) that is the most ^{13}C enriched lipid found in this study. MAGE *m*-17:0 (-18.3‰) follows this isotopic trend and is also present in high concentrations in the soil at the CO_2 vent. However, even minor ethers displayed $\delta^{13}C$ values enriched in ^{13}C, e.g., MAGE *ai*-15:0 (-11.1‰), MAGE 17:1 (-12.5‰) and DAGE *i*-15:0/*i*-15:0 (-8.4‰). Only MAGE *i*-17:0 (-24.7‰) had a carbon isotopic composition similar to that of bacterial lipids from the reference site. The MAGEs and DAGEs with *iso*-methyl branched moieties were prevalent in the soil samples from the reference site. Moreover, all of the MAGEs and DAGEs, such as MAGE *i*-15:0 (-27.8‰), MAGE *i*-16:0 (-28.3‰) and MAGE 16:0 (-32.8‰), displayed a $^{13}C/^{12}C$ ratios similar to that of plant-derived lipids. The major archaeal lipid found in the alcohol fraction was Archaeol (-12.7‰), which was only detected within the CO_2 vent soil.

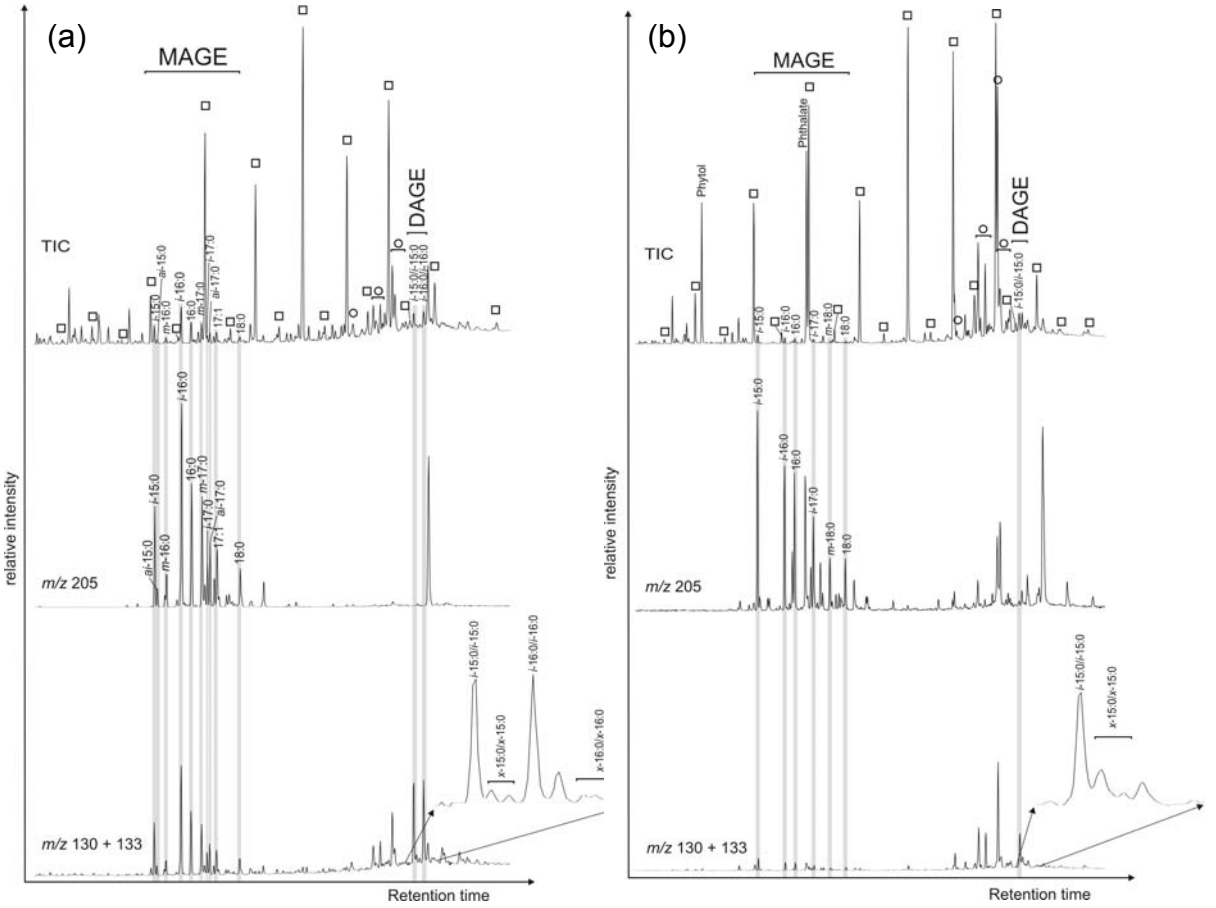


Fig. 3.6. The GC-MS total ion chromatogram of the silylated extract of the alcohol fraction of the CO_2 vent soil (a) and the reference soil (b) and the ion traces indicative of MAGEs and DAGEs. □ C_{16} to C_{34} *n*-alcohol, ○ plant derived sterols, *x*-: possible methyl branched alkyl moieties of DAGEs.

3.3.5.3. GDGTs

In the CO_2 vent soils, the concentrations of most of the GDGTs were up to ten times higher than at the reference site (Fig. 3.7).

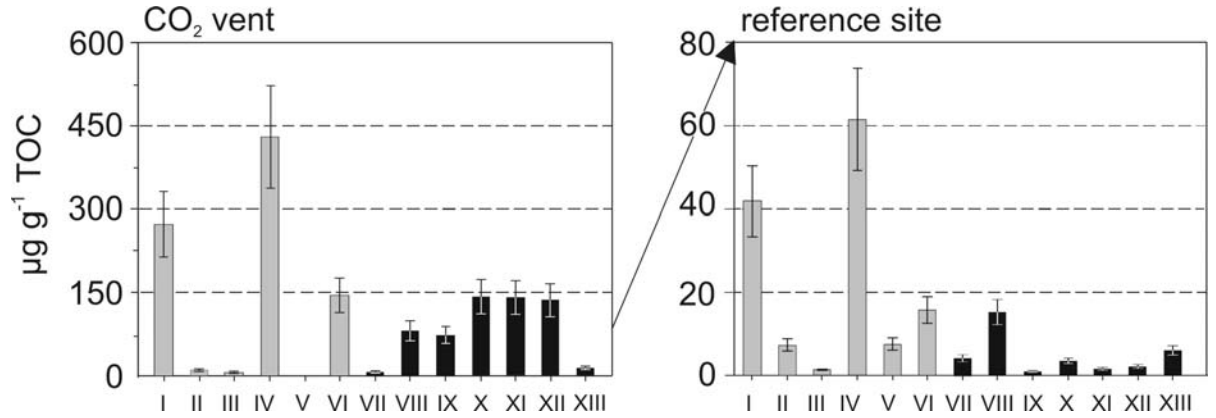


Fig. 3.7. Intact GDGTs detected in the soil samples from the CO_2 vent and the reference site. The hight of bars give the lg of compounds per g TOC Gray bars – bacterial biomarkers, black bars – archaeal biomarkers.

3.3.5.3.1. Branched GDGTs. At the reference site, relatively high concentrations of the branched GDGTs containing cyclopentyl moieties (GDGTs II, III, and V) were detected compared to the GDGTs without cyclopentyl moieties (GDGTs I, IV, and VI) (Fig. 3.7). In the CO_2 vent soil, the GDGTs II, III, and V were found only in minor amounts relative to GDGTs I, IV, and VI. The isotopic values of the C_{30} and C_{31} alkyl moieties released after ether cleavage from the GDGTs in the CO_2 vent soil were enriched in ^{13}C by about 4‰ relative to the same compounds in the reference soil (Fig. 3.8).

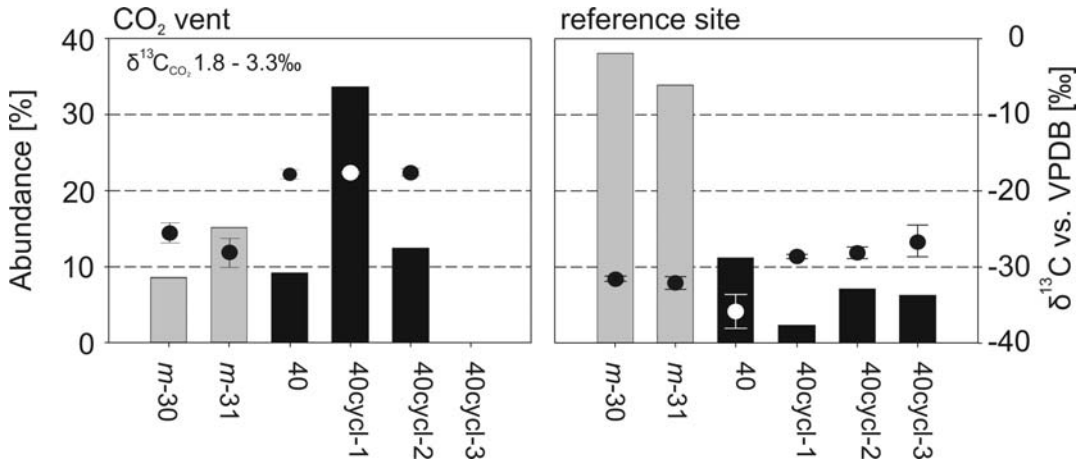


Fig. 3.8. Hydrocarbons released after the ether cleavage of the neutral lipid fraction obtained from the studied soils. The heights of the bars give the relative abundance of the individual compounds. Gray bars – bacterial biomarkers; black bars – archaeal biomarkers.

3.3.5.3.2. Isoprenoidal GDGTs. The GDGTs VIII, IX, X, XI, and XII were all present in the CO_2 vent soil in high concentrations. In the reference site soil, the concentrations of all of the archaeal GDGTs were lower. While the GDGTs IX to XII are lower by a factor of up to 90 (e.g., GDGT XI), the VII, VIII, and XIII were only slightly lower by a factor of 1.5–5. All of the archaeal biphytanes at the CO_2 vent, released after ether cleavage, were more enriched in ^{13}C than the bacterial-derived compounds (Fig. 3.8). At the reference site, the acyclic biphytane was depleted in ^{13}C relative to the biphytanes containing cyclic moieties.

3.3.6. Microbial activities

The determination of the important microbial activities revealed substantial differences between the CO_2 vent and the reference site. The potential rates of the aerobic CO_2 production as well as the methane oxidation were up to five times lower within the CO_2 vent (Fig. 3.9a). In contrast, the sulphate reduction, together with the gross anaerobic mineralisation (CO_2 production), was 10-fold higher in the CO_2 vent than at the reference site (Fig. 3.9b).

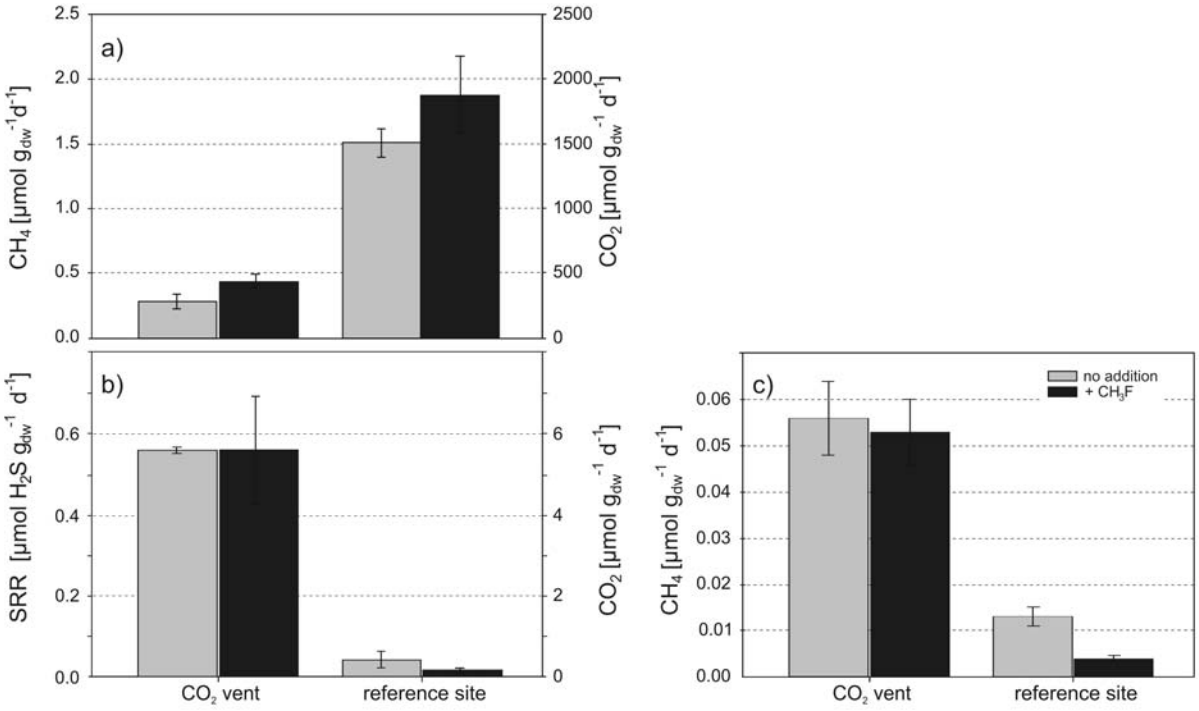


Fig. 3.9. The variations of the microbial activities in the soil samples from the CO_2 vent and the reference site. a) the aerobic processes: aerobic methane oxidation (grey) and the potential rates of aerobic mineralisation (black). The anaerobic processes: b) sulphate reduction (grey) and the potential rates of anaerobic mineralisation (black) SRR: sulphate-reduction rates. c) The variations in the methane production in soil samples from the CO_2 vent and the reference site, the net methane production (grey) and the autotrophic methane production after the addition of CH_3F (black) (mean ± SE, N = 3).

The same pattern was observed for methane production rates (Fig. 3.9c), which were with 0.055 µmol g_{dw}^{-1} d^{-1} at the CO_2 vent, an amount five times higher than that at the reference site. Interestingly, the addition of methyl fluoride to inhibit acetate-dependent methanogenesis led to an 80% reduction at the reference site, while no effect was observed at the CO_2 vent.

3.3.7. Molecular biological community analysis

The compositions of the microbial communities at the CO_2 vent and at the reference site were studied using Q-PCR. The gene copy numbers (16S-rRNA gene) of Bacteria, Archaea, and Eukaryota decreased with increasing CO_2 concentration in the soil gas by 2 orders of magnitude (Fig. 3.10). Opposite to this trend, the communities of anaerobic groups, such as methanogens and SRB, substantially increased in the CO_2-rich vent site. The fingerprinting of the bacterial communities using DGGE indicated complex and varied community compositions at both sites. The diversity of Archaea was limited to 3–5 species (Fig. 3.11).

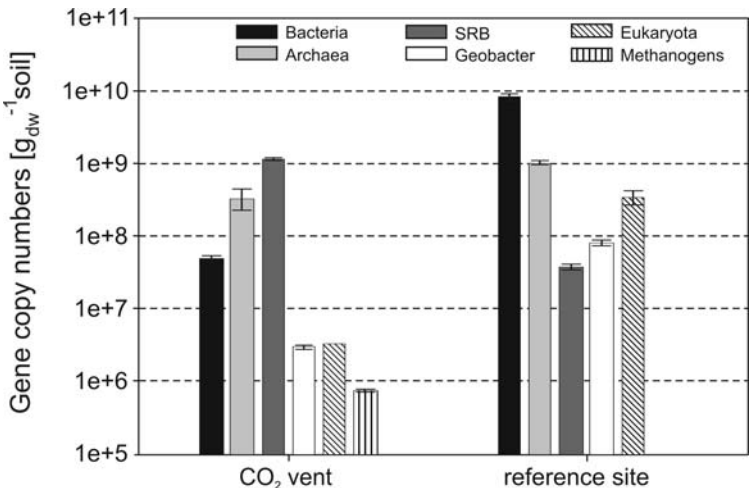


Fig. 3.10. The microbial community composition determined with Q-PCR in the soil samples from the CO_2 vent and the reference site (mean ± SE, n = 8-16).

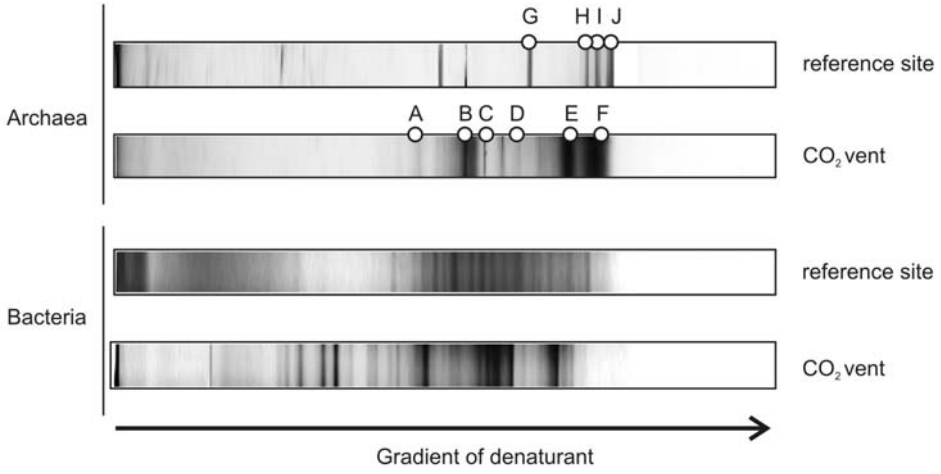


Fig. 3.11. The DGGE of the soil samples from the CO_2 vent and the reference site using 16S-based primers for Archaea.

The sequencing of excised bands from the gel with archaeal DGGE-gel resulted in sequences most closely affiliated to methanogenic or archaeal clones retrieved from acidic environments for the CO_2 vent (Table 3.1), such as fens or acid mine drainage sites. In contrast, the archaeal sequences from the reference site showed no such

tendency for the acidophilic environments. This Blast results were consistent with a maximum likelihood tree calculation with ARB.

Table 3.1. Sequence identity of DGGE Bands (Archaea)

	DGGE Band	Sequence Identity in %	Uncultured representativ (Acc. number)	Sequence origin	References
CO_2 vent	A	99	Methanogenic clone (AJ548932)	oligotrophic fen	Galand *et al.* (2003)
	B	99	Archaeon clone (DQ303254)	acidic river	Garcia-Moyano *et al.* (2007)
	C	99	Archaeon clone (DQ303254)	acidic river	Garcia-Moyano *et al.* (2007)
	D	100	Archaeon clone (AM050410)	anoxic enrichment (rice root)	Penning and Conrad (2006)
	E	89	Archaeon clone (AB161326)	petroleum contaminated soil	Nakaya *et al.* (2009)
	F	99	Archaeon clone (AB300132)	late Pleistocene mud sediment	Takeuchi *et al.* (2009)
Reference site	G	99	Archaeon clone (AB019742)	hydrothermal system	Takai and Horikoshi (1999)
	H	99	Archaeon clone (AB380051)	rice field soil	Nishizawa *et al.* (2008)
	I	99	Archaeon clone (AB300132)	late Pleistocene mud sediment	Takeuchi *et al.* (2009)
	J	98	Archaeon clone (AB300132)	late Pleistocene mud sediment	Takeuchi *et al.* (2009)

Unfortunately, the diversity of bacterial sequences seemed to be too high, thus overloading the DGGE-gel and preventing the reamplification and sequencing of the bands. Nevertheless, the clearly distinct banding pattern for each site shows the presence of a different bacterial community at the high CO_2 levels versus the conditions at the reference site.

3.4. Discussion

In this study, the consequences of high CO_2 fluxes on a soil ecosystem are described. The results of this study show a profound change in the ecology at a natural CO_2 vent as a consequence of the continuous presence of high CO_2 concentrations and the resulting physico-chemical changes. These results might help to evaluate the possible consequences of CO_2 leaks from CCS storage sites before introducing this technology as a wide spread solution for the mitigation of CO_2 emissions. The long-term stability of the CO_2 vent was assessed with repeated gas measurements over a 3-year period. No significant changes in the soil gas composition at the CO_2 vent site or at the reference site were detected. An earlier sampling campaign in 2005 described by Beaubien *et al.* (2008) also did not reveal any major differences compared to our results. Because the microbial community composition that is strongly linked to the soil gas composition changes more slowly than the soil gas composition, we assume that the soil microbial community remained stable during

the 3-year study period. This assumption is strengthened by the fact that the pH values, the isotopic values of the plants and the CO_2 also did not change significantly during this period (data not shown).

3.4.1. General changes in the soil environment due to high CO_2 concentrations

The soil gas composition at the CO_2 vent changed dramatically compared to the reference site (Appendix, Table 3.2). The reference site showed a gas composition similar to the atmosphere. Within the CO_2 vent soil, O_2 was almost absent below 20 cm depth, and CO_2 was the dominating gas. The other important gases in the CO_2 vent soil were methane and H_2S.

Beaubien *et al.* (2008) showed that the sampled soils displayed characteristics typical of young volcanic soils. The clay fraction was dominated by halloysite, accompanied by up to 20% illite. A significant proportion of non-crystalline amorphous material was also observed in the studied soils, and this was most likely derived from volcanic glass. Studies of other CO_2 influenced soils have shown that intense CO_2-induced weathering resulted in a higher soil surface area (Stephens and Hering, 2002).

The water content was more than 10% higher in the CO_2 vent soil as compared to the reference site (Beaubien *et al.*, 2008). Stephens and Hering (2002) reported similar findings for three soils that were exposed to elevated CO_2 concentrations compared to three non-exposed soils in a similar setting at Mammoth Mountain, USA. They proposed that the lack of vegetation, and thus the diminished evapo-transpiration, induced this phenomenon. Beaubien *et al.* (2008) added that the flux of water-saturated gas from the water table probably contributed to the higher water content at the CO_2 vent. Another possibility is that the higher surface area of the CO_2 vent soils could help to capture the moisture.

The pH value was more than two units lower at the CO_2 vent than at the reference site (Fig. 3.3). Below a pH of 5.5, the chemical degradation of potassium-feldspar begins. The resulting Al-cations (Al(III)) are the pH-determining acids in soils at pH values between 3.0 and 5.0 (Vanbreemen *et al.*, 1983). High Al(III)-concentrations are toxic to plants (Kochian, 1995), and low concentration of O_2 at the vent site, can lead to the asphyxiation of plant roots (Hopkins *et al.*, 1950). A high Al(III) and a low O_2 content probably causes the absence of plants in the high-flux core of the CO_2 vent.

At the reference site, the concentrations of organic and carbonate carbon were in the normal range for grassland soils with a relatively low pH (Fig. 3.3). Nitrogen

comprised 0.2% of the total soil [dw] (C/N ration 7.6). The C/N ratio (10.4) was higher in the CO_2 vent soil. This result could have been caused by the incorporation of additional carbon from the geothermal CO_2 into the organic carbon pool. Drissner *et al.* (2007) reported similar findings from soils in free air carbon enrichment experiments, where the amount of CO_2 in the subsoil atmosphere was increased relative to the normal atmospheric value. Another explanation could be the relative depletion of nitrogen in the CO_2 vent. This might arise from lower microbial N_2 fixation activity due to lower N_2 concentrations in the soil gas at the CO_2 vent.

The TOC in the soil samples from the CO_2 vent site was enriched in ^{13}C. This ^{13}C enriched biomass could either derive from microorganisms or from plants. Usually, the microbial biomass contributes to less than 5% to soil TOC (Sparling, 1992). In the CO_2 vent, the Q-PCR analyses (Fig. 3.10) showed lower bacterial and archaeal gene copy numbers, while the TOC concentrations remained similar relative to the reference site. Therefore, we assume that less than 5% of the total organic carbon was of microbial origin. The plant biomass that made up most of the soil TOC was most likely derived from the rim of the CO_2 vent, which is supported by the $\delta^{13}C$ values of soil TOC and by the plant biomass. In the CO_2 vent, the soil TOC is enriched in ^{13}C by 4.6‰ relative to the reference soil; almost the same enrichment was detected (4.2–4.5‰) for the plant biomass from the vent rim compared to the plant biomass 16 m from the CO_2 vent.

3.4.2. Changes in the eukaryota community

The dominating compounds in the hydrocarbon and in the alcohol/ketone fraction in both samples are *n*-alkanes and *n*-alcohols, respectively. Long chain *n*-alkanes and *n*-alcohols are typically found in soils and derive from plants (Bull *et al.*, 2000). The odd over even predominance of *n*-alkanes and the even over odd predominance of *n*-alcohols, as well as their $\delta^{13}C$ values and chain lengths, are in accordance with plant derived lipids reported by Bull *et al.* (2000) and Cayet and Lichtfouse (2001). The vent rim, where the vegetation-free CO_2 vent probably received most of its debris, was dominated by the C3 plant *Agrostis capillaries*. Up to 80% of the reference site was covered by *Trifolium campestre* (Beaubien *et al.*, 2008), another C3 plant. No C4 plants were found in the vicinity of the CO_2 vent. C3 plants typically have $\delta^{13}C$ values between -21‰ and -32‰ (Clark and Fritz, 1997). At the reference site, the plant wax-derived hydrocarbons had $\delta^{13}C$ values typical for C3 plants (Schidlowski, 1987; Glaser, 2005) (Appendix, Table 3.3). In contrast, at the CO_2 vent, the $\delta^{13}C$-values of

these lipids were enriched in ^{13}C relative to the reference site by up to 9.6‰ (HC 31:0), indicating the uptake of ^{13}C enriched CO_2. The enrichment of ^{13}C in the plant derived lipids correlated well with the $^{13}C/^{12}C$ ratio of the plant biomass at the vent rim. The observed shift towards more negative $\delta^{13}C$ values with increasing chain length is a common feature that can be amplified during degradation (Huang *et al.*, 1995).

The abundance of Eukaryota was 100 times lower at the CO_2 vent (Fig. 3.10) compared to the reference site. These low copy numbers in the CO_2 vent suggest lower abundances of fungi, which are usually the most abundant Eukaryota in soils. Fungi are known to produce the diagnostic carboxylic acids 18:2 and 18Δ9 (Beilby and Kidby, 1980), which were absent in samples of the anoxic CO_2 vent, while they were abundant at the reference site.

The saturated carboxylic acids 16:0 and 18:0 are widespread in Eukaryota and Bacteria and, thus, cannot be attributed to any specific source organisms.

3.4.3. Changes in the bacterial community

Several bacterial-derived lipids from the CO_2 vent were enriched in ^{13}C compared to those from the reference soil. These lipids most likely derived from organisms utilising geothermal CO_2 as their main carbon source or from Bacteria feeding on ^{13}C-enriched biomass. Hence, these ^{13}C enriched lipids can be used to track the microorganisms that are profiting from the vent-induced changes. Interestingly, some of the most ^{13}C enriched lipids also increased in quantity, especially the MAGEs and DAGEs. MAGEs and DAGEs are present in both settings (Figs. 3.5 and 3.6). To the best of our knowledge, this is the first report on non-isoprenoidal MAGEs and DAGEs from a soil setting. The potential sources for these uncommon lipids in both the CO_2 vent and the reference site are discussed below.

The high concentration of MAGEs and DAGEs, as well the high concentration of GDGTs within the CO_2 vent, are an indication that ether lipids are valuable adaptations of the cell membrane in this low pH-environment. The MAGEs and DAGEs that were detected at the reference site, however, indicate that bacterial ethers are not restricted to extreme environments and that they might be more widely distributed than was previously thought. Given the extraction methods that were applied, it has to be considered that the MAGE that was analysed may actually originate from hydrolysed alkyl/acyl-lipids as described by Rütters *et al.* (2001).

3.4.3.1. Sulphate-reducing Bacteria

The gene copy numbers of the SRB and sulphate reduction rates (SRR) indicated a higher abundance of SRB in the CO_2 vent (Figs. 3.10 and 3.9b) than at the reference site. Many SRB are known to utilise CO_2 as a carbon source, e.g., members of the *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, and thermophilic SRB (Londry *et al.*, 2004). The presence of ^{13}C enriched biomarkers attributed to SRB, such as *iso*- and *anteiso*-methyl branched CA 15:0 and CA 17:0 (Vainshtein *et al.*, 1992), suggesting that these carboxylic acids are derived from SRB fixing geothermal CO_2 in the vent soil. Furthermore, some of the SRB species (e.g., *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*) are known as sources of MAGEs (Rütters *et al.*, 2001). MAGEs and DAGEs were also described from anaerobic microbial mats rich in SRB (Hinrichs *et al.*, 2000; Pancost *et al.*, 2001, Blumenberg *et al.*, 2004). Although these compounds have been reported from other organisms (e.g., Caillon *et al.*, 1983; Langworthy and Pond, 1986; Wagner *et al.*, 2000; Ring *et al.*, 2006), they are likely form a source within the CO_2 vent.

3.4.3.2. Myxobacteria

The most prominent MAGE and DAGE at the reference site was DAGE *i*-15:0/*i*-15:0, which has been reported from the spores of the aerobic *Myxcoccus xantus* (Ring *et al.*, 2006). Other Myxobacteria, such as *Stigmastella aurantiaca,* are known to produce MAGE and DAGE (with $C_{14–18}$ alkyl moieties) after sporulation (Caillon *et al.*, 1983). Myxobacteria are very common in soils, and they form spores under unfavourable conditions. Therefore, they are a likely source of the majority of the MAGEs and the DAGEs at the reference site, but they may also contribute to the glycerol ether lipids in the CO_2 vent.

3.4.3.3. Acidobacteria

In the CO_2 vent soil, the carboxylic acid *i*-15:0 showed a strong enrichment of ^{13}C. Acidobacteria are a possible source for *iso*-C_{15} components, and perhaps even for the MAGEs and the DAGEs containing these moieties. Eichhorst *et al.* (2007) found that the carboxylic acid inventory of the soil derived *Terriglobus* strains and *Acidobacteriaceae* strains consisted of 36–47% of CA *i*-15:0, but the ether lipids have not yet been described from Acidobacteria. Acidobacteria are widely distributed in soils, and in some soils they are even the dominating microorganisms (Dunbar *et al.*, 2002).

3.4.3.4. Unknown autotrophic soil Bacteria

At the CO_2 vent site, MAGEs and DAGEs with C_{16}-alkyl moieties dominated. The strong enrichment in ^{13}C in some of the MAGEs and DAGEs, especially DAGE *i*-16/*i*-16 (Fig. 3.5), implies an autotrophic source organism that is utilising geothermal CO_2. To date, no mesophilic Bacteria are known to produce DAGE and MAGE with *i*-16:0 moieties. Bacteria containing DAGEs with a certain alkyl moiety sometimes also contain the analogous MAGEs and vice versa, but strong isotopic differences between the MAGE and the DAGE that are produced by one organism have not been reported. Therefore, the different isotopic values of MAGE *i*-16:0 (-11.1‰) and DAGE *i*-16:0/*i*-16:0 (-2.6‰) at the CO_2 vent indicate two different source organisms. Currently the thermophilic Bacteria *Aquifex pyrophilus* and *Thermodesulfobacterium commune* are the only known sources of DAGE *i*-16:0/*i*-16:0 (Langworthy and Pond, 1983; Huber *et al.*, 1992). Thermophilic Bacteria such as *Clostridium thermocellum* (Langworthy and Pond, 1986) and *Aquifex pyrophilus* (Huber *et al.*, 1992) contain MAGE 16:0, but are an unlikely source for bacterial ethers in this mesophilic environment. It is possible, however, that some close relatives of these thermophilic Bacteria have adapted to this mesophilic environment. In the CO_2 vent, with its low pH and anoxic conditions, an ether-lipid containing relatively rigid cell membrane might be an advantage. Other likely sources for MAGE 16:0 are SRB species (see Section *3.4.3.1.*).

3.4.3.5. Unknown heterotrophic soil Bacteria

High concentrations of branched GDGTs were detected in both studied soil samples (Fig. 3.7). Although the alkyl moieties are bound to the glycerols via ether bonds in the GDGTs, they clearly possess characteristics of bacterial lipids, such as straight alkyl chains and the stereoconfiguration at the C-2 position of the glycerol (see Weijers *et al.*, 2006b). Pancost and Sinninghe Damsté (2003) and Weijers *et al.* (2007) proposed that these compounds derive from yet unknown anaerobic soil Bacteria based on the predominance of branched GDGTs in anoxic compartments of soils and peat bogs. This information fits well with our study and the more than six times higher concentrations of bacterial GDGTs in the anoxic CO_2 vent (e.g., GDGT I 264.1 µg g^{-1} TOC) compared to the oxic reference site (e.g., GDGT I 43.7 µg g^{-1} TOC; Fig. 3.7).

Branched C_{30}- and C_{31}-alkanes (Fig. 3.8) were released after ether cleavage from the TLC dialcohol fraction, indicating that they represent the alkyl chain of branched GDGTs. Interestingly, the $\delta^{13}C$ values of these C_{30}- and C_{31}-alkanes in the CO_2 vent and the reference soil mimicked the $\delta^{13}C$ values of the plant derived lipids. Therefore, stable carbon isotopic values of C_{30}- and C_{31}-hydrocarbons point to heterotrophic source organisms.

At the reference site, higher concentrations of cyclic bacterial GDGTs II, III, and V, relative to the saturated GDGTs I, IV, and VI were detected. Weijers *et al.* (2007) found that higher soil pH values correlated well with higher amounts of cyclic GDGTs. Applying the cyclisation ratio of the branched GDGTs (CBT, Appendix, formula 3.1A) proposed by Weijers *et al.* (2007) to calculate the soil pH of our sampling sites (Appendix, formula 3.1B), it suggests that the pH should be 3.9 at the CO_2 vent and 6.7 at the reference site. These values are slightly higher than the measured values (3.3 and 6.1, respectively), but the overall relationship observed by Weijers *et al.* (2007) is apparent.

In the CO_2 vent, the concentrations of the branched GDGTs with additional methylation at the C-5 and C-5' position are higher relative to the GDGTs I–III. Weijers *et al.* (2007) found a correlation between the methylation index of branched GDGTs and the mean air temperature and the precipitation, and to a lesser extent, the soil pH. Because the studied samples were located only 50 m apart, the same temperatures and precipitation can be expected for both samples and the differences in the relative concentrations of GDGTs I–III to IV–VI are presumably only influenced by soil pH.

3.4.3.6. Methanotrophic Bacteria

High potential rates of aerobic methane oxidation were detected at the reference site (Fig. 3.9a), but no lipid biomarkers of methanotrophs were identified. On the other hand, ^{13}C enriched biomarkers typical of type II methanotrophs (Hanson and Hanson, 1996 and references therein), i.e., CA 18:1Δ10, were found in the CO_2 vent soil (Fig. 3.4). Bowman *et al.* (1991) and Dedysh *et al.* (2002) showed that type II methanotrophs produce CA 18:1Δ10 and 18:1Δ11 in considerable amounts. The similar $\delta^{13}C$ values (Fig. 3.4) of these two lipids at the CO_2 vent indicate a possible common source organism for both compounds. Methanotrophic Bacteria are aerobic; therefore, it is likely that in the CO_2 vent this biomarker was transported downwards from the top soil layers. Knoblauch *et al.* (2008) found the methanotrophic community

to be the most abundant in the top layer of the four different arctic bogs, where they could receive methane from the underlying anoxic soil and oxygen from the atmosphere. Lipid biomarkers of type I methanotrophic Bacteria (e.g., 16:1Δ8) were not detected. Amaral *et al.* (1995) proposed that type II methanotrophs prefer higher CH_4 concentrations and lower O_2 concentrations, whereas type I methanothrophs favour low CH_4 and high O_2 conditions. Depending on the concentration of methane in the environment, the $\Delta\delta^{13}C_{lipids}$ relative to source methane varies between -2.5 and -24.2‰, yielding lower fractionation factors (-2.5 to -11.4‰) as methane becomes limited (Jahnke *et al.*, 1999). The $\delta^{13}C$ value of the CA 18:1Δ10 (-15.9‰ ±1.1) at the CO_2 vent therefore implies the utilisation of the relatively ^{13}C enriched CH_4 at approximately +8.3 to -13.4‰. However, the thermogenic methane from an underlying carbon pool should result in $\delta^{13}C$ values between -20 to -50‰ (Whiticar, 1999) that are progressively approaching the $\delta^{13}C$ values of the organic source with increasing maturity. The activity measurements and the higher gene copy numbers for methanogenic Archaea indicated that *in situ* production of methane also took place (Fig. 3.9c; see also 3.4.4.1.2 below), which could lead to more ^{12}C enriched methane. Therefore, the heavy $\delta^{13}C$ value of the CA 18:1Δ10 suggests either a different source organism for this carboxylic acid or a complex methane cycling system in the CO_2 vent that is insufficiently understood.

3.4.3.7. Geobacter

Gene copy numbers (Fig. 3.10) showed the presence of *Geobacteraceae* in both of the sampled soils. However, in the CO_2 vent, the gene copy numbers were lower two orders of magnitude than at the reference site. Members of the family *Geobacteraceae* are anaerobes using Fe(III) or other metals as electron acceptors. Lin *et al.* (2004) showed that *G. sulfurreducens* can tolerate oxygen concentrations of 10% under certain circumstances and can even use oxygen as an electron acceptor. They postulated that *Geobacter* species preferentially live close to the oxic-anoxic interface, where Fe(III) concentrations are relatively high due to the supply of Fe(II) and its oxidation to Fe(III). The low concentrations of metals in the CO_2 vent (Beaubien *et al.*, 2008) are likely the reason for lower *Geobacter* cell numbers in the CO_2 vent. The most abundant carboxylic acid of *Geobacter* species is the specific 10-*methyl*-16:0 (Lovley *et al.*, 1993; Zhang *et al.*, 2003), which was absent in the CO_2 vent soil (Fig. 3.4). The CAs *i*-15:0, *i*-17:0, *ai*-15:0, *ai*-17:0, 17cycl-1, and 19cycl-1

have also been described from *Geobacter*. These lipids were equally abundant in the CO_2 vent soils and at the reference site, which could be related to the longer stability of lipids compared to the DNA, or because other organisms also produce these general carboxylic acids and mask the *Geobacter* signal.

3.4.3.8. Other soil Bacteria

Methyl branched C_{17} and C_{18} carboxylic acids were present in high quantities in the CO_2 vent soil, and the $\delta^{13}C$ values indicate that they originate from Bacteria utilising geothermal CO_2. A number of mostly aerobic Bacteria are known to produce the carboxylic acids *m*-17:0 and *m*-18:0. They have been found in *Actinomycetales,* which mainly synthesise 10-*methyl*-carboxylic acids, e. g., *Streptomyces* sp. (Brown *et al.*, 1985), *Mycobacterium* sp. (Julák *et al.*, 1980), *Corynebacterium amycolatum* (Yagüe *et al.*, 2003) and 9-*methyl*-carboxylic acids *Corynebacterium* spp. (Niepel *et al.*, 1998). Corynebacteria, Streptomycta and Mycobacteria are widely distributed in soils, and some *Actinomycetales* are known to live under anaerobic conditions. Hence, they may account for these compounds in the studied samples. *Rhizobium* sp. (Gerson *et al.*, 1974) and *Sphingomonas echinoides* (Barnes *et al.*, 1989) also contain CA 10-*methyl*-18:0, but these bacteria are aerobic and therefore not likely to be abundant in the anoxic CO_2 vent soil.

Methyl-branched C_{17} carboxylic acids are also known from *Nitrospira moscoviensis*, which contains 10-*methyl*-17:0 (Lipski *et al.*, 2001). Under anaerobic conditions *Nitrospira moscoviensis* uses hydrogen as electron donor (Ehrich *et al.*, 1995). Since nitrite-oxidising Bacteria are common in soils and *Nitrospira moscoviensis* can also use CO_2 as a carbon source, they are a possible source for 10-*methyl*-17:0 and structurally related compounds here.

The parasitic *Mycoplasma fermentans* (Wagner *et al.*, 2000) contains MAGE 16:0, which is abundant in the CO_2 vent soil. Nevertheless, this bacterium is not ubiquitous in soils, and we assume that the ^{13}C-enriched MAGE 16:0 in the CO_2 vent soil derives from SRB (see Section *3.4.3.1*) or from mesophilic relatives of thermophilic Bacteria (see Section *3.4.3.4*).

3.4.4. Changes in the archaeal community

The gene copy numbers of Archaea were slightly lower in the CO_2 vent soil than at the reference site (Fig. 3.10), which does not correlate with the concentrations of archaeol (Fig. 3.5). Archaeol is widely distributed in Archaea and is therefore used as

a biomarker for this domain. However, some Archaea possess only minor amounts of archaeol. This is especially true of the thermophilic Archaea that contain as little as 0.1% diethers in their lipid fraction (Langworthy and Pond, 1986). The dominant archaeal lipids in our samples were GDGTs VII–XIII (Figs. 3.2, 3.7, and 3.8).

3.4.4.1. Crenarchaeota

Crenarchaeota are especially widespread in marine environments (Karner *et al.*, 2001; Sinninghe Damsté *et al.*, 2002), but are also found in terrestrial settings (Bintrim *et al.*, 1997; Jurgens *et al.*, 1997). A diagnostic biomarker for Crenarchaeota is crenarchaeol, GDGT XIII built of one dicyclic and one tricyclic biphytane (Fig. 3.2). In addition to crenarchaeol, Crenarchaeota are also known to contain other GDGTs (DeLong *et al.*, 1998). De Rosa *et al.* (1980) showed that the numbers of cyclisations within the biphytanes of the archaeal GDGTs strongly depend on the growth temperature. The number of cyclisations within GDGTs of *Sulfolobus solfataricus*, which has a growth optimum of 75°C, decreased as the growth temperature decreased. Therefore, Crenarchaeota are a possible source of considerable quantities of GDGTs found in the studied soil samples.

3.4.4.1.1. Mesophilic Crenarchaeota. The isotopic values of the biphytanes released after ether cleavage from the dialcohol fraction provided information on the source organisms. In the reference-site soil, crenarchaeol-derived dicyclic (-28.1‰) and tricyclic (-26.6‰) biphytanes had similar $\delta^{13}C$ values (Fig. 3.8) to the monocyclic biphytane (-28.6‰), thus indicating that they all derived from mesophilic Crenarchaeota thriving in the soil. The $\delta^{13}C$ value of the acyclic biphytane (-35.9‰) from the reference soil sample differed strongly from the cyclic biphytanes, suggesting another, possibly methanogenic, archaeal source. However, crenarchaeol was present in higher amounts in soil samples of the reference site relative to other archaeal biomarkers. Together with the lower rate of methanogenesis at the reference site and the higher gene copy numbers of Archaea this showed that Crenarchaeota were the dominating Archaea at the reference site.

3.4.4.2. Euryarchaeota

3.4.4.2.1. Thermophilic Euryarchaeota. The DGGE analyses showed that Archaea that are closely related to the thermophilic genera *Thermoplasma* were prominent in the CO_2 vent. The GDGTs, which are present in high amounts in the vent soil, were first found in *Thermoplasma acidophilum* (Langworthy, 1977). Since the early discovery of GDGTs in environmental samples (Michaelis and Albrecht, 1979), GDGTs have lately been found in many mesophilic environments originating from mesophilic Crenarchaeota (DeLong *et al.*, 1998; Schouten *et al.*, 2000; Weijers *et al.*, 2006a; Escala *et al.*, 2007). Since the studied environment had mesophilic ambient temperatures, thermophilic Archaea seem to be an unlikely source of GDGTs. However, the solfatara fields from which *Thermoplasma* has been isolated (Segerer *et al.*, 1988) exhibit a low pH and a high H_2S content, which were very similar to the study site. Thus, *Thermoplasma* might posses some mesophilic relatives that are able to thrive at lower temperatures.

3.4.4.2.2. Methanogenic Euryarchaeota. The gene copy numbers, the DGGE, and the microbial activity measurements indicate that methanogens were more abundant at the CO_2 vent than at the reference site (Fig. 3.9c–3.11 and Table 3.1). In fact, the functional genes specific for methanogenic Archaea were only detected in the CO_2 vent soil. Nevertheless, the biomarker concentrations and the methane production rates demonstrate that methanogenic Archaea were also present at the reference site, although to a lesser extent. Many methanogenic Archaea contain high amounts of acyclic GDGT VIII (De Rosa and Gambacorta, 1988; Koga *et al.*, 2006 and references therein). In addition to GDGT VIII methanogenic, Euryarchaeota were also described to produce smaller amounts of GDGTs bearing cyclopentyl moieties (De Rosa and Gambacorta, 1988). Consequently, the core lipids, which are biphytanes with up to two pentacyclic groups, have been used as biomarkers for methanogenic Archaea in mesophilic environments (Michaelis and Albrecht, 1979; Chappe *et al.*, 1980, 1982; Hoefs *et al.*, 1997). Recent studies, however, have demonstrated that most GDGTs bearing cyclopentyl moieties originate from ubiquitous mesophilic Creanarchaeota (DeLong *et al.*, 1998). GDGTs VIII–XII were prominent in the CO_2 vent soil. The similar $\delta^{13}C$ values (Fig. 3.8) of the acyclic and the cyclic biphytanes released after ether cleavage of the dialcohol fraction (acyclic: -17.9‰; monocyclic: -17.7‰ and dicyclic: -17.6‰) emphasise that they derive from a

single ^{13}C enriched carbon source. Therefore, the ^{13}C enriched carbon utilised for the GDGT production at the CO_2 vent is probably at least partly geothermal CO_2. Hence, it can be concluded that the majority of the archaeal GDGTs are synthesised by autotrophic methanogenic Archaea. This was also confirmed by inhibitor experiments (Fig. 3.9c), showing that autotrophic methanogenesis dominated in the CO_2 vent. At the reference site, the acetoclastic methanogenesis was equally important as the autotrophic methanogenesis (Fig. 3.9c). The acyclic C_{40} (-35.9‰ ±2.2) at the reference site exhibited a strong ^{13}C depletion relative to the crenarchaeol derived 40cycl2 (-28.1‰) and 40cycl3 (-26.6‰). It can therefore be assumed that the acyclic C_{40} was produced by a different Archaeon. Methanogenic Archaea are the most likely source for the acyclic C_{40}, but it was not possible to reveal to what extent the autotrophic and/or heterotrophic methanogens contribute to the acyclic C_{40} that was detected in the reference soil.

3.5. Conclusions

In the Latera Caldera, strong changes in the soil microbial community were observed as an apparent response to the exhaustion of geothermal CO_2. Eukaryota such as fungi and plants were lacking or only present in reduced numbers due to geochemical conditions induced by the high concentrations of CO_2. Microbial carbon fixation changed from heterotrophic, utilising plant derived debris in the reference soil, to autotrophic pathways, utilising the geothermal CO_2 within the CO_2 vent soil. Anaerobic and acidophilic microorganisms profited most from the environmental conditions at the CO_2 vent, and aerobic groups such as Crenarchaeota were diminished. In addition, anaerobic Bacteria such as *Geobacteraceae* were diminished due to the altered geochemical conditions that were induced by the high concentrations of CO_2 in the vent soil. The presence of autotrophic methanogenic Archaea and sulphate-reducing Bacteria was stimulated at the vent. This led to the enhanced production of methane and H_2S, which might result in feedback mechanisms that potentially amplify reducing conditions in the soil. Moreover, the utilisation of the greenhouse gas CO_2 at the vent site enhanced the production of methane by a factor of five compared to the reference site. This process needs special attention in the relation to the mitigation of greenhouse gases and CCS since methane has a global warming potential that is 25 times that of CO_2.

Appendix

Figure 3.12. The procedure for extraction of analytes.

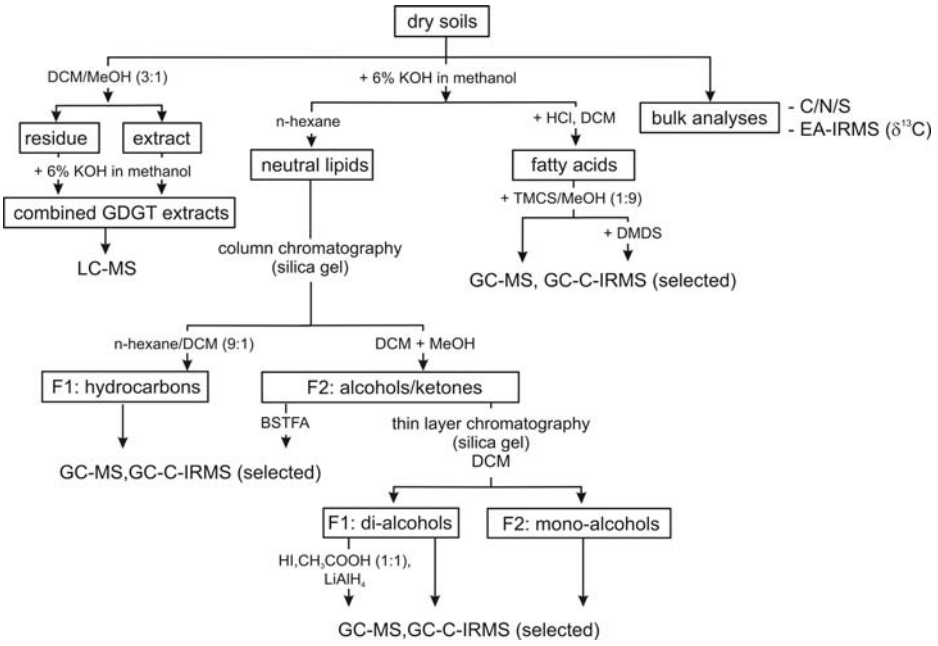
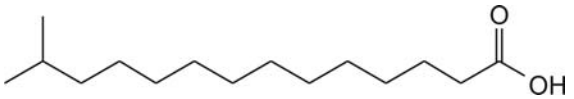


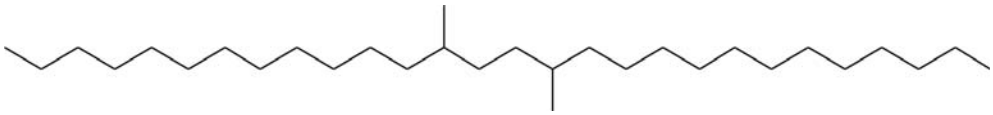
Figure 3.13. If not stated in the text the substituents are given as HC, OH, and CA for hydrocarbons, alcohols, and carboxylic acids. The lipids were named by their number of carbon atoms (e.g., 16) and the number of undersaturations (e.g., two undersaturations 16:2). The position of double bounds is counted from the carboxylic acid end of the molecule (Δ).

OH 16:1Δ11

The methyl branches were abbreviated with *i*-, a*i*- and *m*- for iso-, anteiso-, and mid chain methyl branched, respectively; or with the number of the carbon atom where the methyl branch is located, counted from the functional group, e.g., CA *i*-15:0 and HC 13,16-*m*-30:0.



CA *i*-15:0



HC *m*-30:0

GDGTs and GDGT derived hydrocarbons are abbreviated according to Fig. 3.2.

Table 3.2. The concentrations of selected soil gases at the CO_2 vent and the reference site

	Depth [cm]	CO_2 [%]	CH_4 [ppm]	H_2 [2] [ppm]	H_2S [ppm]	O_2 [%]	N_2 [%]
CO_2 vent	20 [1]	93.1	2.7	n.a.	16	1.0	5.8
	80	95.6	1014.9	900	>200	0.9	4.4
Reference site	20 [1]	0.4	0.9	n.a.	0	21.0	78.5
	80	1.2	0.3	0	0	20.6	76.3

n.a. not analysed;

[1] Beaubien *et al.* (2008)

[2] Sampled in 2005

Table 3.3. The concentrations and $\delta^{13}C$ values of selected hydrocarbons and alcohols

	CO_2 vent		Reference site	
	µg g^{-1} TOC	[‰] $\delta^{13}C$ vs. VPDB	µg g^{-1} TOC	[‰] $\delta^{13}C$ vs. VPDB
Hydrocarbons				
20:0	0.1	-24.2	0.2	n.d.
21:0	0.2	-22.7	0.4	-29.5
22:0	0.2	-24.7	0.3	-29.0
23:0	0.3	-27.9	0.7	-29.9
24:0	0.2	-26.7	0.5	-28.3
25:0	0.8	-28.7	2.9	-29.4
26:0	0.4	-28.3	1.3	-29.2
27:0	2.3	-28.3	8.4	-31.0
28:0	1.0	-32.2	2.5	-31.7
29:0	5.0	-31.1	20.5	-33.5
30:0	0.1	-33.0	1.9	-32.1
31:0	4.4	-24.3	20.7	-33.9
32:0	0.4	-26.6	0.9	-34.0
33:0	0.7	-31.5	10.3	-35.2
Alcohols				
16:0	16.2	-26.8	17.0	-28.5
17:0	2.1	-29.6	2.6	-30.8
Phytol	9.1	-24.5	38.8	-32.8
18:0	8.1	n.d.	17.5	-32.0
19:0	0.8	-29.6	3.3	n.d.
20:0	30.4	-28.6	51.9	-34.1
21:0	0.9	-26.7	6.3	-34.0
22:0	104.7	-29.6	97.1	-35.2
23:0	5.2	-31.6	2.3	-34.5
24:0	66.8	-29.8	0.3	-33.6
25:0	6.4	-31.9	7.9	-33.0
26:0	153.3	-32.1	222.6	-35.0
27:0	6.0	-30.0	2.1	-34.0
28:0	76.9	-33.0	204.1	-34.3
29:0	6.3	-30.4	30.0	-29.9
30:0	92.0	-32.8	185.2	-34.1
31:0	24.8	n.d.	3.0	-31.7
32:0	9.0	-31.4	57.1	-28.7
33:0	104.7	n.d.	14.0	-34.3
34:0	5.2	-28.4	19.0	-35.2
ββ-Bishomohopanol	7.5	-30.3	14.0	-29.9

n.d. – not detected

Formula 3.1. The formulas to calculate CBT (A), pH B modified after Weijers *et al.* (2007).

$$CBT = -\log [(II + V) / (I + IV)] \quad (A)$$

$$pH = - (CBT - 3.33) / 0.38 \quad (B)$$

Structures of abbreviated GDGTs are found in Fig. 3.2.

Anaerobic microbial methane consumption in a soil overlying a natural CO_2 vent

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Abstract

Even though great progress was made in the last years to assess the anaerobic oxidation of CH_4 (AOM) in marine environments, the role and mode of AOM in soils is yet poorly studied. Recent studies demonstrate that beside sulphate, electron acceptors like nitrite, manganese(IV) and iron(III) can be used for AOM. These additional AOM pathways are of great interest in terrestrial environments were sulphate concentrations are usually low. To assess the possible, microbial consumption of CH_4 in an anaerobic soil, a natural CO_2 vent was studied and compared to an undisturbed soil. Although the soil at the CO_2 vent was permanently anoxic, high rates of aerobic CH_4-consumption were detected *in vitro*. To resolve this contradiction, methanotrophic organisms were investigated using lipid biomarker studies combined with compound-specific stable carbon isotope analyses, assessment of microbial activities, determination of concentrations of possible electron acceptors and molecular biological methods.

Sulphate reducing Bacteria (SRB) were more common in the CO_2 vent compared to the reference site as indicated by *in vitro* measured sulphate reduction rates and biomarker analyses, but SRB were most likely not involved in the AOM. Biomarker distributions and $\delta^{13}C$-values indicate that bacterial groups connected to denitrification and/or to the metal cycle are involved in the degradation of CH_4 and mitigation of methane emissions. This indicates a more diverse methanotrophic community in terrestrial systems than previously assumed. Our results might also be of interest for other acidic terrestrial environments, like boreal peat bogs, where CH_4-cycling will be of increasing global relevance due to the predicted thawing of permafrost soils.

4.1. Introduction

Natural CO_2 vents are excellent sites to study the impact of enhanced gas fluxes on soil macro- and microorganisms. Although they often exhibit extreme conditions, like a low pH-regime and permanent anoxia, their natural isotope labelling of CO_2 and CH_4 as well as their limited spatial extent make them good natural laboratories for studying anaerobic processes in soils. Following the destruction in the stratosphere soils are the main global sink of CH_4 (Forster *et al.*, 2007 and references therein). Considering the high global warming potential of CH_4 the modes of its consumption in these terrestrial environments are of great interest.

The aerobic oxidation of CH_4 was first described by Leadbetter & Foster (1958). Since Whittenbury *et al.* (1970) isolated the responsible Bacteria it was studied in detail. 20 years later Barnes & Goldberg (1976) and Reeburgh (1976) described CH_4-consumption from anaerobic marine sediments. Before Raghoebarsing *et al.* (2006) described the anaerobic oxidation of CH_4 coupled to denitrification the only known anaerobic process consuming CH_4 was AOM coupled to sulphate reduction (Hoehler *et al.*, 1994; Michaelis *et al.,* 2002). AOM coupled to sulphate is conducted by a consortium of SRB and methanotrophic Archaea (Boetius *et al.*, 2000; Michaelis *et al.,* 2002). While the microorganisms responsible for the oxidation of CH_4 in the denitrification coupled AOM are Bacteria, which gain oxygen for CH_4 oxidation from nitrate and nitrite (Ettwig *et al.*, 2008, 2010). Beal *et al.* (2009) revealed another AOM pathway utilizing manganese(IV) and iron(III) as electron acceptors. In fresh water environments and soils these additional electron acceptors are of great importance since sulphate concentrations are usually low in these environments.

The site of this study is located at an oxygen-depleted soil at a volcanic CO_2 vent (>90% of CO_2 in soil gas). The study site was first described by Beaubien *et al.* (2008) giving detailed soil gas, geochemical and botanical data. The described high CO_2-fluxes (2000-3000 g m^{-2} d^{-1}) led to changes of the soil microbiota, with low cell numbers and a shift to anaerobic processes (Beaubien *et al.*, 2008). Oppermann *et al.* (2010) revealed further details of CO_2-induced changes in the microbial community. They found microorganisms producing robust membrane lipids, also known from other extreme environments like hydrothermal vents, to profit from the conditions at the CO_2 vent. The combination of stable isotope analyses with biomarker studies and microbiological methods further showed that volcanic CO_2 is

used as a carbon source by autotrophic methanogens, leading to enhanced microbial production of CH_4. However, to estimate the CH_4 flux into the atmosphere also CH_4 consuming processes need to be assessed.

In the present study, we combined biogeochemical, geochemical, microbiological and molecular biological methods for a detailed investigation of processes and organisms involved in the CH_4-cycle at a constantly anaerobic soil influenced by high CO_2 fluxes.

4.2. Materials and methods

4.2.1. Site description and sampling

The study area is located in Central Italy in the Vulsinian volcanic district within the Latera Caldera (Fig. 4.1a) on pasture land. The studied CO_2 vent is fed by the thermo-metamorphic degradation of underlying carbonate-rocks (Barberi *et al.*, 1984). Beaubien *et al.* (2008) found high CO_2-fluxes (2000-3000 g m^{-2} d^{-1}) and significant CO_2-induced changes, such as anoxia, low pH, reduced microbial activities, lower concentrations of oxides in the soil and a lack of vegetation at the studied CO_2 vent. Moreover, a shift towards autotrophic (CO_2 and CH_4), anaerobic and acidophilic organisms in the microbial community was detected (Oppermann *et al.,* 2010).

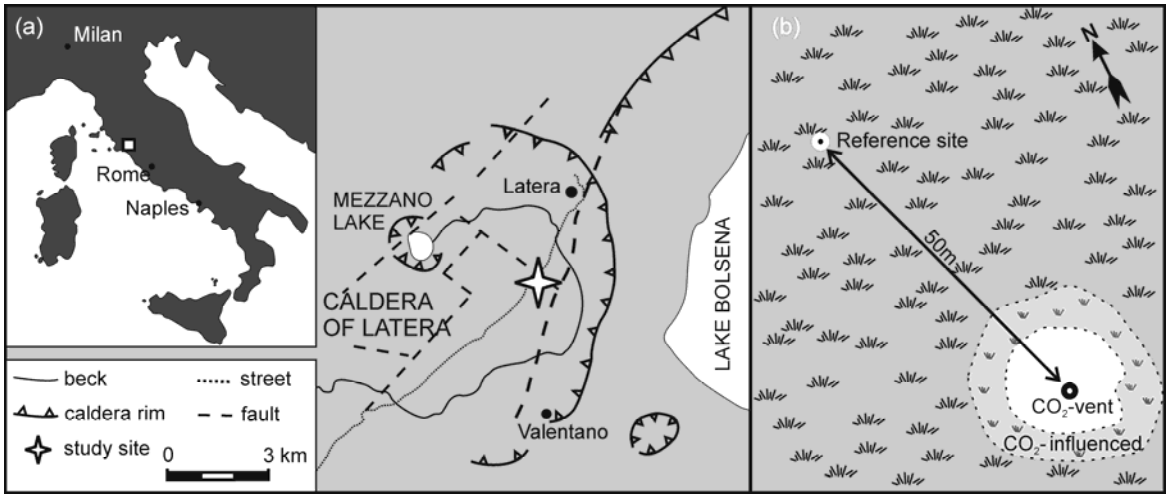

Fig. 4.1. (a) Map of the study area, modified after Palladino and Agosta, (1997), Beaubien *et al.* (2008) and Oppermann *et al.* (2010). (b) Sketch of the sampling site, showing the CO_2 vent with reduced vegetation, the CO_2-influenced-zone with acidophilic grasses and the reference site.

The soil samples were taken in the centre of the CO_2 vent and at a reference site in 50m distance not notably affected by gas venting (Fig. 4.1b). At both sampling sites a 60-70-cm-deep-hole was opened to assess, describe and sample the different soil horizons. Soil samples for lipid biomarker and microbial analyses were stored at -10°C and 5°C, respectively.

4.2.2. Soil analysis

Total organic carbon (TOC) and total nitrogen were measured with an elemental analyser (VarioMAX Elementar Analysensysteme GmbH, Germany) after the soil samples were sieved (>2 mm) (not for isotopic analyses) dried at 105°C, milled and, for the isotopic analyses, the inorganic carbon was removed with HCl. Repeated measurements showed an error of less than ±0.05%. An aliquot of the samples were taken for the isotopic analyses of TOC. The $\delta^{13}C$-values were determined using a Finnigan MAT 252 mass spectrometer after high-temperature combustion (1020°C) to CO_2 in a Carlo Erba NA-2500 elemental analyser (Erba Science, Italy). After the combustion sulphur was removed with Sulfix (WAKO Chemicals, Japan), using a method described by (Carlson *et al.*, 1998). Based on replicate measurements of reference standards IAEA-CH-6 (IAEA, Austria) and IVA-Sediment (IVA, Germany), the analytical precision was better than 0.1‰. Duplicate measurements showed a mean deviation of < 0.2‰.

From a suspension of 10 g of fresh soil in 25 ml of distilled water soil pH and electrical conductivity were measured in the field using a pH-redoxmeter GPRT 1400 AN equipped with a redox electrode GE 105 (GSG Greisinger Electronic GmbH, Germany). Dried and sieved soil was used for several extraction methods to quantify the amount of cations and anions dissolved in the pore water. To quantify nitrite, nitrate and sulphate concentrations in the soil solution, 5 g soil was extracted with 20 ml of a 12.5 mM $CaCl_2$ solution. Nitrate and nitrite were quantified by HPLC (Agilent Technol, 1200 series) and sulphate was quantified photometrically after precipitation with $BaCl_2$ according to van Reeuwijk (2002). Concentrations of available phosphorous and potassium were determined by extracting 1 g dried soil sample with a calcium-lactate solution (0.2 M, pH 3.6) for 90 minutes. The extract was filtered and photometrically analysed for phosphorous and by atomic absorption spectroscopy for potassium. Soil texture was determined by the pipette method (van Reeuwijk, 2002) using a Sedimat 4-12 (Umwelt-Geräte-Technik GmbH, Germany). Soil porosity was quantified in undisturbed soil samples, 100 ml soil sample (Richards & Fireman, 1943). Subsequently the total pore volume was determined by an Hg-pycnometer (Eijkelkamp, The Netherlands). The volumetric water content in the undisturbed samples was calculated from the weight difference of fresh and dried samples. Ammonium, nitrite, nitrate and sulphate concentrations in the pore water were calculated from the total amount of ions extracted from the dried soil and the

volumetric water content under field conditions. Total elemental composition was determined by X-ray fluorescence spectrometry using a MagixPRO equipped with a Rh-Anode (Pananalytical, The Netherlands) as described by Kikkert (1983).

4.2.3. Gas sampling and analyses

To sample soil gas, a thick-walled, stainless steel tube, sealed by a rubber stopper was pounded to 1 m sampling depth. The gas was extracted from this tube using an airtight plastic syringe fitted with a Teflon valve. The sampled gas was rapidly transferred into airtight glass containers, sealed with airtight caps. Stable carbon isotopes of CH_4 and CO_2 were analysed after a protocol of Michaelis *et al.* (2002). Standard deviations for replicate injections of CH_4 and CO_2 were less than 0.4 and 0.7‰, respectively. The precision, as determined by a repeated analysis of air, was 0.9‰.

4.2.4. Biomarker analyses

4.2.4.1. Extraction, isolation and derivatisation

Carboxylic acids and alcohols were extracted after a protocol by Oppermann *et al.* (2010) (see Appendix, Fig. 4.9). In short, to release ester-bound lipids, lyophilised and homogenised samples were hydrolysed with 6% KOH in methanol (MeOH) in excess for 2 h at 80°C with additional ultrasonication. Alcohols were extracted with *n*-hexane and separated from the ketones and hydrocarbons using column chromatography (Merck silica gel 60) with organic solvents of increasing polarity (*n*-hexane, DCM, MeOH). Carboxylic acids were extracted from the remaining, acidified (pH 1-2) aquatic solution with *n*-hexane and DCM. An aliquot of the alcohol fraction was separated according to the method of Michaelis *et al.* (2002) by thin layer chromatography (TLC) yielding a di- and a mono-alcohol fraction. To analyse the alkyl moiety of ether bound lipids, an ether cleavage with HI was conducted after a protocol of Kohnen *et al.* (1992). Before further analyses alcohols were silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide for 2 h at 80°C and carboxylic acids were converted to their methyl esters (trimethylchlorosilane in MeOH 1:9; v:v; 2 h 80ºC).

Intact bacteriohopanepolyols were analysed and quantified by liquid chromatography-mass spectrometry (LC-MS) using an acetylated aliquot of a total organic extract (DCM/MeOH; 1:1; v:v) as previously published (Blumenberg *et al.* 2010). Briefly, LC-MS was performed using a Prostar Dynamax HPLC-system interfaced to a 1200L triple-quadrupole mass spectrometer (both Varian). The mass spectrometer

was equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode.

4.2.4.2. Identification and Quantification

Tentative identification of compounds in the silylated alcohol- and methylated carboxylic acid-fractions was accomplished using gas chromatograph (GC) retention times, mass spectra, published and from the NIST Mass Spectral Search Program 2.0.0.26 (U.S. Secretary of Commerce, USA). For this, GC-mass spectrometry (GC-MS) was conducted with a Thermo DSQ II spectrometer interfaced with a Trace GC Ultra with a 30-m fused silica capillary column (DB5-MS, 0.32 mm i.d. 0.25 µm film thickness). The carrier gas was helium and the temperature program was: 3 min at 80ºC; from 80ºC to 310ºC at 6ºC min^{-1}; 20 min at 310ºC. The software used for these analyses was Xcalibur 2.0.7. (Thermo Fisher Scientific Inc., USA). The concentration of alcohols and carboxylic acids was calculated relative to an authentic, internal standard (Squalane, Fluka, Switzerland) using the GC-FID signals processed in ChromStar 6.3 (Chromatography Software, Germany). For quantification of bacteriohopanepolyols sample peaks were compared with responses of external standards (bacteriohopanetetrol for non-amino- and 35-aminobacteriohopanetriol for amino-containing BHPs).

Lipids were named by their number of carbon atoms (e.g., 16) and the number of unsaturations (e.g., two unsaturations: 16:2). Positions of methyl branches were abbreviated with *i*-, *ai*-, *m*- and *x*-for iso-, anteiso-, mid chain and unknown position, respectively. The exact position of methyl branches of mid chain methylated compounds is counted starting at the functional group, e.g., 12-*m*-17:0. If not stated in the text the substituents are given as OH and CA for alcohol group and carboxylic acid, respectively.

4.2.5. Compound specific stable isotope analyses

The stable carbon isotope compositions are given in the delta notation $\delta^{13}C$ versus the Vienna Pee Dee Belemnite (V-PDB). The coupled GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was conducted using a Finnigan DeltaPlus XL mass spectrometer coupled to a HP 6890 GC via a CuO/Ni/Pt combustion furnace (GC-combustion III) operated at 940ºC. Samples were applied using splitless injector. The GC temperature programme and column were the same as for the GC-MS (see

above). The precision was checked daily using a standard alkane mix (C_{15} to C_{29}) with a known isotopic composition. Based on these measurements, the analytical precision was determined to be better than 0.6‰ (three replicates). To be able to calculate the $\delta^{13}C$-values of the underivatized compounds, the $\delta^{13}C$-values of the carbon atoms introduced into the molecule by the derivatisation reagent were determined and the $\delta^{13}C$-values of the derivatized compounds was corrected using a formula by Goñi and Eglinton (1996).

4.2.6. Determination of the microbial activities

As an indicator for the overall microbial activity in the soil, CO_2-production rates were determined *in vitro*, both with and without oxygen (gross mineralisation). More specifically, the aerobic CH_4-oxidation, the anaerobic CH_4-production and the sulphate reduction were measured.

These experiments were carried out in glass tubes (20 ml) sealed with butyl-rubber stoppers and screw caps. The soil samples were mixed 1:1 with an artificial mineral medium after Widdel and Bak (1992) to obtain homogenous slurries. Subsequently, 9 ml of medium were added to 3 ml of soil slurry. All manipulations were performed under an atmosphere of nitrogen in a glove box. The headspace of the incubation tubes consisted either of CH_4 (100%) or N_2/CO_2 (90/10; v:v) in the control and in the CH_4-production experiments. The potential aerobic CH_4-oxidation rates in the soil samples were determined *in vitro* as described in Krüger *et al.* (2002). The slurries were prepared under oxic conditions as noted above. Aliquots (20 ml) were transferred into sterile glass bottles (175 ml) and supplemented with 5% CH_4. CH_4-depletion was quantified by sampling the headspace after the thorough shaking of the bottles for subsequent GC-FID analysis.

To check for nitrate/nitrite-dependent AOM, incubations were carried out under helium atmosphere with ^{15}N-nitrate (5 mM) or ^{15}N-nitrite (0.1 mM) and $^{13}CH_4$ and checked for the production of $^{15}N_2$ and $^{13}CO_2$ by GC-IRMS. Another experiment was conducted to look for sulphate-dependent ammonium oxidation. Sediment slurries were incubated with $^{15}NH_4$ (5 mM) in the presence and absence of sulphate (10 mM) and again the formation of $^{15}N_2$ was followed.

For all experiments, triplicate tubes were incubated horizontally at 20ºC and gently shaken once per day to ensure an even distribution of CH_4 and sulphate within the

medium. The rates were calculated per gram of dry weight (g_{dw}). The dry weight was determined after drying at 80ºC for 48 h.

The sulphide content was determined using the formation of copper sulphide (Cord-Ruwisch, 1985). CH_4 and CO_2 were determined using a GC 14B (Shimadzu, Japan) additionally equipped with a methaniser to quantify the CO_2, as described in Nauhaus *et al.* (2002).

4.2.7. Quantification of microbial populations

For the Q-PCR, high-molecular-weight DNA was extracted from 450 mg of the frozen soil samples. Cell lysis was performed with 10% sodium dodecyl sulphate (SDS) and horizontal shaking for 45 s after the addition of zirconium-silica beads. The DNA was purified using NH_4-acetate and isopropanol precipitations as described in detail by Henckel *et al.* (1999). To determine the 16S rRNA copy numbers of Archaea (Takai *et al.*, 2000) and Bacteria (Nadkarni *et al.*, 2002), Q-PCR (ABI Prism 7000, Applied Biosystems, USA) was used. The eukaryotic 18S rDNA was quantified using a commercially available Q-PCR assay from Applied Biosystems (TaqMan® ribosomal RNA control reagents, VIC™ probe). Iron reducing Bacteria (*Geobacteraceae*), SRB and methanogenic Archaea were quantified after Holmes *et al.* (2005), Schippers and Neretin (2006) or Wilms *et al.* (2006) using either the 16S rRNA (*Geobacter*) or the functional genes in cases of SRB and methanogens. The detection limit for the Q-PCR analyses was at 10^3 DNA copies per g of sample or lower, depending on the specific assay. The purified PCR products obtained from the DNA of the pure culture cell extracts were used as DNA standards for the Q-PCR assays. The amplification efficiency of the Q-PCR reactions was between 85 and 100% depending on the particular Q-PCR assay, with average standard deviations between 5 and 15%.

4.3. Results and discussion

In this study methanotrophic microorganisms and the impact of elevated CO_2 concentrations on the soil microbial communities at a natural CO_2 vent in Italy were studied.

4.3.1. Changes in the soil chemistry and structure

4.3.1.1. Soil characteristics

Changes in abiotic parameters like pH, nutrient concentrations, carbon sources and available electron donors and acceptors control the composition of microbial communities. Similarly as previously published, but with higher resolution we detected a set of CO_2-induced changes in the soil chemistry and structure within the soil profile of a CO_2 vent compared to the reference soil.

The soil at the reference site developed on weathered and relocated unconsolidated sediments of previously volcanic origin. It showed a strong accumulation of organic matter at the soil surface but no further pedogenetic changes in the soil profile (Fig. 4.2). The water table during sampling (June 2009) was 58 cm below the soil surface and no redoximorphic features could be detected. According to the US soil taxonomy (USDA, 2010) the soil was classified as Oxyaquic Xerorthent. At the CO_2 vent site, the parent material of the reference site was present only in the deeper soil horizon (Cg-horizon, Fig. 4.2). The latter material was overlain by lacustrine sediments alternating with organic soil material of Holocene age. These organic rich layers are the major not CO_2-induced difference between the two sampling sites; they might offer additional carbon sources for heterotrophic organisms fostering heterotrophic growth. Especially at depths between 12 and 20 cm several layers of organic soil material were present (C_{org} 6.6%) indicating soil development under water saturated, probably anaerobic conditions, during a wetland phase. This phase was followed by terrestrial soil formation with an accumulation of organic carbon in the Ah-horizon.

The water table at the CO_2 vent site was at a depth of 48 cm and redoximorphic features could be found below 20 cm. According to the US soil taxonomy the soil was classified as Aquic Xerorthent (USDA, 2010). Therefore, the differences in classification are a direct consequence of oxygen displacement by CO_2. Below a depth of 20 cm the soil at the CO_2 vent is permanently anoxic as indicated by the redoximorphic features. Soil pH differed considerably between the two sites being moderately acidic at the reference site (pH 6.3-5.7) and extremely acidic at the CO_2

vent site (pH 3.7-4.1). The electrical conductivity (Fig. 4.2) and plant-available K^+- and PO_4^{3-}-concentrations (Appendix, Table 4.4) were considerably higher at the CO_2 vent site compared to the reference site. Both sites were used as a sheep pasture but the vent site was characterised by the absence of vegetation and abundant sheep excrements.

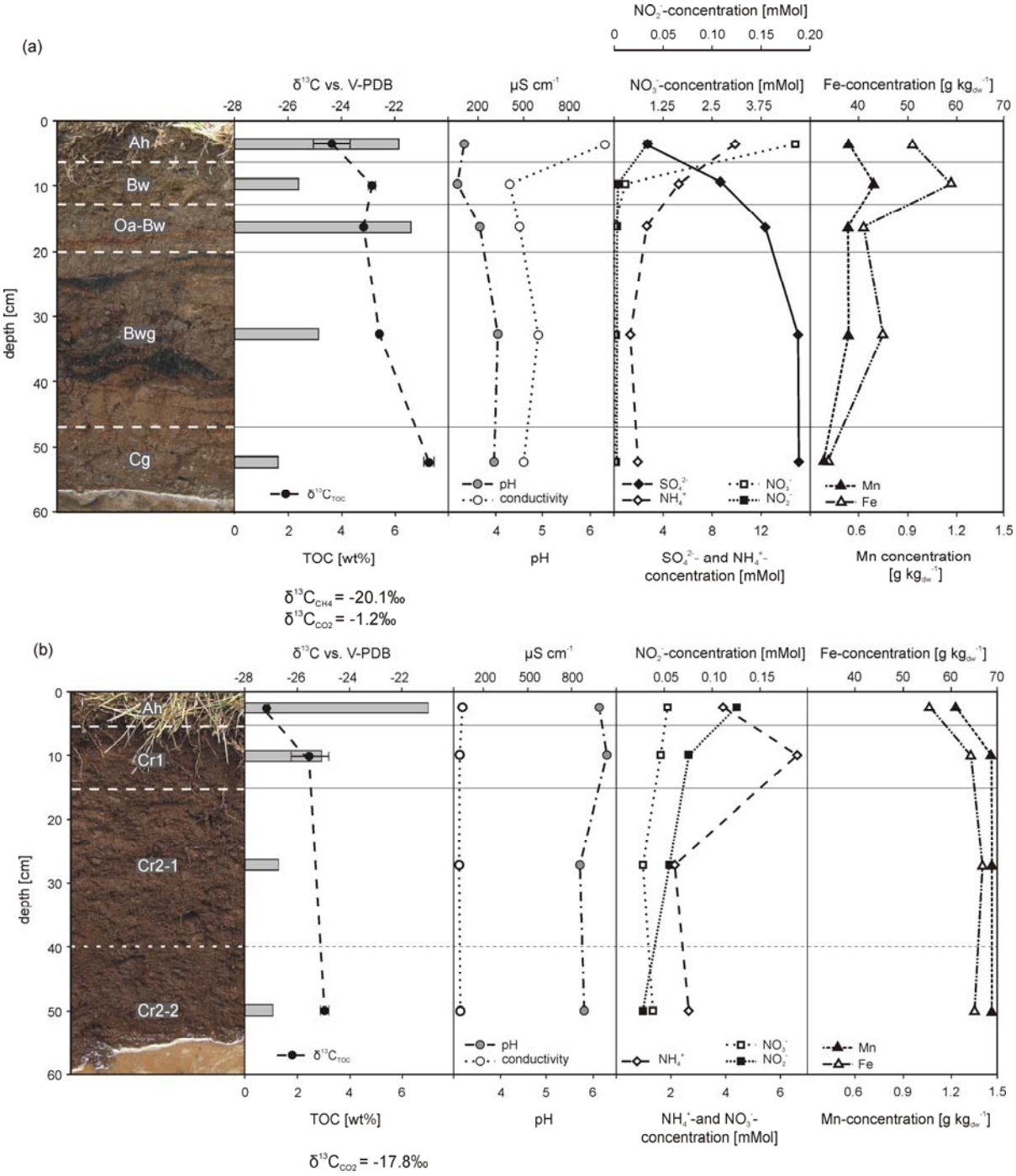

Fig. 4.2. Soil profiles from the CO_2 vent site (a) and the reference site (b). Pictures of the studied soil profiles, abbreviations of soil types, the concentrations of TOC (grey bars), the $\delta^{13}C$-value of TOC, pH-values, conductivity, sulphate-, ammonium-, nitrite-, nitrate-, manganese- and iron-concentrations in the studied soil horizons. Consider different scales of the x-axis.

At the CO_2 vent, the $\delta^{13}C$-values of the soil TOC became gradually enriched in ^{13}C with increasing soil depth (-24.4‰ at 0-7 cm and -20.7‰ below 48 cm). Also the $\delta^{13}C$-values of CO_2 in the soil gas samples (-1.2‰) from the CO_2 vent showed enrichment of ^{13}C, relative to the $\delta^{13}C$-value detected at the reference site (-17.8‰). Although no CO_2-induced alteration of the TOC concentration was observed the $\delta^{13}C$-values showed incorporation of volcanic CO_2 into organic material in the soil at the CO_2 vent, corresponding to the findings of Oppermann *et al.* (2010). Even though, the $\delta^{13}C$-values of the CO_2 at the CO_2 vent are depleted in ^{13}C relative to the earlier study. Oppermann *et al.* (2010) showed variations of the $\delta^{13}C_{CO2}$ between +1.8 and +3.3‰ over depth. Possibly, the normal range of fluctuation over the soil profile is higher than previously thought. CH_4 in the soil gas sample from the CO_2 vent had a $\delta^{13}C$-value of -20.1‰ (Fig. 4.2). According to (Whiticar, 1999) such $\delta^{13}C$-values indicate a geothermal source as already assumed by Chiodini *et al.* (2007). Oppermann *et al.* (2010) showed that the CH_4 concentrations were lower at 20 cm (2.7 ppm) than at 80 cm (1014.9 ppm) soil depth at the CO_2 vent. If CH_4 derives from a hydrothermal source it migrates upward in the soil and is, as the concentrations show, almost completely consumed before it enters the oxic environments (Ah-horizon/atmosphere).

At the CO_2 vent site, high sulphate concentrations were present. They increased from 2.7 mM in the surface Ah-horizon to a maximum of 15.1 mM in the Cg-horizon (Fig. 4.2). The declining sulphate concentrations in the upper soil horizons are indicating sulphate to derive either from the lower soil horizons at the CO_2 vent or from areas below the studied profile. Although the same parent material was detected in the lower horizon at the reference site no sulphate was contained there, hinting to a sulphate source below the studied soil horizon. According to Duchi *et al.* (1992) the sulphate originates from the same Mesozoic carbonates as the CO_2 and rises up together with the CO_2 to the near surface environment.

In contrast, highest ammonium concentrations were measured at the soil surface (9.9 mM) decreasing to 2.0 mM at the bottom of the profile. Despite high ammonium concentrations only relatively low nitrate concentrations (>0.9 mM) and low nitrite concentrations (<0.2 mM) were present. Concentrations of dissolved ammonium and nitrate in soil pore water of the reference site were relatively high with maximum values of 6.6 mM and 4.4 mM respectively (Fig. 4.2). Nitrite concentrations were low throughout the profile (≤0.05 mM) and sulphate concentrations were below the

detection limit (<1 mM). The high NH_4^+ concentration in the top layer at the CO_2 vent most probably results from the higher input of sheep faeces at the vent area and the low oxidation rates in the strongly O_2-depleted vent. Nevertheless, the concentrations of NO_2^- and NO_3^- in the Ah-horizon at the CO_2 vent show some influence of atmospheric O_2 within the uppermost centimetres of the soil, as NH_4^+ is very stable under strict anaerobic conditions and low pH-values. The decrease of NH_4^+ concentrations below the Ah-horizon could be caused by lateral transport in the groundwater flow.

Fe- and Mn-concentrations were lower in the soil at the CO_2 vent. Especially, in the lower part of the soil profile of the CO_2 vent the Fe- and Mn-concentrations decreased compared to the reference site (e.g., Fe: 33.29 vs. 65.11 g kg_{dw}^{-1}, CO_2 vent vs. reference soil, respectively). The relatively low concentrations of Mn and Fe are most likely caused by enhanced mobilisation, conversion to reduced Mn and Fe species and subsequent transport out of the CO_2 vent. This mobilisation can be either a consequence of abiotic processes, like lower pH values and reductive conditions, or of biogenic reduction of metals.

Soil analyses and field observation did not reveal any significant differences between the two layers (Cr2-1 and Cr2-2) within the Cr2 horizon of the reference site; hence for the microbial and biomarker studies only material from the lower part of this horizon was used.

4.3.2. Microbial CH_4-cycling at the CO_2 vent

Rate measurements of microbial processes showed a significant increase of methanogenic and methanotrophic activities at the CO_2 vent compared to the reference site (Table 4.1 and Fig. 4.3). Interestingly this included particularly the potential of aerobic oxidation of CH_4, exceeding the microbial CH_4-production by a factor of 1000.

Table 4.1. Microbial activities detected in selected soil horizons. Consider different scales of units

	autotrophic methanogenesis [nmol g_{dw}^{-1} d^{-1}]	heterotrophic methanogenesis [nmol g_{dw}^{-1} d^{-1}]	aerobic CH_4-oxidation [µmol g_{dw}^{-1} d^{-1}]	sulphate reduction [µmol g_{dw}^{-1} d^{-1}]	anaerobic CO_2-production [µmol g_{dw}^{-1} d^{-1}]
CO_2 vent site					
7-12 cm	1.53 ± 0.7	n. d.	1.26 ± 0.2	0.43 ± 0.1	11.9 ± 0.4
48-60 cm	3.34 ± 0.4	0.31 ± 0.6	1.19 ± 0.2	0.69 ± 0.1	14.89 ± 0.3
Reference site					
5-15 cm	0.45 ± 0.2	0.49 ± 0.2	0.27 ± 0.2	n. d.	3.05 ± 1.0
40-60 cm	0.26 ± 0.2	0.43 ± 0.2	0.33 ± 0.1	0.04 ± 0.1	4.5 ± 0.2

Also compared to the microbial activities reported by Oppermann *et al.* (2010) the aerobic CH_4-oxidation in the studied samples is strongly increased (by a factor of 4). These differences can be explained with spatio-temporal heterogeneity; especially gas flux and gas composition in volcanic systems are known to change over time. However, *in vitro* studies revealed capabilities for the aerobic oxidation of CH_4 in both sample sets (from 2006 and 2009) at the CO_2 vent. This indicates a high potential of aerobic CH_4 turnover for microbial communities in the respective soils although they are permanently oxygen-deficient below 20 cm as demonstrated by the redoximorphic features (see also 4.3.1). Anaerobic oxidation of CH_4 connected to denitrification was detected. Denitrification rates detected during incubation of $^{15}NO_3^-$ and $^{13}CH_4$ where significantly higher than CH_4 oxidation. Ettwig *et al.* (2010) suggested a ratio of 2:1 in the nitrate coupled AOM, but even if this ratio is applied, denitrification rates are more than five times higher than CH_4 oxidation. Apparently additional, no CH_4 coupled denitrification takes place in the studied samples, probably consuming TOC derived carbon. Therefore, the $\delta^{13}C$ values of CO_2 present a mixture between CH_4 and TOC derived CO_2.

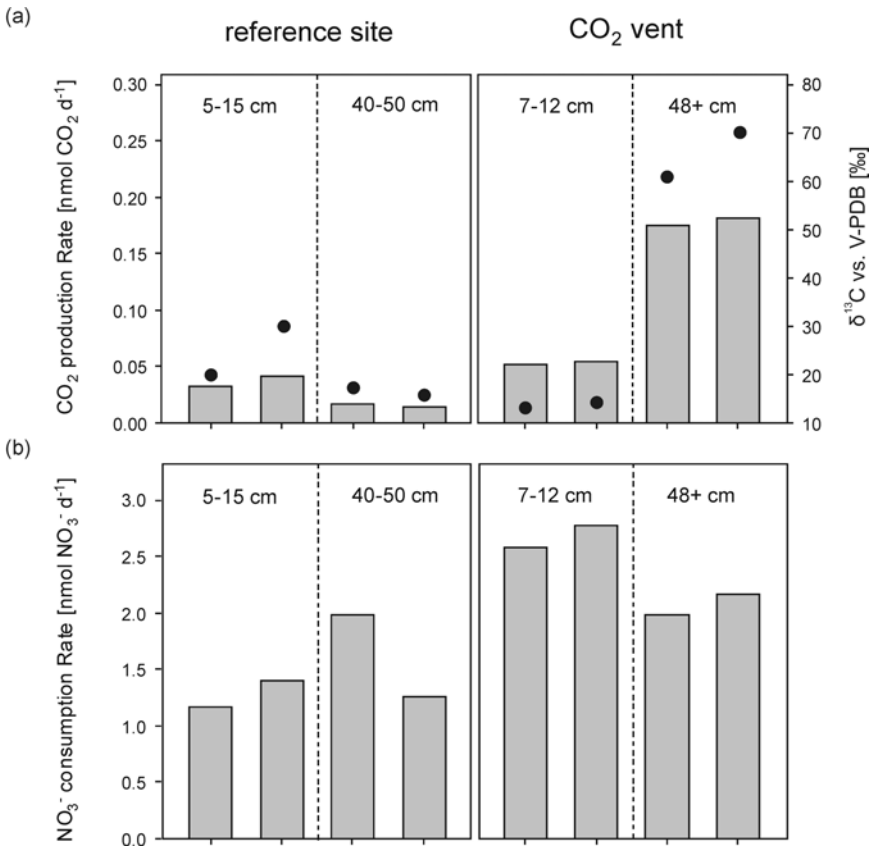

Fig. 4.3. Microbial activities of selected soil horizons, detected in a growth experiment with ^{13}C labelled CH_4 and ^{15}N labelled NO_3^- as only added electronen donor and acceptor, respectively. (a) Anaerobic $^{13}CO_2$ production (bars) and $\delta^{13}C$ values of CO_2. (b) $^{15}NO_3^-$ consumption, calculated from the $^{15}N_2$ production.

The CO_2-production rates under anaerobic conditions, as indicators of gross microbial activity, were substantially higher at the CO_2 vent than in reference site samples (Table 4.1). The opposite trend was observed for the aerobic CO_2-

production rates. This again confirms a stronger adaptation of the microbial populations in the vent to the permanently anoxic conditions.

4.3.2.1 General shifts in microbial communities

First indications for differences in the composition of the microbial community are already found in the gene copy numbers detected in the soils from the CO_2 vent and the reference site (Table 4.2). In the lower horizon of the CO_2 vent soil higher numbers of Archaea (2435.5 x 10^4 DNA copies g_{dw}^{-1}) compared to numbers of Bacteria (1474.2 x 10^4 DNA copies g_{dw}^{-1}) were detected, while the opposite was true for the reference site soil (61.7 x 10^4 DNA copies g_{dw}^{-1} vs. 1421.8 x 10^4 DNA copies g_{dw}^{-1}), respectively. These finding are in accordance with those from Oppermann *et al.* (2010). Interestingly, gene copies of SRB at the CO_2 vent were relative stable throughout the soil profiles (23.2 x 10^4 DNA copies g_{dw}^{-1} at 0-7 cm and 20.00 x 10^4 DNA copies g_{dw}^{-1} at 48-60 cm) and higher compared to the reference site (by a factor of 4 to 10). Whereas, gene copy numbers of *Geobacter* were lower at CO_2 vent (factor of 2) and decreased in the upper soil horizons.

Table 4.2. Community composition determined by Q-PCR in selected soil horizons from the CO_2 vent soil and the reference soil

	Bacteria [DNA copies x 10^4 g_{dw}^{-1}]	Archaea [DNA copies x 10^4 g_{dw}^{-1}]	SRB [DNA copies x 10^4 g_{dw}^{-1}]	Geobacter [DNA copies x 10^4 g_{dw}^{-1}]
CO_2 vent site				
0-7 cm	2609.19	1.11	23.16	0.57
7-12 cm	527.45	1.64	3.15	2.95
12-20 cm	908.01	1205.58	19.40	19.55
48-60 cm	1474.15	2435.49	20.00	66.07
Reference site				
0-7 cm	54.55	11.02	0.29	n.d.
40-60 cm	1421.75	61.66	5.44	140.40

Carboxylic acids and their $\delta^{13}C$-values (Fig. 4.4 and Table 4.3) reveal further details about the CO_2-induced community shift. At the CO_2 vent, concentrations of carboxylic acids were highest in the top layer (5000 µg g^{-1} TOC), minimum concentrations were detected between 7 and 20 cm (1740 and 1010 µg g^{-1} TOC). Below 20 cm the concentration was only half of that in the top layer (1990 µg g^{-1} TOC). At the reference site, the highest concentration was found between 5 and 15 cm (3920 µg g^{-1} TOC).

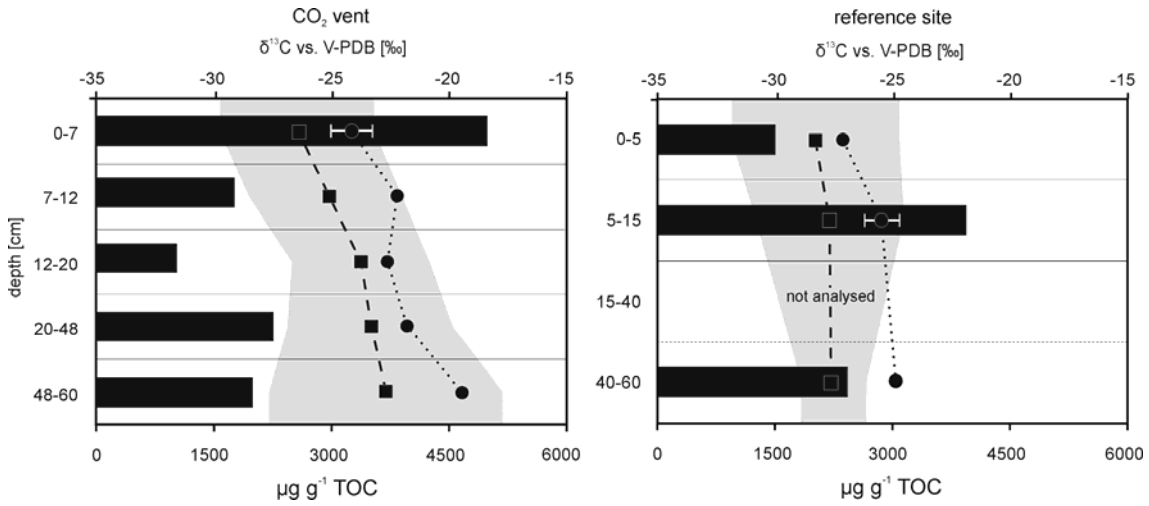

Fig. 4.4. Concentrations of C_{12} to C_{19} carboxylic acids (black bars) their mean of $\delta^{13}C$ values (black squares). The grey area indicates the range of $\delta^{13}C$ values of C_{12} to C_{19} carboxylic acids. Closed circles give the $\delta^{13}C$-values of soil TOC from the studied soil.

To trace uptake of CH_4 by microorganisms the isotopic values of lipids can be used. Heterotrophic biosyntheses display relatively low $\Delta\delta^{13}C_{substrate\ to\ lipid}$ values, while autotrophic biosyntheses (CO_2 or CH_4) yield higher fractionation factors. In both cases, carboxylic acids are depleted in ^{13}C relative to the total biomass of an organism (Hayes, 2001). The $\delta^{13}C$-values of lipids from heterotrophic assimilation therefore mimic the $\delta^{13}C$ values of soil TOC. Since the organic material in the Ah-horizon is usually relatively pristine it offers a wider set of carbon sources for heterotrophic organisms, e.g., sugars and amino acids. Also the Ah-horizon is in contact with the atmosphere and offers access to atmospheric CO_2 with a $\delta^{13}C$-value of -6‰ to -10‰. Since the $\delta^{13}C$-values of available carbon sources for the autotrophic or heterotrophic formation of microbial biomass should be wider in the Ah-horizon than in the lower soil horizons, the range of $\delta^{13}C$-values of carboxylic acids should decrease. This was observed in the soil at the reference site. At the CO_2 vent, the $\delta^{13}C$-values of the carboxylic acids disperse, especially in the lower horizon. This indicates additional carbon species (like CO_2 and CH_4) and consequently also additional fixation pathways are being used for biosyntheses in this anaerobic environment.

Table 4.3. Concentrations and $\delta^{13}C$-values of selected lipid biomarkers

CO_2 vent site

	0-7 cm		7-12 cm		12-20 cm		20-48 cm		48-60 cm	
	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]
MAGE										
i-15:0	8.2		0.3		1.2	-14.2	1.0	-17.5	7.6	-12.8
ai-15:0	5.4	-29.3	0.3				0.2		2.5	-19.4
15:0	2.9	-25.1	0.1				2.5	-16.2	3.6	-16.2
16:0	5.0	-26.6	1.6	-33.4	2.3	-27.2	3.1	-22.9	17.3	-19.9
i-17:0	1.5		0.2		0.7		1.4		8.0	-16.1
ai-17:0	2.6	-19.0	0.2		0.5	-19.3	1.4	-26.0	13.6	-18.5
DAGE										
i-15:0/*i*-15:0			1.6	-24.9			3.4	-19.5	18.2	-19.9
i-16:0/*i*-16:0							5.7	-16.4	32.6	-7.1

Reference site

	0-5 cm		5-15 cm		40-60 cm	
	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]
MAGE						
i-15:0	3.0		1.2		0.6	
ai-15:0	1.4		0.6	-33.8		
15:0	0.7		0.3	-35.9		
16:0	6.2	-28.0	1.9	-29.7	0.7	
i-17:0	1.3		1.0		2.0	
ai-17:0	1.2		0.1		0.9	
DAGE						
i-15:0/*i*-15:0			32.1	-28.3	3.5	
i-16:0/*i*-16:0						

* versus V-PDB

Table 4.3. (continued). Concentrations and $\delta^{13}C$-values of selected lipid biomarker

CO_2 vent site

	0-7 cm		7-12 cm		12-20 cm		20-48 cm		48-60 cm	
	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]
Carboxylic acids										
i-14:0	37.5	-32.6	11.9	-24.6	7.3	-22.9	42.3	-32.3	56.8	-34.4
14:0	172.5	-31.0	44.0	-27.2	34.8	-29.4	64.3	-25.8	56.2	-25.4
i-15:0	280.0	-25.1	98.4	-22.9	51.6	-21.7	142.6	-20.6	117.4	-18.2
ai-15:0	288.7	-25.2	94.2	-24.3	27.0	-22.1	145.6	-27.7	180.3	-30.6
15:0	82.2	-31.1	21.6	-26.6	17.5	-25.3	26.9	-25.2	18.1	-22.6
i-16:0	206.4	-25.2	117.3	-23.6	75.2	-22.7	116.3	-23.6	111.2	-22.9
16:1Δ8	16.4		4.7						3.5	
16:1Δ9	157.4	-27.3	23.6	-24.4	8.1	-25.8	46.1	-22.9	47.8	-27.1
2-OH-*i*-15:0	4.4		0.1		0.6		1.0		0.6	
16:1Δ10			9.9		6.4	-23.6	13.5		21.6	-29.4
16:1Δ11	18.8		6.2		3.8	-19.4	5.7		4.7	
16:0	1073.7	-27.2	361.7	-26.7	226.4	-27.3	396.8	-25.2	252.7	-25.2
3-OH-16:0	18.3		20.7		12.4		55.1		90.1	
m-17:0	40.1		2.5							
m-17:0	137.9	-23.3	112.8	-23.9	81.2	-23.4	175.1	-21.4	186.4	-19.8
m-17:0	33.7		18.3		13.1	-18.7	40.2	-21.1	13.5	-19.0
i-17:0	191.4	-24.6	77.2	-23.7	29.7	-24.3	81.1	-20.5	55.2	-20.4
ai-17:0	128.7	-24.5	43.9	-22.8	15.0	-24.0	45.3	-21.1	37.3	-20.0
cycl-17	61.9	-23.7	30.3	-24.4	15.1	-25.9	27.6	-23.9	27.6	-26.5
17:0	55.9		11.6		9.2		10.2		11.5	
m-18:0	156.9	-26.1	82.4	-25.4	26.9	-23.6	52.4	-22.7	53.6	-20.6
m-18:0	10.9		5.2		21.2	-20.1	37.8	-20.2	10.1	-18.5
m-18:0	8.7		9,6				8.8		57.2	-24.4
i-18:0	37.9	-22.6	8.8	-22.1					20.8	
ai-18:0	23.6	-23.0	6.8		6.2		10.3	-22.7		
18:1Δ9	548.3	-25.0	66.7	-24.5						
18:1Δ10			23.0	-23.0	39.4	-22.8	138.3	-25.0	157.6	-24.4
18:1Δ11	126.7	-25.4	15.5	-22.2	17.1	-21.6	159.0	-17.1	162.0	-18.6
18:1Δ13	23.4				3.9		25.4		46.7	
18:0	643.8	-30.6	142.3	-26.8	100.3	-26.3	130.0	-21.6	106.9	-20.8
3-OH-17:0	15.2				6.5		0.4		0.1	

* versus V-PDB

Table 4.3. (continued) Concentrations and $\delta^{13}C$-values of selected lipid biomarker

Reference site

	0-5 cm		5-15 cm		40-60 cm	
	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]	µg g^{-1} TOC	$\delta^{13}C$* [‰]
Carboxylic acids						
i-14:0	4.4	-26.0	16.8	-27.6	17.8	-26.3
14:0	22.0	-33.3	61.3	-31.3	50.6	-29.5
i-15:0	42.5	-26.0	127.0	-27.0	113.4	-26.4
ai-15:0	33.7	-26.7	111.4	-24.8	111.9	-24.8
15:0	10.4	-25.8	27.0	-29.0	20.6	-27.9
i-16:0	56.8	-28.5	119.4	-28.4	77.7	-27.2
16:1Δ8	2.4		19.3		13.6	-29.2
16:1Δ9	115.6	-30.8	387.9	-30.1	169.4	-28.3
2-OH-*i*-15:0	0.1		17.8		1.7	
16:1Δ10						
16:1Δ11	178.2	-31.3	304.4	-28.8	170.1	
16:0	383.7	-31.0	963.2	-28.3	547.1	-28.4
3-OH-16:0			9.4		13.9	
m-17:0	10.6	-21.8	26.8	-26.8	21.2	-26.7
m-17:0	22.9	-30.4	82.6	-26.7	121.2	-27.2
m-17:0	5.9		11.8			
i-17:0	23.0	-26.2	46.9	-24.5	41.3	-26.6
ai-17:0	10.3	-26.1	49.2	-22.8	53.6	-26.1
cycl-17			39.3	-24.1	17.7	-26.7
17:0	12.7		14.8		9.1	
m-18:0	7.1		29.7	-28.8	43.3	-28.6
m-18:0			27.4	-27.4		
m-18:0						
i-18:0	11.7	-29.6			13.4	-27.4
ai-18:0						
18:1Δ9	263.4	-29.7	538.1	-26.1	273.3	-28.6
18:1Δ10						
18:1Δ11	92.0	-26.3	193.3	-26.4	119.5	-26.7
18:1Δ13	4.5	-21.3	11.6		9.4	-24.8
18:0	99.0	-31.0	185.6	-27.5	109.7	-27.9
3-OH-17:0	2.5		1.1		0.3	

* versus V-PDB

4.3.2.2 Sulphate dependent anaerobic CH_4-oxidation

At the CO_2 vent, high sulphate concentrations and sulphate reduction rates were measured in the lower soil horizons (e.g., below 48 cm: 15.0 mM and 0.69 µmol g_{dw}^{-1} d^{-1}, respectively; Fig. 4.2 and Table 4.1), leading to a decline in sulphate concentrations (e.g., 0-7 cm: 2.7 mM; Fig. 4.2). SRB are a very divers group, capable of using a wide set of carbon sources. But at the CO_2 vent, mainly CO_2-fixating SRB are present. This was already shown by Oppermann *et al.* (2010) for the lower soil horizon with the help of $\delta^{13}C$-values of SRB-specific biomarkers (e.g., MAGE *ai*-15:0, DAGE *ai*-15:0/ *ai*-15:0 as well as CA 16Δ11, *i*- and *ai*-15:0 and 17:0). Indications for SRB involved in AOM were not observed. The known $\Delta\delta^{13}C_{substrate\ to\ lipid}$ values for SRB associated to the sulphate-dependent AOM can be relative high (up to -34‰; Niemann & Elvert, 2008 and reference therein) compared to e.g., heterotrophic $\Delta\delta^{13}C_{substrate\ to\ lipid}$. At the CO_2 vent, CH_4 has a $\delta^{13}C$-value of -20.1‰ but lipid biomarkers of SRB did not show depletion of ^{13}C in the range expected for SRB involved in AOM. In this study, these findings were confirmed also for the upper soil horizons, either by the absence of specific biomarkers (e.g., DAGE *ai*-15:0/ *ai*-15:0) or by their $\delta^{13}C$-values (e.g., 12-20 cm: CA 16Δ11 -19.4‰) (Table 4.3).

The lack of hydroxyarchaeol and the stable isotope signatures of archaeal tetraether, derived biphytanes, confirm absence or minor relevance of sulphate-dependent AOM. The $\delta^{13}C$-values of biphytanes indicate that they originate from methanogenic and other, but not from methanotrophic archaea (Oppermann *et al.,* 2010).

4.3.2.3 Other anaerobic pathways of CH_4-oxidation

Until recently, methanotrophic Bacteria were believed to require oxygen for CH_4-oxidation. But Raghoebarsing *et al.* (2006) were able to cultivate an enrichment culture capable of oxidising CH_4 anaerobically by connecting it to denitrification. After the first implication for Bacteria as the responsible CH_4-oxidiser in this process (Ettwig *et al.*, 2008), Ettwig *et al.* (2010) isolated Bacteria, '*Candidatus* Methylomirabilis oxyfera', able to conduct denitrification utilising the liberated oxygen for CH_4-oxidation.

Methanotrophic Bacteria are phylogenetically divided into groups after the respective pathway used for CH_4-oxidation: type I (ribulose monophosphate pathway (RuMP)-pathway), type II (serine-pathway) and type X (RuMP- and serine-pathway) (Hanson & Hanson, 1996). According to Ettwig *et al.* (2010) the newly discovered anaerobic

methanotrophic Bacteria form an additional cluster. However, labelling experiments with ^{13}C marked CH_4 revealed common biomarkers of aerobic methanotrophs (CA 14:0, 16:1Δ9, 18:1Δ10, 18:1Δ11 and diplopterol) also incorporated ^{13}C-labelled CH_4 during AOM coupled to denitrification (Raghoebarsing *et al.*, 2006). Apparently, denitrifying anaerobic methanotrophs contain lipid biomarkers known from the aerobic type I and type II methanotrophs. Type I methanotrophs can synthesise 3β-methylbacteriohopanetetrol and 35-aminobacterio-hopanepentol (aminopentol) (Neunlist & Rohmer, 1985a; Zundel & Rohmer, 1985) whereas type II can synthesise 35-aminobacteriohopanetriol (aminotriol) and 35-aminobacteriohopanetetrol (aminotetrol) (Neunlist & Rohmer, 1985b; Talbot *et al.*, 2001).

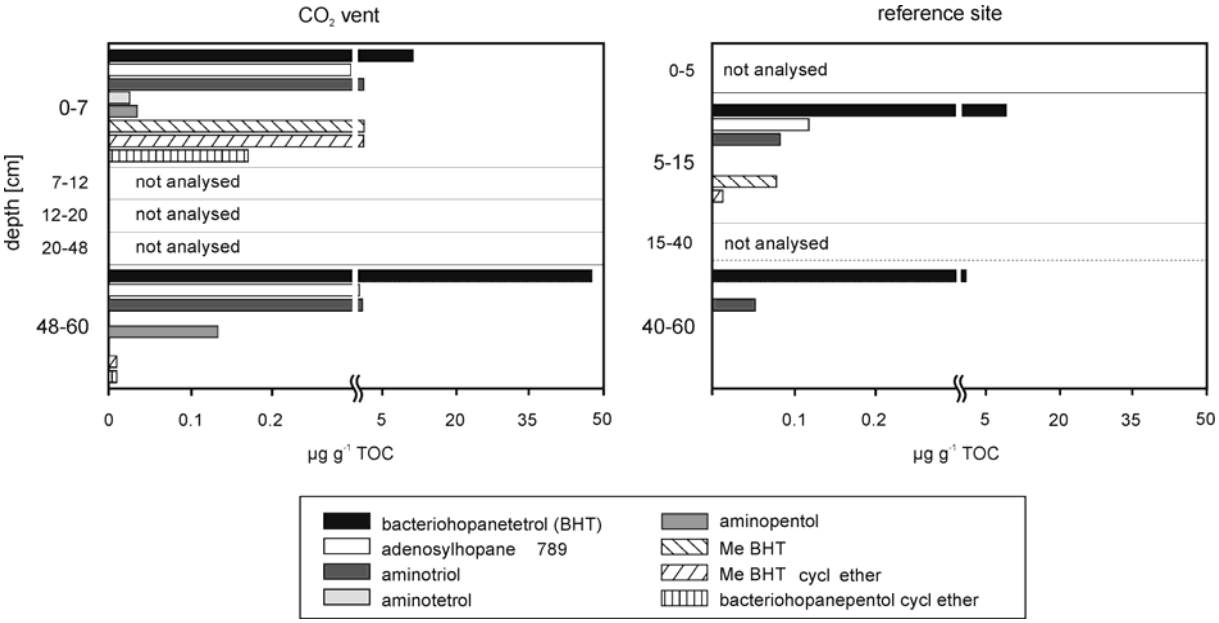

Fig. 4.5. Concentrations of hopanoids extracted from the CO_2 vent site and the reference site.

In soil of the CO_2 vent, higher concentrations of bacteriohopanepolyols (BHPs) compared to the reference site were found (Fig. 4.5). Especially the concentration of aminotriol (1.24-1.06 vs. 0.05-0.08 µg g^{-1} TOC, respectively) were significantly higher at the CO_2 vent, moreover aminotetrol was only detected at the CO_2 vent (0.02 µg g^{-1} TOC, 0-7 cm). The higher concentrations of type II methanotroph-associated hopanoids indicate a higher relevance of type II methanotrophs in the soil at the CO_2 vent. Besides characteristic BHPs methanotrophic Bacteria contain diagnostic carboxylic acids. Type I methanotrophs produce CA 14:0, 16:1Δ8, 16:1Δ9 and 16:1Δ11, while type II methanotrophs contain considerable amounts of CA 18:1Δ10 and 18:1Δ11 (Nichols *et al.*, 1985; Bowman *et al.*, 1991, 1993; Dedysh *et al.*, 2002). The concentrations of type II methanotroph-associated CA 18:1Δ10 were high at the CO_2 vent (up to 157.6 µg g^{-1} TOC) while this carboxylic acid was absent at the

reference site (Fig. 4.6). Another carboxylic acid, 18:1Δ11, also found in type II methanotroph, increased in concentration with soil depth at the CO_2 vent. But the highest concentrations were found at the reference site (5-15 cm: 193.3 µg g^{-1} TOC). The 18:1Δ11 is found in other Bacteria as well (e.g., SRB; Vainshtein *et al.*, 1992) and therefore less specific than the CA 18:1Δ10. Hence, where 18:1Δ10 is missing, the 18:1Δ11 is most likely not type II methanotroph derived.

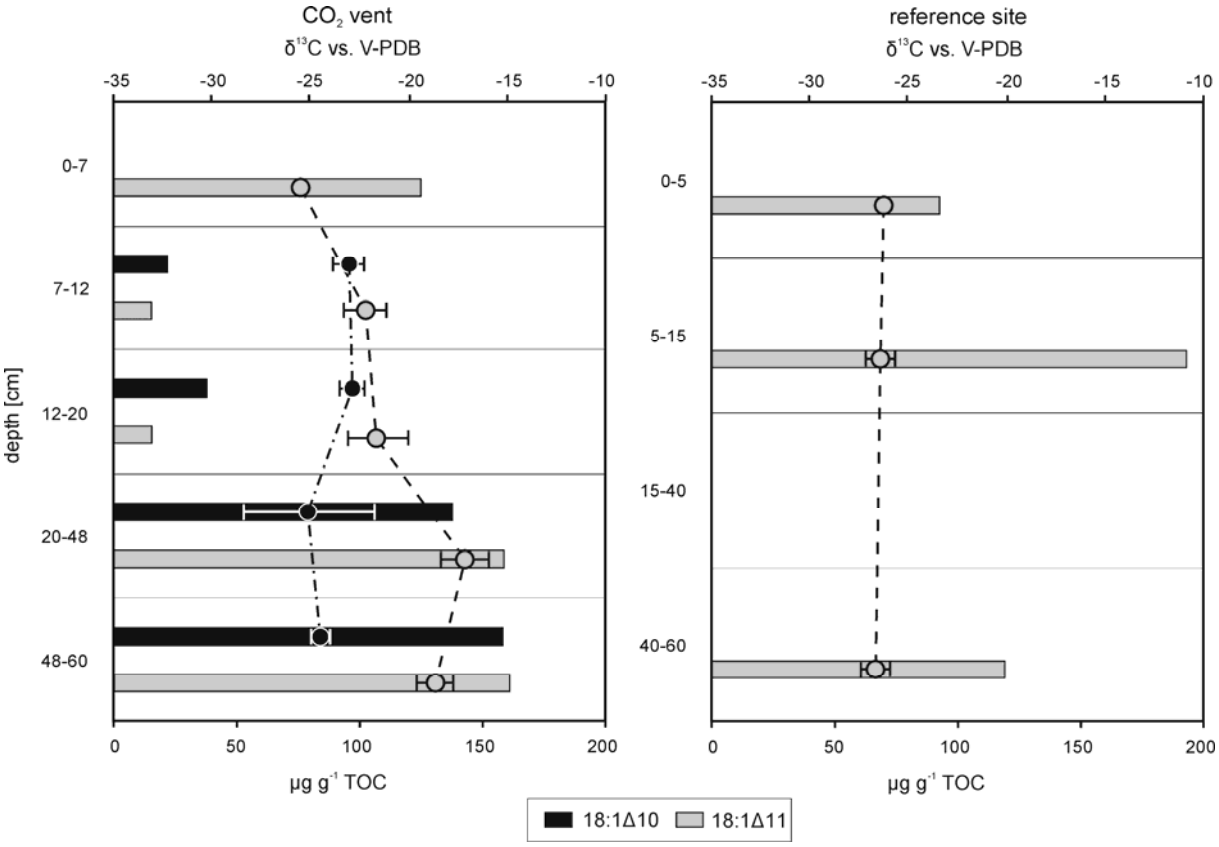

Fig. 4.6. Carboxylic acids assigned to methanotrophic Bacteria (type II), extracted from the CO_2 vent site and the reference site. Concentrations and $\delta^{13}C$-values of the octadec-10-enoic acid (black bars and circles, respectively) of the octadec-11-enoic acid (grey bars and circles, respectively).

The $\delta^{13}C$-values of CA 18:1Δ10 and CA 18:1Δ11 were inconsistent. The CA 18:1Δ10 showed a trend of ^{13}C-depletion with increasing soil depth at the CO_2 vent, hinting to an uptake of CH_4 by its source organisms. Since not only type II methanotroph contributed to the CA 18:1Δ11 pool, its ^{13}C-values do not follow the $\delta^{13}C$-values of CA 18:1Δ10. Therefore biomarker distribution and the $\delta^{13}C$-values of CA 18:1Δ10 suggests active CH_4-consumption by methanotrophs in the lower part of the soil at the CO_2 vent. Enhanced growth of normal aerobic type II methanotrophs is unlikely in the permanently anoxic part of the CO_2 vent. Considering the detection of AOM coupled to nitrate (Fig. 4.3), the newly discovered anaerobic methanotrophs of the '*Candidatus* Methylomirabilis oxyfera' cluster conducting AOM coupled to denitrification are a more likely source for the type II methanotroph-associated

hopanoids and the ^{13}C depleted CA 18:1Δ10. Although the fractionation factor between CH_4 and lipids was not extensively determined, results by Raghoebarsing *et al.* (2006) indicate a lower fractionation compared to sulphate coupled AOM (Niemann & Elvert, 2008 and references therein, see also 4.3.2.2). The results in this study show for the first time implications for the applicability of these lipid biomarkers in natural samples. Hence, findings of type II methanotrophs associated biomarkers should be considered carefully before attribution to aerobic CH_4-oxidation.

Moreover, since these newly discovered Bacteria utilise the liberated oxygen from the denitrification for CH_4-oxidation they might also be capable of aerobic CH_4-oxidation. This might explain the high potential aerobic oxidation rates measured (1.19-1.26 μmol g_{dw}^{-1} d^{-1}, found *in vitro*; Tab. 1) in the permanently oxygen free CO_2 vent. Maybe, if oxygen becomes accessible the step of oxygen production can be bypassed and the oxygen be used directly.

Biomarkers of type I methanotrophs showed a more complex distribution. Aminopentol was absent at the reference site but detected at the CO_2 vent (e.g., 0.03-0.13 μg g^{-1} TOC; Fig. 4.5) with higher concentrations in the lower soil horizon like the type II-derived biomarkers. The 3β-methyl-BHT (1.25 μg g^{-1} TOC at 0-7 cm, not detected below 48 cm; Fig. 4.5) and the CA 16:1Δ8 (16.4 μg g^{-1} TOC at 0-7 cm, 3.5 μg g^{-1} TOC at 48-60 cm; Fig. 4.7), are very diagnostic for type I methanotrophs. But both biomarkers decreased in concentration with soil depth at the CO_2 vent. Also the $\delta^{13}C$-value the CA 14:0 did not indicate an enhanced uptake of ^{13}C in the lower horizon of the CO_2 vent (Fig. 4.7). In summary, strong evidence for type I-related methanotrophic Bacteria in the Ah-horizon were found. As oxygen can enter this horizon these biomarkers most likely derive from aerobic type I methanotrophs. Knoblauch *et al.* (2008) found the methanotrophic community to be most abundant in the top layers of four different permafrost-affected peats, where CH_4 from the underlying anoxic soil and oxygen from the atmosphere meet. Some of the type-I associated biomarkers in the lower soil horizons might also derive from Bacteria conducting denitrification coupled AOM as shown for the previously type II associated lipids.

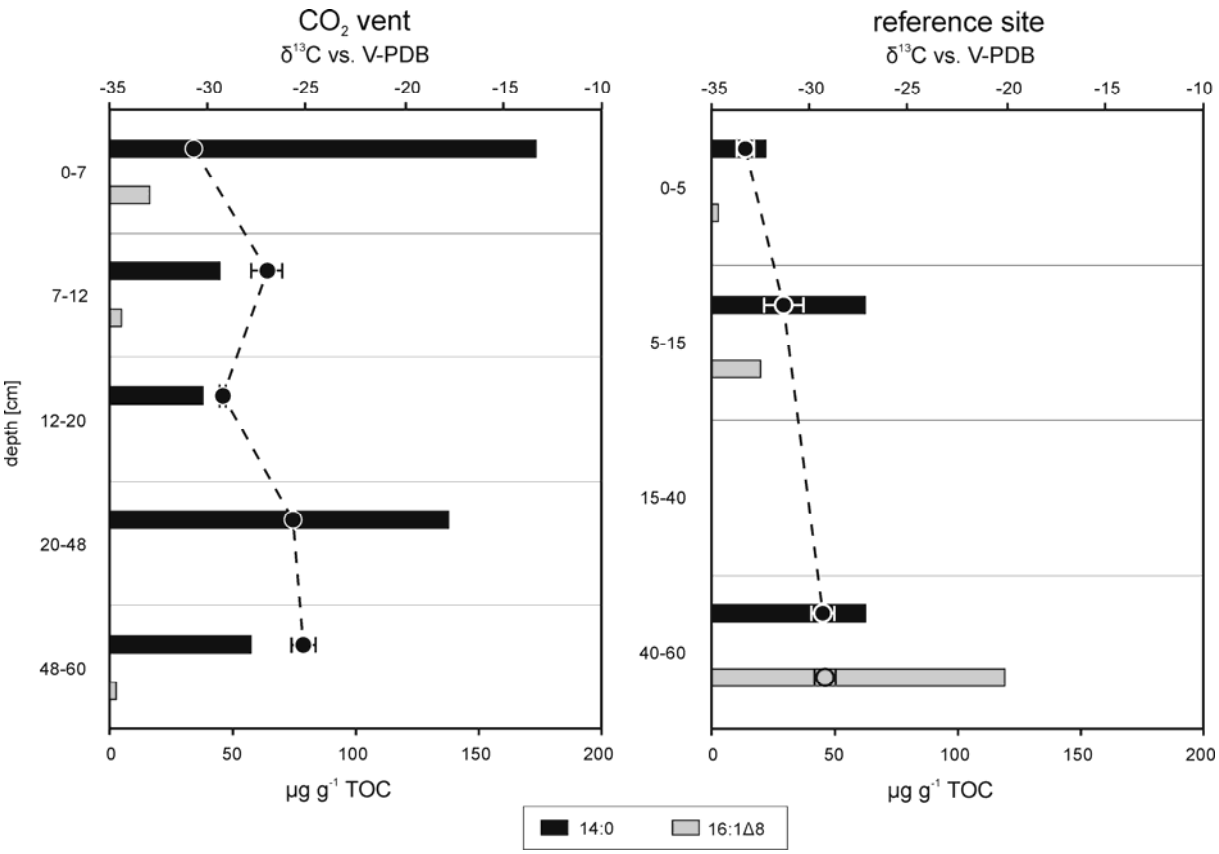

Fig. 4.7. Carboxylic acids, assigned to methanotrophic Bacteria (type I), extracted from the CO_2 vent site and the reference site. Concentrations and $\delta^{13}C$-values of the tetradecanoic acid (black bars and circles, respectively) and of the hexadec-8-enoic acid (grey bars and circles, respectively).

Besides sulphate, nitrate and nitrite also manganese and iron can serve as electron acceptors in AOM (Beal *et al.*, 2009). Interestingly, enzymes of the denitrification are also able to reduce Fe(III) (Ottow, 1970). Compared to the soil at the reference site, the concentrations of these metals are lower at the CO_2 vent (Fig. 4.2, e.g., for manganese 10.07-14.23 g kg_{dw}^{-1} vs. 14.05-18.45 g kg_{dw}^{-1}, at the CO_2 vent vs. the reference soil, respectively). Initially the manganese and iron concentrations should have been similar since the source materials in the lower soil horizons are the same at both sites (see section 3.1.1). Due to the low pH value at the CO_2 vent the metals were reduced, either abiotically or biologically. At low pH values manganese and iron are more bio-available and could have been reduced by manganese and iron reducing Bacteria. As the reduced species of manganese and iron are more soluble and therefore more easily transported they were most likely transported out of the CO_2 vent.

The microorganisms responsible for AOM coupled to manganese and iron are not yet identified. Beal *et al.* (2009) proposed that either ANME-1, maybe together with ANME-3 and a syntrophic bacterial partner or solely Bacteria (similar to the AOM

coupled to denitrification) are responsible for the metal-dependent AOM. But as described above (Section 4.3.2.2) biomarker of Archaea involved in AOM are not present at the CO_2 vent soil. The other possibility suggested by Beal *et al.* (2009) is that solely Bacteria are responsible for this process. Stable isotope fractionation factors of this process have not been reported. Therefore one can only speculate. According to Beal *et al.* (2009) the potential bacteria performing metal-linked AOM might include *Bacteriodes*, Proteobacteria (including *Geobacter*), *Acidobacteria* and/or Verrucomicrobia. Gene copy numbers indicate that *Geobacter* are less prominent at the CO_2 vent than at the reference site, e.g., 60.07 vs. 140.40 x 10^4 DNA copies g_{dw}^{-1} respectively (Table 4.2).

Terminal branched carboxylic acids are wide spread in anaerobic Bacteria. *Acidobacteria* and *Bacteriodes* contain high amounts of CA *i*-15:0 (Mayberry, 1980; Eichorst *et al.*, 2007). This compound is found at the CO_2 vent (51.6-280.0 µg g^{-1} TOC; Fig. 4.8) in slightly higher concentrations than at the reference site (42.5-127.0 µg g^{-1} TOC; Fig. 4.8). The highest concentration of CA *i*-15:0 was detected in the top soil layer at the CO_2 vent. *Bacteriodes* also posses high amounts of CA *ai*-15:0 and 3-hydroxy-17:0; this compound was also detected in the CO_2 vent soils. But the $\delta^{13}C$-values of CA *i*-15:0 (-18.2‰ at 48-60 cm) are hinting to an uptake of volcanic CO_2, rather than CH_4 (Oppermann *et al.,* 2010).

Only few studies have been published studying species of Verrucomicrobia. Therefore our knowledge of Verrucomicrobia concerning their distribution and role in different environments is incomplete. But Verrucomicrobia seem to play an important role in soils as they account, on average, for 5.0% of all genes found by 16S rRNA genes studies (Sangwan *et al.*, 2004 and references therein). Only recently, three independent studies revealed that Verrucomicrobia isolated from hydrothermal fields were capable of oxidizing methane (Dunfield *et al.*, 2007; Pol *et al.*, 2007, Islam *et al.*, 2008). These newly discovered aerobic methanotrophs do not belong to the Proteobacteria, like all previous described methanotrophic bacteria. Interestingly, Verrucomicrobia contain the most acidophilic methanotrophs known yet. No other known methanotrophic Bacteria are able to oxidise CH_4 at a pH below 4. This is of particular interest in the very acidic soil of the CO_2 vent (pH 3.2-4.1; Fig. 4.2).

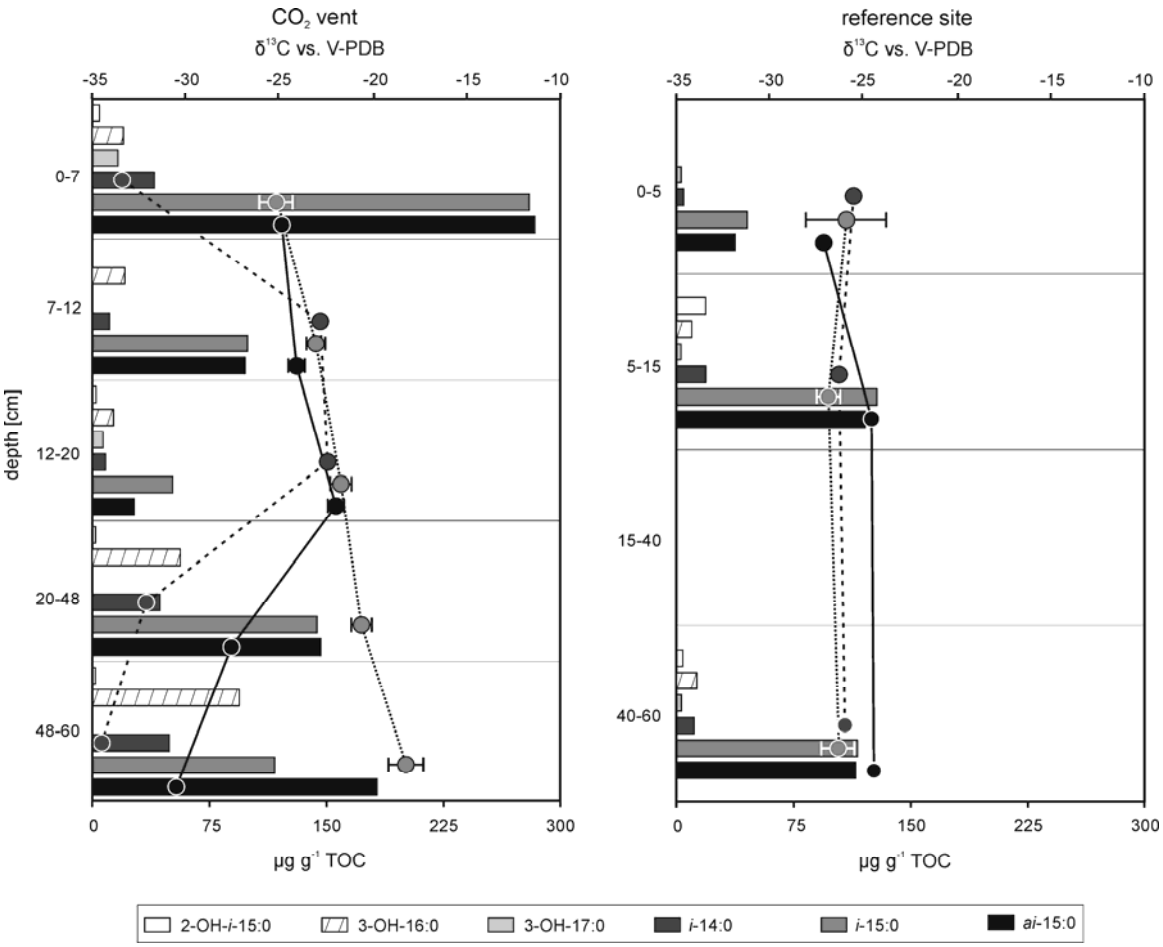

Fig. 4.8. Carboxylic and hydroxyl-carboxylic acids, extracted from the studied soils. Concentrations of 2-hydroxy-*i*-pentadecanoic acid (black bars); concentrations of 3-hydroxy-heptadecanoic acid (white bars) and concentrations and $\delta^{13}C$ values of *i*-pentadecanoic acid (grey bars and circles, respectively).

The rare surveys studying the carboxylic acid inventory of methanotrophic Verrucomicrobia showed them to contain CA *i*-14:0 and *ai*-15:0 (up to 22% and 32% of total fatty acids, respectively) as most diagnostic lipids (Dunfield *et al.*, 2007; Op den Camp *et al.*, 2009). Additionally, Op den Camp *et al.* (2009) found Verrucomicrobia *Kam*1 to produce 3-OH-16:0 in minor amounts (1.2% of total fatty acids). In the lower part of the soil profile of the CO_2 vent, CA *i*-14:0 and *ai*-15:0 show $\delta^{13}C$- values indicative of CH_4-utilizing organisms (-30.6‰ for CA *ai*-15:0 and -34.4‰ for CA *i*-14:0 at 48-60 cm; Fig. 4.8). Although these finding indicate the possibility of Verrucomicrobia participating in the consumption of CH_4, further research is needed to assess the capability of Verrucomicrobia to thrive under anaerobic condition.

These results would also be of great importance for other oxygen depleted, acidic environments like peat bogs being the mayor natural source for atmospheric CH_4 (Gorham, 1991). A better understanding of methanotrophic microorganisms mitigating CH_4-emissions from those environments will help to improve prediction on net emissions from such environments.

4.4. Conclusion

Strong differences in the microbial CH_4-cycle were detected at a natural volcanic CO_2 vent compared to an undisturbed reference soil. Within the CO_2 vent higher CH_4-production and consumption rates were measured. Together with high capability of aerobic methanotrophy also anaerobic oxidation of CH_4 coupled to nitrate was detected at the anoxic CO_2 vent soil. The high rates of aerobic CH_4 consumption, detected *in vitro*, can only be explained either by aerobic microorganisms outliving anaerobic conditions in the soil or by microorganisms capable of using multiple electron acceptors for CH_4-oxidation. Potential microbial groups for the utilization of multiple electron acceptors for CH_4-oxidation were investigated using biomarker analyses, $\delta^{13}C$-values of biomarkers and microbiological methods. As other possible acceptors sulphate, nitrate, manganese and iron were considered for CH_4-oxidation.

Both partners in the sulphate coupled AOM, SRB and methanotrophic Archaea, are very sensitive to oxygen. Moreover, $\delta^{13}C$-values and concentrations of lipid biomarkers revealed no evidence for involvement of Archaea in the consumption of CH_4 at the CO_2 vent soil. Correspondingly, $\delta^{13}C$-values of lipid biomarkers for SRB did not show involvement in the CH_4-cycle. The high sulphate reduction rates detected at the CO_2 vent are associated with autotrophic SRB.

At the CO_2 vent, high denitrification rates were detected *in vitro*. In the lower soil profile a significant proportion of this denitrification was coupled to AOM. In addition, Biomarkers of methanotrophic Bacteria, until recently only known to consume CH_4 in the presence of oxygen, were found at the anoxic CO_2 vent. They are possibly related to the anaerobic oxidation of CH_4 coupled to denitrification. As this group utilises the liberated oxygen from the nitrate reduction for CH_4-oxidation, it could be possible that this group is also capable to proceed with their metabolism under aerobic conditions. But, the *in situ* relevance of both processes, O_2 and NO_3^- coupled AOM in the lower part of the soil profil remains questionable since O_2 and NO_3^- are absent or contained in very low amounts.

The responsible organisms in the newly discovered AOM coupled to reduction of Mn and Fe are yet unknown. Low concentrations of these metals in the CO_2 vent compared to a reference soil are indicating Mn and Fe reduction and subsequent mobilisation.

Verrucomicrobia are the only methanotrophic Bacteria known to survive in a low pH environment like at the studied CO_2 vent. Biomarker and stable carbon isotope

evidence indicates an involvement of acidophilic Verrucomicrobia in the AOM at the CO_2 vent.

Our findings strongly suggest that the CH_4 cycle in terrestrial systems is much more complex than previously assumed. Consequently, more research is needed to investigate the importance and distribution of Verrucomicrobia and other methanotrophic groups in soils.

Appendix

Figure 4.9. The schematic diagram for the procedure of biomarker extraction.

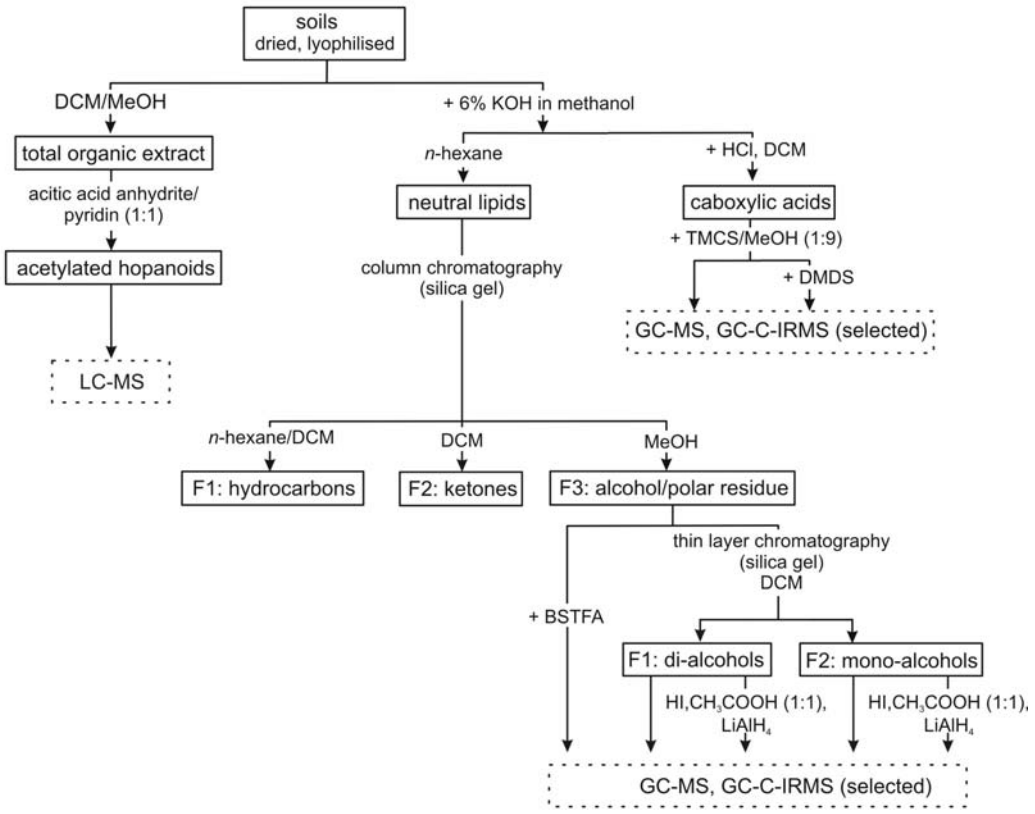

Table 4.4. Soil characteristics of the two sampling sites

Reference site (Oxyaquic Xerorthent)[a]

horizon	depth (cm)	C (%)	N (%)	pH	conductivity (µS cm^{-1})	texture	porosity (%)	K^+ (mg kg^{-1})	PO_4^{2-}P (mg kg^{-1})
Ah	0-5	7.3	0.72	5.8	58	loam	67.9	553	12.2
Cr1	5-15	2.9	0.31	5.7	35	loam	58.4	291	2.0
Cr21	15-40	1.3	0.14	6.3	34	loam	53.9	68	3.1
Cr22	40-60	1.1	0.11	6.1	39	loam	53.6	50	5.1

CO_2 vent site (Aquic Xerorthent)[a]

horizon	depth (cm)	C (%)	N (%)	pH	conductivity (µS cm^{-1})	texture	porosity (%)	K^+ (mg kg^{-1})	PO_4^{2-}P (mg kg^{-1})
Ah	0-7	6.1	0.70	4.0	1040	silt loam	70.4	1027	117
Bw	7-12	2.4	0.29	4.1	410	silt loam	54.2	314	77
Oa-Bw	12-20	6.6	0.65	3.7	473	silt loam	67.6	226	161
Bwg	20-48	3.2	0.32	3.2	598	clay loam	67.7	153	162
Cg	48-60	1.6	0.16	3.3	500	clay loam	63.7	138	130

[a]Soil classification according to (USDA, 2010)

Effects of elevated CO_2 concentrations on the vegetation and microbial populations at a terrestrial CO_2 vent at Laacher See, Germany

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Abstract

CO_2 capture and geological storage offers an option for reducing man-made greenhouse gas emissions. But one major concern related to geological CO_2 storage is the possibility of leakage from the reservoir and subsequent effects on the environment, which cannot completely be excluded. This study aims at investigating the environmental impact of CO_2 release from reservoirs into near surface terrestrial environments. To understand the effect of CO_2 leakage on such an ecosystem, detailed knowledge on the abundance and diversity of plants and microorganisms is essential. Therefore, an ecosystem study has been conducted within the Network of Excellence "CO_2GeoNet" on a natural CO_2 vent at the Laacher See, Germany. Near surface CO_2 conditions and CO_2 fluxes of the venting area were described by means of conventional soil gas measurement equipment, and brought up the difference between the CO_2 anomalies and their surroundings. A comparison of the soil columns between control sites and the centre of the venting area showed a small but significant change in the soil pH below 10 cm. The botanical survey revealed some remarkable vegetation changes like the investigation of important soil microbial communities showed significant differences between the CO_2-rich sites (up to 90% and more of soil gas), medium CO_2 sites (~20%), and control locations with background CO_2 concentrations. The ecosystem appears to be adapted to the different conditions through species substitution or adaptation, showing a shift towards anaerobic and acidotolerant to acidophilic species under elevated CO_2 concentrations. At the end, this ongoing study should identify possible candidates in the botanical and microbial kingdoms, whose presence or absence provide easily detectable indicators for the leakage of CO_2 from deep reservoirs into near surface terrestrial ecosystems.

5.1. Introduction

The fourth IPCC report on global warming states once again that the rise in average global temperatures observed over the last century is most likely due to the release of anthropogenic greenhouse gases (IPCC, 2007b). It turns out that large-scale solutions are needed immediately to quickly reduce greenhouse gas emissions and to mitigate their subsequent environmental effects. CO_2 capture and geological storage in deep saline aquifers or depleted gas and oil reservoirs offers a new option for reducing greenhouse gas emissions in large quantities. To proceed with a responsible large-scale deployment of this technology, all potential risks should have been studied, understood and, finally, minimized to exclude harm to the environment including humans. For this, it is a priori important to assess the potential risks associated with the unlikely leakage of significant volumes of CO_2 from the reservoir into the near surface environment (West *et al.*, 2005; West *et al.*, 2006). Although several studies have been published regarding the effect of increased atmospheric CO_2 concentrations on ecosystems (Jossi *et al.*, 2006), there are only very few that have examined the effects of increasing CO_2 concentrations in the soil column due to upwardly migrating gas. These include a detailed study of a terrestrial CO_2 vent at Latera, Italy (Beaubien *et al.*, 2008; Oppermann *et al.*, 2010), a survey at Mammoth Mountain (California, USA) for the influence of volcanic CO_2 on soil chemistry and mineralogy (Stephens and Hering, 2004) and research at Stavesinci (NE Slovenia) for the influence of high soil-gas concentrations of geothermal CO_2 on plants (Macek *et al.*, 2005; Pfanz *et al.*, 2007).

In order to address some of the above-mentioned issues, this study investigates the potential environmental impact of CO_2 release from deep reservoirs on near surface terrestrial environments. Particularly the effect of CO_2 leakage on the abundance and diversity of plants and microorganisms is investigated in an ecosystem study conducted as a joint activity within the European network “CO2GeoNet” at a natural CO_2 vent at Laacher See, Germany.

The Laacher See volcanic centre is located in the core of the East Eifel volcanic field, and comprises of about 100 eruptive centres that cover an area of approximately 330 km². The East Eifel volcanic field is located west of the Rhine River in the still uplifting Paleozoic Rhenish Massif. The Laacher See eruption is the only known large explosive eruption that took place in central Europe during late Quaternary time

(~12,900-12,880 yrs BP; e.g., Boogard and Schmincke, 1985). The Laacher See volcanic centre is morphologically characterized by a basin filled by a lake (Laacher See), with 3.3 km² area, surrounded by a steep ringwall rising 90 to 240 m above the basin. The ringwall, which can be classified as an extinct volcanic caldera, is made up by different basanitic/tephritic cinder cones and the tephra deposits of the Laacher See eruption. The internal structure of the Laacher See basin is dominated by an Eastnortheast-Westsouthwest striking thrust and four other geological lineaments: 2 running more or less North-South, the other Northeast-Southwest. CO_2 is produced below the caldera, it emerges from degassings of the upper earth mantle and migrates along faults and fractures to the surface (Möller *et al.*, 2008). Release to the atmosphere typically occurs from gas vents, characterised by a small core of elevated gas flux.

One defined gas vent was chosen for this study, located in an almost naturally-vegetated pasture field on the western side of the lake (Fig. 5.1). The vent is situated in an area which became dry land very recently. As a consequence of two tunnel constructions in 1164 and 1844, the water level of the lake was artificially lowered by about 10 plus 5 m. The terrestrial development of the studied site is therefore very young. Some organic material from former lake deposits is still to be found in deeper soil horizons. The vent is clearly visible due to a 5 m wide core of nearly bare soil surrounded by an approximately 40 m wide area of variably-impacted vegetation.

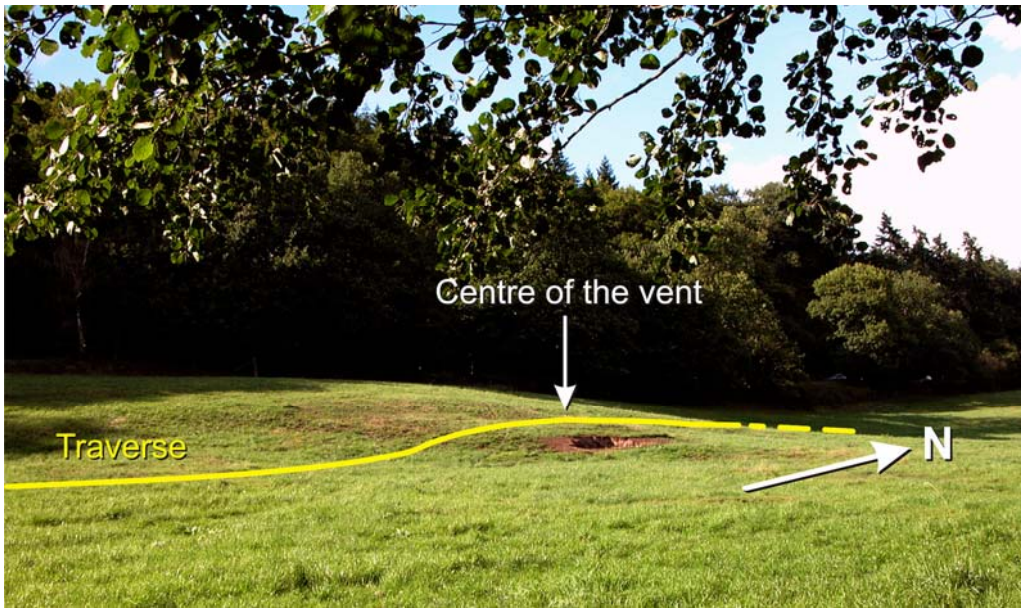

Fig. 5.1. Sketch of the investigated CO_2 vent close to the western shore of the lake Laacher See; note the vegetation change from closed, green grassland to patchy *Polygonum arenastrum*-mats towards the centre of the vent.

5.2. Methods and materials

Surveys were conducted in September 2007 and July 2008 along a 60 m long transect across the vent (Fig. 5.1), providing a spectrum of different CO_2 flux rates, soil gas concentrations and compositions. In addition to the detailed survey of these conditions (soil gas concentrations and gas fluxes), intensive botanical studies and sampling for microbiological, mineralogical and geochemical analysis were performed at the same time.

5.2.1. Characterisation of the near surface CO_2 conditions

The applied techniques are potential methods for the near surface monitoring of geological CO_2 storage sites; they were used during both field campaigns in 2007 and 2008. First, a rapid surveying of the whole study area was undertaken by means of a newly developed, mobile open path laser system which was mounted at about 30 cm above the ground on a quad bike (for details see Jones *et al.*, 2009). The system detected already known CO_2 vents, and confirmed and discovered suspicious or unknown degassing sites. Afterwards, the 60 m long traverse across the strongest vent was intensively investigated with conventional soil gas concentration and flux measurement equipment: Steel probes and handheld infrared gas sensors (Li-Cor and Dräger instruments) for soil CO_2 concentration, and commercial and custom-made accumulation chambers for the CO_2 flux quantifications. The measurements were carried out in 0.5 m intervals. Some additional gas samples taken along the traverse for comparative laboratory analyses were also used for the determination of carbon isotope ratios ($\delta^{13}C_{CO2}$; by means of a Thermo Delta plus XL mass spectrometer) which give hints on the origin of the CO_2.

5.2.2. Botanical impact survey

The botanical survey was conducted along the entire length of the transect. The investigations registered the percentage cover of identified plant species and groups at 0.5 m intervals using a 0.5 m x 0.5 m quadrat levels. Field flora books were used to identify critical plant taxa (Blamey and Grey-Wilson, 2003; Fitter *et al.*, 1984) and digital photographs were taken of each quadrat for a complete visual record.

5.2.3. Microbiological analyses

Basic soil related field work and lab analyses of soil samples followed conventional approaches. Soil pH was measured in a suspension of 10 g of fresh soil in 25 ml of distilled water with a pH-redoxmeter GPRT 1400 AN (GSG Greisinger Electronic). Prior to organic carbon measurements the soil was dried at 105°C and grounded. Inorganic carbon was removed with 50 µL 1N HCl followed by drying the sample on a 40°C heating plate (repeated three times). The content of organic carbon was finally determined using an elemental analyser (VarioMAX Elementar Analysensysteme).

5.2.3.1. Determination of microbial activities

The collected soil samples were first mixed 1:1 with artificial mineral medium to obtain homogenous slurries (Widdel & Bak, 1992). Subsequently, 9 ml of medium were added to 3 ml of soil slurry into sterile glass tubes (20 ml) which were afterwards sealed with butyl-rubber stoppers and screw caps. The headspace was either flushed with N_2 for methane and anaerobic CO_2 production as well as sulphate reduction measurements, with air for aerobic CO_2 production or with air and 2% CH_4 for aerobic methane oxidation.

As important indicators of the gross mineralisation in the soil the CO_2 production (CPR; under aerobic and anaerobic conditions), the anaerobic methane production (MPR), and the sulphate reduction rates (SRR) were quantified. The potential aerobic oxidation of methane rates in the soil samples were determined *in vitro* as described previously by Krüger *et al.* (2002). Triplicate tubes were incubated horizontally at 20°C and gently shaken once per day to ensure an even distribution of gases or sulphate within the microcosms. The rates were calculated per gram of dry weight (g_{dw}) as determined after drying at 80°C for 48 h and deviations are expressed as 95% confidence intervals unless stated otherwise. The sulphide content was determined using the formation of copper sulphide after Cord-Ruwisch (1985). Methane and CO_2 were determined using a GC 14B gas chromatography (Shimadzu) as described in Nauhaus *et al.* (2002), which was additionally equipped with a methaniser to quantify the CO_2.

5.2.3.2. DNA extraction and quantitative Real Time PCR (qPCR)

The DNA was extracted from 0.5 to 1 g of a frozen soil sample following the manufactory's manual of the FastDNA Spin Kit for Soil (Bio 101) with addition of 200 µg of poly-adenylic acid (poly A) to the lysis mixture (Webster *et al.* 2003). The resulting DNA was dissolved in 100 µl ultrapure PCR water and used as target for PCR based analysis.

DNA standards for quantitative real time PCR (qPCR) were prepared as described previously by Engelen *et al.* (2008). Specific fluorescent probes were used targeting the ubiquitous 16S rRNA genes of bacterial or archaeal organisms (Takai & Horikoshi, 2000, Nadkarni *et al.,* 2002). The assays were carried out using the TaqMan PCR Master Mix (Applied Biosystems). Each DNA extract was measured in triplicate and in two to tree dilutions to check for PCR inhibition. Conversion factors for DNA copy numbers to cell numbers were: 4.1 for Bacteria, and 1.5 for Archaea (Lee *et al.,* 2009). The detection limits for qPCR analyses were 10^3 DNA copies g^{-1} dry weight for the assays specific for Bacteria and 10^1 DNA copies g^{-1} dry weight for the assays specific for Archaea.

5.2.3.3. Lipid biomarker studies

The microbiological analyses were supplemented by lipid biomarker studies as described in detail by Oppermann *et al.*, 2010.

5.3. Results and discussion

5.3.1. Gas monitoring and soil chemistry

The results of the gas surveys are generally very similar from year to year. The soil CO_2 concentration and flux data series of the two years are very homogeneous and well correlated as shown by their coefficients of determination (see Table 5.1).

Table 5.1. Correlation matrix (coefficients of determination, r^2) of soil CO_2 concentrations in 15 and 60 cm depth and CO_2 fluxes for 2007 and 2008

CO_2 … (r^2)	15cm, 2007	60cm, 2007	Flux, 2007	15cm, 2008	60cm, 2008	Flux, 2008
15cm, 2007						
60cm, 2007	0.92					
Flux, 2007	0.96	0.87				
15cm, 2008	0.93					
60cm, 2008		0.83		0.82		
Flux, 2008			0.88	0.92	0.73	

Hence, the spatial patterns observed in 2007 could be confirmed in their shapes in 2008. Figure 5.2 illustrates the soil CO_2 concentrations in 15 and 60 cm depth while Figure 5.3 shows the CO_2 fluxes from the underground to the atmosphere for the different years.

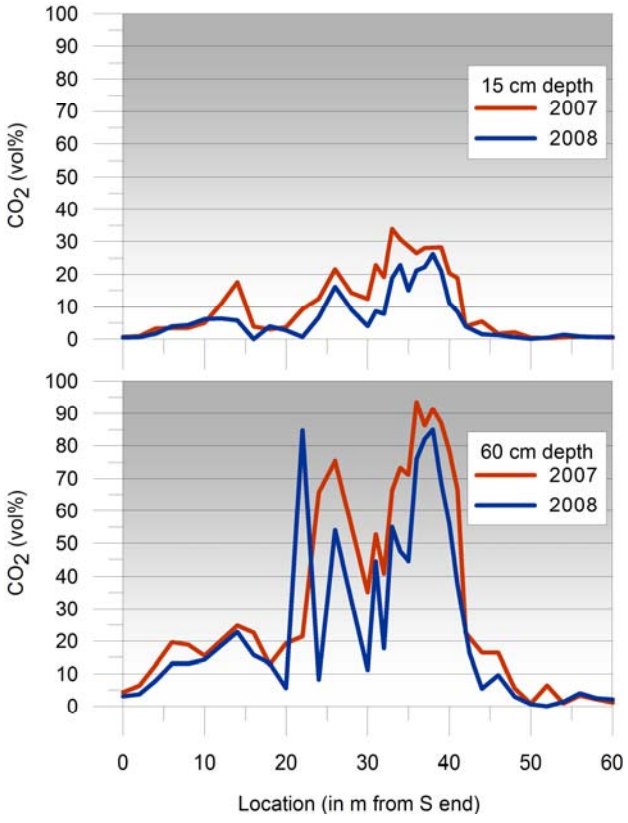

Fig. 5.2. Comparison of 2007 and 2008 CO_2 concentrations in soil gas along the traverse across the studied vent (September 2007 and July 2008, using only points measured in both years).

Three main zones of higher CO_2 concentrations and fluxes could be identified along the traverse: Between locations 11-17 m, 21-28 m, and 30-42 m, the later representing the so-called centre of the vent where peak concentration values of more than 90 vol% CO_2 were registered in 60 cm depth (Fig. 5.2). But already in a very short distance from these distinct anomalies the CO_2 concentrations and fluxes drop back to background values; demonstrating the limited size of natural CO_2 vents. Furthermore, particularly the figures of the CO_2 concentrations show also the relatively high small scale variability which could be quite marked between adjacent measurement points.

There certainly are also some differences in detail between the two years, but this is to be expected given that the sampling locations will not precisely match within a few centimetres, and there could be changes in the migration pathways of gas to the surface owing to changing underground conditions. One clear difference between the two years are the generally higher gas concentrations and fluxes in 2007 (see Figs. 5.2 and 5.3). Data correlations with meteorological parameter suggest that factors

such as lower atmospheric pressure and higher wind speed drew slightly larger amounts of gas from the ground in the autumn and overrode any impeding effect of higher soil moisture.

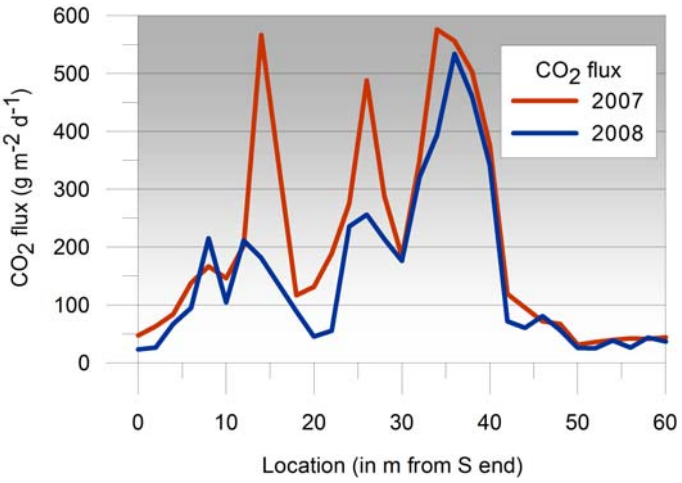

Fig. 5.3. Comparison of 2007 and 2008 CO_2 flux data for the traverse across the vent.

Carbon isotope analyses ($\delta^{13}C_{CO2}$) were helpful for the characterisation of the venting areas since the isotope ratios differ from CO_2 rich sites (-4.1 to -2.7‰ PDB) to those with medium (-1.7 to -0.2‰) to low concentrations (-1.0 to 0.8‰). The $\delta^{13}C_{CO2}$ values for the CO_2 rich sites point directly to the upper earth mantle and/or lower earth crust as origin of the CO_2. Contrastingly, the CO_2 gas from medium to low concentration sites is already affected by mixing processes and isotope fractionation, probably under the influence of some underground carbonate levels.

In terms of bulk mineralogical compositions and soil chemistry, the analysed samples from the centre of the vent (35 m) and the control site (55 m) were relatively similar in the top 70 centimetres. Going deeper into the soil, a small but significant change in the soil pH was observed below 10 cm (Fig. 5.4). This might influence the activity and composition of the microbial communities, as well as the soil mineralogy as also seen at Latera (Beaubien *et al.*, 2008).

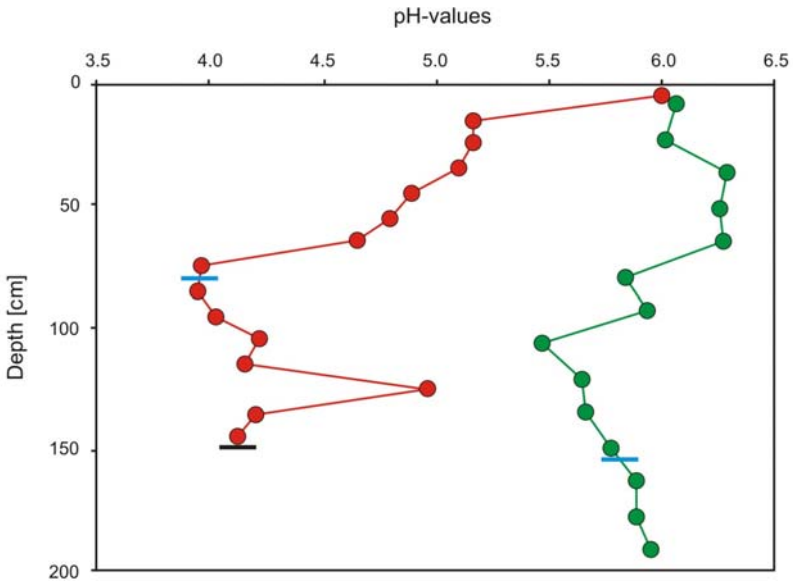

Fig. 5.4. Acidity of two sediment cores (pH profiles); in red = area of highest CO_2-seepage, in green = control site; the blue lines show the top of the water table, the black the rock base.

5.3.2. Botanical investigations

The botanical survey showed that CO_2 soil gas concentrations influence vegetation types with grasses predominating below 20% CO_2. Above this concentration two predominant dicotyledonous plant species were observed and could be used as bioindicators of high CO_2 soil gas concentrations.

Main results of the botanical survey are summarised in Figure 5.5, which shows the percentage coverage for total moss, total grass (monocotyledonous plants), *Polygonatum arenastrum* and 'other' dicotyledonous flowering plants. *P. arenastrum* is the only observed dicotyledonous plant between 25 and 50 m along the transect where CO_2 concentrations are between ~10-35% at 15 cm depth and ~35-90% at 60 cm depth. Where CO_2 concentrations are below 20% at 15 cm depth, grasses predominate and *P. arenastrum* is not observed (0-25 m and 40-60 m) although other dicotyledonous plants are present. These results can be compared to observations from another natural CO_2 gas vent site at Latera, Italy where only grasses were observed when concentrations of CO_2 were between 5-40% at 10 cm depth (Beaubien *et al.*, 2008). Indeed, dicotyledonous plants did not appear to be able to tolerate CO_2 concentrations over 5% at this site. Other observations at a controlled injection site in an English pasture also suggested that grasses were more tolerant to higher concentrations of CO_2 than dicotyledonous plants (West *et al.*, 2008). The observation of a dicotyledonous plant as a bioindicator of increased soil gas CO_2 is therefore unexpected and also demonstrates that botanical changes are site specific, depending also on other factors such as soil moisture, pH influencing plant ecology, etc. However, monocotyledonous plants appear, in general, to be more tolerant to increased soil gas CO_2.

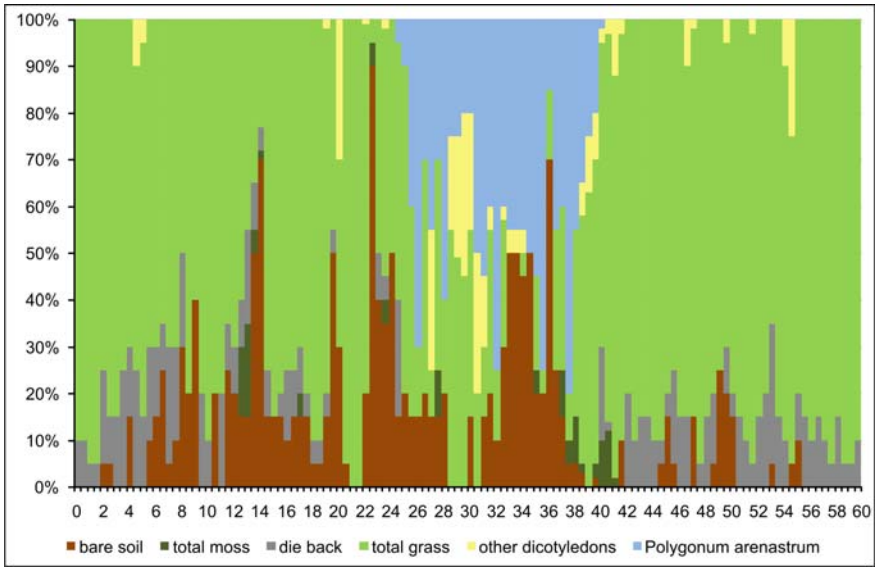

Fig. 5.5. Effect of CO_2 emissions on the distribution of different botanical groups/species along the transect across the CO_2 vent (centre at approx. 30-35 m); x-axis: location in m from S end, y-axis: coverage.

5.3.3. Microbial community composition and activities

The determination of environmentally important microbial activities in the soil samples showed significant differences between the CO_2-rich sites (>90% of soil gas), medium CO_2 sites (20%) and control locations with background CO_2 concentrations. To get some more detailed information, potential sulphate reduction rates as well as methane production and oxidation were determined in sediment samples from the different sites. These measurements with samples from vent and non vent sites should also provide first information on the influence of elevated carbon dioxide concentrations on selected microbial populations (Fig. 5.6). Gross CO_2 production was under aerobic conditions about 100-fold higher than under anaerobic conditions. Under anaerobic conditions CO_2 production was similar at the vent and the control site, with 4.34 ± 0.25 and 1.03 ± 0.32 µmol g_{dw}^{-1} d^{-1}. In contrast, aerbic rates were with 432 ± 57 µmol g_{dw}^{-1} d^{-1} significantly higher at the control site than in the vent centre with 121 µmol g_{dw}^{-1} d^{-1}.

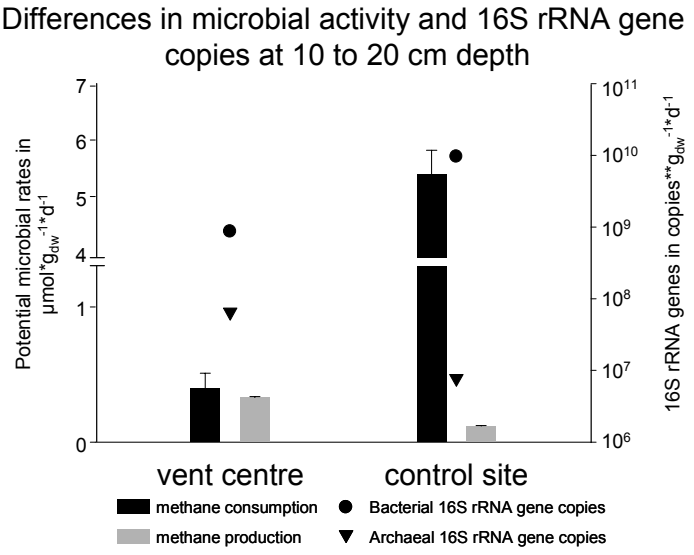

Fig. 5.6. Differences in microbial activity and 16S rRNA gene copies at 10 to 20 cm depth.

Potential methane production rates without substrate addition in the sediment samples from 10-20 cm depth were at about 0.33 ±0.007 µmol CH_4 g_{dw}^{-1} d^{-1} and therefore much higher than at the vent centre (location 36 m) than in the control site samples (55 m) where they reached 0.12 ±0.002 µmol CH_4 g_{dw}^{-1} d^{-1}. Data for methane oxidation under aerobic conditions showed the opposite picture: Higher rates of 5.4 ±0.42 µmol CH_4 g_{dw}^{-1} d^{-1} at the control site compared to 2.2 ±0.35 µmol CH_4 g_{dw}^{-1} d^{-1} in the intermediate CO_2 positions (14 m) and 0.4 ±0.11 µmol CH_4 g_{dw}^{-1} d^{-1} at the centre of the vent. Finally, a remarkable aerobic methane oxidation acitivity was found even in sediment samples down to 1.5 m depths with an identical pattern (data not shown). This points towards a methane supply for the methanotrophic bacteria from deeper sources present in the deepest oxygen-poor, organic deposits of the soil column and tracing back to the limnic evolution of the study site.

Sulphate reduction rates were relatively high, between 1.5 to 2.2 µmol g_{dw}^{-1} d^{-1} in the samples from the centre of the vent, with the highest activity observed in deeper sediment layers below 50 cm depth. Interestingly, sulphate reduction was also detected in deeper samples from the control site, albeit at a much reduced rate. The sources of sulphate and substrates for the sulphate-reducing bacteria is yet to be determined, but might be originating from underground water streams or the decomposition of organic material from lake sediment deposits in the deeper soil horizons.

In accordance with the microbial activities, total numbers of microorganisms showed also significant differences between the individual sites. Cell numbers of *Bacteria* were determined using quantitative PCR (qPCR, Krüger *et al.* 2008): They were highest at the control site and substantially lower towards the vent centre; the values decreased from 9.6 x 10^9 to 8.7 x 10^8 gene copies g_{dw}^{-1} of soil. For *Archaea* in contrast, the values increased from control site towards the centre, with 7.7 x 10^6 and 6.5 x 10^7 gene copies g_{dw}^{-1} of soil. One explanation for the observed changes in the community composition might be the replacement of oxygen in the soil gas with CO_2, leading to first microaerobic and then to anaerobic conditions. This would thus favour e.g., methane-producing *Archaea* or sulphate-reducing bacteria. To analyse this in more detail, group-specific qPCR assays are carried out currently to reveal, whether certain functional groups, like the methane oxidising or sulphate reducing bacteria, were absent or stimulated at the CO_2-rich sites.

Another implication for strong changes in the microbial community came from the lipid biomarker studies. Although the cell numbers of bacteria decreased, the biomarker studies showed that in the CO_2 vent, bacterial non isoprenoidical tetraethers lipids were contained in higher quantities then at the control site (e.g., in 50-60cm depth 503 µg g^{-1} TOC at the CO_2 vent site and 302 µg g^{-1} TOC at the control site). Even though the source organisms of bacterial tetraethers are not known yet, they most likely derive from anaerobic bacteria (see Oppermann *et al.*, 2010 and references therein). This finding is of special interest since ether lipids are more stable than ester lipids that are commonly found in bacteria. Bacteria able to synthesis etherlipids are therefore probably better adapted to the low pH conditions found at the CO_2 vent (Fig. 5.4).

5.4. Conclusions

CO_2 gas fluxes into the Laacher See are roughly estimated in the range of about 5,000 tons of CO_2 per year (Aeschbach-Hertig *et al.*, 1996). Additional CO_2 gas seepages from the underground occur permanently at the fringes of the lake. Even if a CO_2 gas release of up to 600 g m^{-2} d^{-1} could be registered along the studied vent, our results indicate that the effects of the gas vents are spatially limited. Nevertheless, some significant effects of high CO_2 concentrations on the terrestrial ecosystem were observed. The ecosystem appears to have adapted to the different conditions through species substitution or adaptation, showing a shift towards anaerobic and acidotolerant to acidophilic species under elevated CO_2 concentrations. The present results have stimulated future research activities which will include an extensive investigation campaign with gas, water and sediment sampling both for the Laacher See and carbonic springs nearby. At the end, this study should identify possible candidates in the botanical and microbial kingdoms, whose presence or absence provide easily detectable indicators for the leakage of CO_2 from deep reservoirs into near-surface terrestrial ecosystems.

Hopanoid production by sulphate reducing Bacteria

For the characterisation of complex microbial communities with the help of biomarker studies, it is necessary to possess detailed knowledge on the distribution of potential biomarkers in certain organisms.

Hopanoids are important lipid components of many bacterial groups and according to Ourisson and Albrecht (1992) hopanoids are the most abundant organic molecules on Earth until recently the synthesis of hopanoids was believed to be restricted to at least microaerophilic Bacteria. But after the finding of hopanoids in an AOM performing microbial mat (Thiel *et al.*, 2003) it became apparent that also anaerobic Bacteria are capable to synthesise hopanoids. The microbial mat studied by the authors was recovered from the Black Sea. Since the early Holocene the water column of the Black Sea is permanently stratified (Ross *et al.*, 1970; Ryan *et al.*, 1997). Due to this stratification the lower water body is cut of from oxygen supply and therefore permanently oxygen free. Numerous CH_4 seeps are located at the sea bottom and the Black Sea is the largest surface water reservoir of dissolved CH_4. According to Cicerone and Oremland (1988) the amount of CH_4 contained in the Black Sea is equivalent to the amounts annually emitted from wetland and rice paddies. The CH_4 in the Black Sea derives almost completely from the sediments and interestingly nearly 99% of the CH_4 are anaerobically oxidised (Reeburgh *et al.*, 1991). Hence, the Black Sea provides ideal conditions to study anaerobic methanotrophic communities. Moreover, the Black Sea is considered to be a contemporary analogue of early Earth oceans and it offers the possibility to supply information on life in such bygone environments.

Shortly after the discovery of Thiel *et al.* (2003), Sinninghe Damsté *et al.*, (2004) and Fischer *et al.* (2005) were able to show biosynthesis of hopanoids by anaerobically grown Planctomycetes and some *Geobacter* species, respectively. But these findings could not explain the occurance of hopanoids in AOM performing microbial consortia.

To reveal the source of hopanoids in AOM performing microbial mats we analysed pure cultures of sulphate reducing Bacteria, isolated from these mats.

In this study (Chapter VII), we show production of diploptene, diplopterol and intact BHPs, mainly tetrafunctionalised BHPs (bacteriohopanetetrol and aminobacterio-hopanetriol) by SRB, isolated from an AOM performing microbial consortia. However, the BHT cyclitol ether which was found in the microbial mat and the sediment was

absent in the studied *Desulfovibrio* strains. Consequently, additional anaerobic hopanoid-producing Bacteria are likely present in AOM perfoming mats. But marine deposits are apparently dominated by tetrafunctionalised BHPs (Farrimond *et al.*, 2000; Bednarczyk *et al.*, 2005), while in freshwater environments penta- and hexafunctionalized hopanoids prevail (Farrimond *et al.*, 2000). According to Bisseret *et al.* (1985) and Talbot *et al.* (2003a) these penta- and hexafunctionalized hopanoids originate from type I and type II methanotrophic Bacteria and/or cyanobacteria.

Since Desulfovibrionaceae are found in almost all anoxic environments that contain sulphate (Madigan *et al.*, 2002) and tetrafunctionalised BHPs are ubiquitous in sulphate containing sediments it could be assumed that tetrafunctionalised BHPs are biomarker of sulphate reduction rather than AOM. To test this assumption four additional SRB strains were examined for hopanoids biosysthesis.

D. vulgaris Hildenborough, *D. africanus* and *D. halophilus* did not contain hopanoids. *D. bastinii*, isolated from an oil reservoir, also contained high amounts of non-extended hopanoids and bacteriohopanepolyols, showing that hopanoids in anoxic environments are not restricted to CH_4 consuming communities, but can also be produced by heterotrophic SRB. Consistently, to the Desulfovibrionaceae isolated from the AOM performing microbial mat, *D. bastinii* produced bacteriohopanetetrol and aminobacteriohopanetriol. Nevertheless, the absence of BHPs in three of the four studied species highlights the restriction of hopanoid production to distinct species within the Desulfovibrionaceae. Further studies are needed to reveal the mechanisms controlling this distribution. Nevertheless, the finding of tetrafunctionalised BHPs in Desulfovibrionaceae can help to draw conclusions on the composition of the sulphate reducing community at natural CO_2 vents.

In any case, the presence of hopanoids can no longer be used as an indicator for aerobic conditions in recent or fossil sediments.

Biosynthesis of hopanoids by sulphate-reducing bacteria (genus *Desulfovibrio*)

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Abstract

Sulphate reduction accounts for about a half of the remineralization of organic carbon in anoxic marine shelf regions. Moreover, it was already a major microbial process in the very early ocean at least 2.4 billion years before the present. Here we demonstrate for the first time the capability of sulphate-reducing bacteria (SRB) to biosynthesize hopanoids, compounds that are quantitatively important and widely distributed biomarkers in recent and fossil sediments dating back to the late Archean. We found high concentrations (9.8–12.3 mg per gram of dry cells) of non-extended and extended bacteriohopanoids (bacteriohopanetetrol, aminobacteriohopanetriol, aminobacteriohopanetetrol) in pure cultures of SRB belonging to the widely distributed genus *Desulfovibrio*. Biohopanoids were found – considered as membrane rigidifiers – in more than 50% of bacterial species analysed so far. However, their biosynthesis appeared to be restricted to aerobes or facultative anaerobes with a very few recently described exceptions. Consequently, findings of sedimentary hopanoids are often used as indication for oxygenated settings. Nevertheless, our findings shed new light on the presence of hopanoids in specific anoxic settings and suggests that SRB are substantial sources of this quantitatively important lipid class in recent but also past anoxic environments.

7.1. Introduction

Triterpenoids of the hopane series (biohopanoids) are widely distributed components in the biosphere and therefore account for the ubiquity of their diagenetic products, the geohopanoids (Ourisson and Albrecht, 1992). These compounds are among the oldest molecular biomarkers, dating back to late Archean times [2.77 billion years (Gya)] (Brocks *et al.*, 1999; Brocks *et al.*, 2003). Biohopanoids occur in several higher plants, ferns, mosses, fungi, protists, and particularly in bacteria (Ourisson *et al.*, 1987). However, bacteria are the only known source of C_{35} hopanepolyols (bacteriohopanepolyols; BHPs), which are thought to act as cell membrane rigidifiers analogues to sterols in eukaryotes (Kannenberg and Poralla, 1999).

In a comprehensive study of bacteria, diverse phyla including cyanobacteria, methylotrophs, purple non-sulfur bacteria, and Gram-negative and Gram-positive chemoheterotrophs were found to synthesize hopanoids (Rohmer *et al.*, 1984).

Although the biosynthesis of hopanoids in general does not require the presence of free oxygen, there have been very few reports of hopanoid-producing, facultative anaerobic prokaryotes. These include photosynthetic purple non-sulfur bacteria or the fermentative *Zymomonas mobilis* (Kleemann *et al.*, 1990; Rohmer *et al.*, 1992; Rosa-Putra *et al.*, 2001). Accordingly, occurrences of hopanoids are interpreted as evidence for an input of lipids from oxygen-respiring organisms and thus for oxygenated environments (e.g., Innes *et al.*, 1997). Interestingly, recent studies showed that two anaerobically grown Fe(III)-reducing *Geobacter* species (Fischer *et al.*, 2005; Härtner *et al.*, 2005) and enrichment cultures of specific planctomycetes, which anaerobically oxidize ammonium (Sinninghe Damsté *et al.*, 2004), generate hopanoids. Particularly because elevated occurrences of hopanoids in recent and fossil anoxic deposits can often not be explained by the bacteria mentioned above (Elvert *et al.*, 2000; Pancost *et al.*, 2000; Thiel *et al.*, 2003), these findings raise the question whether additional groups of anaerobic bacteria are also able to biosynthesize hopanoids. Sulphate-reducing bacteria (SRB) are likely candidates as they are very common and are key players with respect to the remineralization of organic matter in anoxic marine shelf sediments (Jørgensen, 1982).

To date, reports on the search for hopanoids in SRB exist only for *Desulfovibrio desulfuricans.* This organism showed no detectable amounts of hopanoids (Rohmer *et al.*, 1984). However, in fact *D. desulfuricans* (Rohmer, 1999) and in addition

D. vulgaris Hildenborough (Heidelberg *et al.*, 2004) were found to contain at least enzymes that are essential for the biosynthesis of isoprenoids such as hopanoids.

During our work on hopanoid-containing Black Sea microbial mats which oxidize methane anaerobically (Michaelis *et al.*, 2002; Thiel *et al.*, 2003; Blumenberg *et al.*, 2004), we studied pure cultures of mesophilic bacterial isolates for their lipid content. These bacteria reduce sulphate and were anaerobically grown on lactate (with CO_2 in the gas phase).

Here we show that SRB of the genus *Desulfovibrio* are indeed rich sources for hopanoids, giving evidence that SRB contribute to the recent and also to the fossil sedimentary hopanoid pool.

Knowledge about carbon assimilation and biosynthetic pathways within eubacteria has increased in recent years. However, data on lipid biosynthesis in anaerobic bacteria are still fragmentary (e.g., Teece *et al.*, 1999; Zhang *et al.*, 2003). As information on this topic can be obtained from stable carbon isotope signatures (Jahnke *et al.*, 1999; Hayes, 2001; Zhang *et al.*, 2003), we performed detailed $\delta^{13}C$ analyses of lipid components, biomass, and the substrates used in the cultivation experiments.

7.2. Results and discussion

7.2.1. Hopanoids in new isolates of the genus Desulfovibrio

Isolated bacteria from a Black Sea microbial mat were used for our lipid study. Physiological investigations of these bacteria showed that they grow under strictly anoxic conditions and perform dissimilatory sulphate reduction for energy generation. 16S rRNA gene sequences revealed that these bacteria belong to the genus *Desulfovibrio* (family Desulfovibrionaceae) of the δ-proteobacteria (Fig. 7.1). We found three strains (*Desulfovibrio* strain BSS2, BSS3 and BSS6) containing considerable amounts of diploptene (**I**) and diplopterol (**II**) (Table 7.1; Fig. 7.2), which are both common hopanoids in phylogenetically diverse bacteria (Rohmer *et al.*, 1984). Desulfovibrionaceae are not restricted to specific habitats and are relevant members of the microbial community in almost all aquatic anoxic environments that contain sulphate (Madigan *et al.*, 2002). They are physiological versatile, using autotrophic, heterotrophic and mixotrophic carbon assimilation pathways and can even tolerate exposure to oxygen (Cypionka, 2000), although no sustainable aerobic growth in pure cultures has been observed. Within the phylogenetic tree of life, the Desulfovibrionaceae had their major node at 2.38 Gya (Sheridan *et al.*, 2003), notably the time for which also a fundamental increase in marine sulphate reduction was proposed (Habicht *et al.*, 2002).

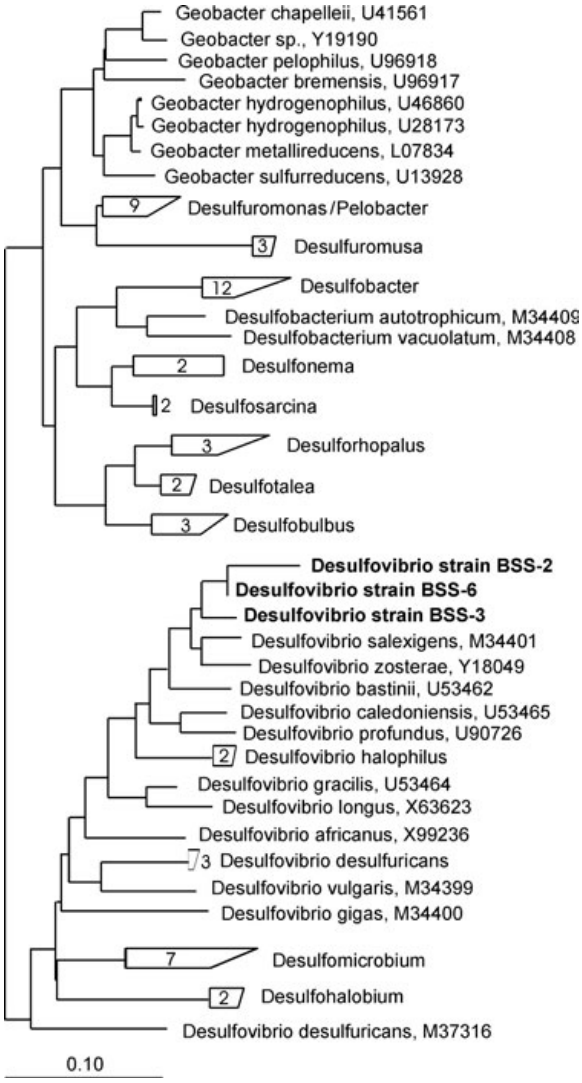

Fig. 7.1. Phylogenetic tree showing the affiliation of the new BSS isolates based on 16S rRNA gene sequences. The tree was calculated using the Neighbour-Joining algorithm. Tree topology was evaluated by Maximum Likelihood analyses. Accession numbers are indicated. Bar: 10% estimated sequence divergence.

Table 7.1. Distributions and concentrations of hopanoids within the *Desulfovibrio* species

	Diploptene[a]	Diplopterol[a]	M^+ 683[ab]	BHT[a]	Aminotriol[a]	Aminotetrol[a]	Total hopanoids[c]
Desulfovibrio BSS2	2	8	2	68	21	< 1	9.8
Desulfovibrio BSS3	< 1	2	< 1	40	56	1	12.3
Desulfovibrio BSS6	1	5	< 1	21	71	1	11.1

a. Amounts are given in percentage of total hopanoids.
b. Indicates an unidentified BHP with a molecular mass of 683 atomic mass units.
c. Total hopanoid concentrations in mg g^{-1} calculated on cell dry weight (dw); diploptene and diplopterol were quantified by GC-MS using 5α(H)-cholestane as internal standard; concentrations of the other acetylated components were calculated from the major mass fragment compared with *m/z* 345 of the acetylated internal standard pregnane-3β, 20β-diol by LC-MS. Response factors for LC-MS quantifications are given in *Experimental procedures*. The concentrations were also calculated for GC-amenable-acetylated bacteriohopanepolyols (BHT and aminotriol; pregnane-3β, 20β-diol diacetate as internal standard) using gas chromatography demonstrating a difference of ±10–15% between GC- and LC-MS quantifications. For structures see Fig. 6.2.

Analyses of the acetylated total extracts with coupled gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) showed that the *Desulfovibrio* species contain, in addition to diploptene (**I**) and diplopterol (**II**), intact BHPs (for structures see Fig. 7.2). By far the most dominant hopanoids are bacteriohopanetetrol (BHT, **III**) and aminobacteriohopanetriol (aminotriol; **IV**, Fig. 7.3 and Table 7.1), hopanoids that are ubiquitous in recent sediments and which are putative precursors of hopanoidal alcohols, ketones, acids and hydrocarbons diagenetically formed in sediments (Ourisson and Albrecht, 1992). Bacteriohopanetetrol and the aminotriol account for 89–96% of the total hopanoids of the three *Desulfovibrio* strains (Table 7.1). Hopanoids of strain BSS2 are clearly dominated by BHT, whereat in strain

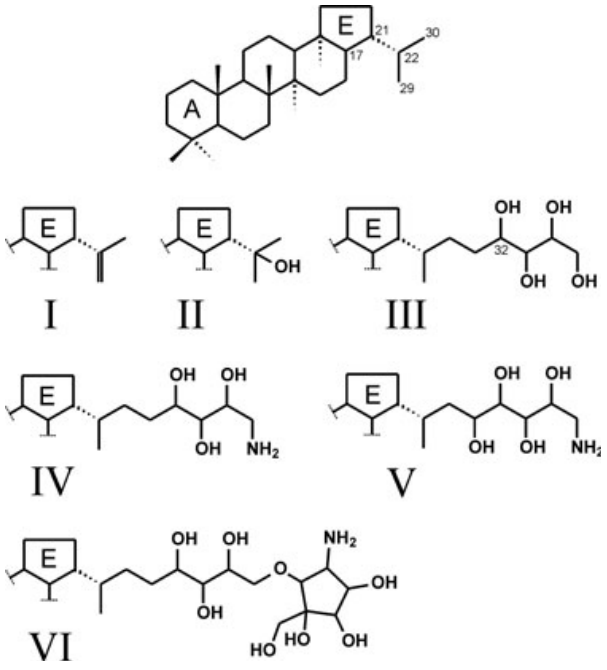

Fig. 7.2. Structures of hopanoids found in the *Desulfovibrio* strains, the Black Sea microbial mat and the sediment. All hopanoid side-chains are linked to the ring system of the basic hopanoid structure. Roman number **I** indicates diploptene [hop-22(29)-ene], **II** diplopterol, **III** bacteriohopanetetrol [17β(H), 21β(H)-bacteriohopane-32,33,34,35-tetrol; BHT)], **IV** aminobacteriohopanetriol [17β(H),21β(H)-35-aminobacteriohopane-32,33,34-triol; aminotriol], **V** aminobacteriohopanetetrol [17β(H),21β(H)-35-aminobacteriohopane-31,32,33,34-tetrol; aminotetrol] and **VI** BHT cyclitol ether. Compounds **V** and **VI** were only identified using liquid chromatography mass spectrometry.

BSS3 and BSS6 the amount of aminotriol exceeds that of BHT by up to threefold. Additionally, LC-MS analyses showed minor amounts of other extended hopanoids [aminobacteriohopanetetrol (aminotetrol) and an unknown structure with mass-spectral characteristics indicative of BHPs and a molecular mass of 683 atomic mass units (M^+)]. Biohopanoids with a hexafunctionalized side-chain and A-ring-methylated hopanoids were not found. Therefore, the SRB shown here are producers mainly of tetrafunctionalized BHPs. Low amounts of aminotetrol (**V**) were also found in all three strains. Notably, very high amounts of BHPs were quantified from the acetylated total extracts maximizing at 12.3 mg g^{-1} dry weight (dw) of *Desulfovibrio* strain BSS2 (Table 6.1; up to 23% of the total extracted lipids). These amounts are comparably high as a range of 1–5 mg g^{-1} dw was found to be common in other hopanoid-producing bacteria (Rohmer *et al.*, 1984).

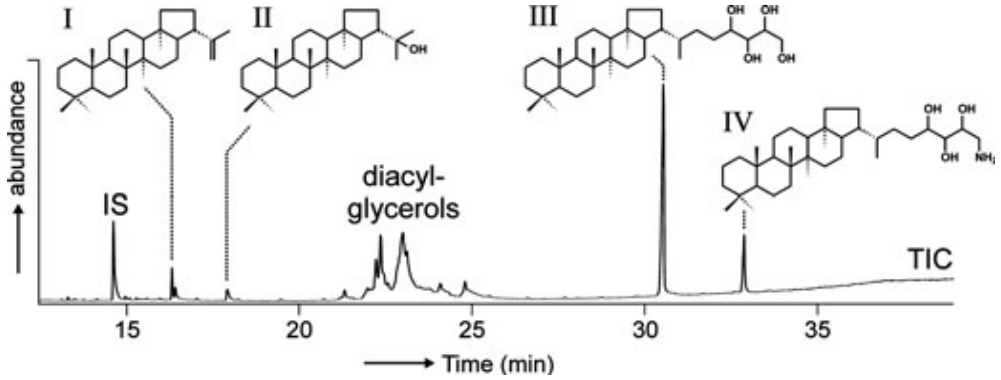

Fig. 7.3. The reconstructed total ion chromatogram of the acetylated total extract of *Desulfovibrio* strain BSS2 showing the distribution and abundance of gas chromatography amenable compounds. For structures see Fig. 6.2. IS indicates the internal standard.

Nevertheless, our data show for the first time that anaerobically grown members of the genus *Desulfovibrio* are sources for hopanoids. Moreover, the wide distribution of the Desulfovibrionaceae and its early appearance in the tree of life suggests that this group is likely an important source of sedimentary hopanoids.

7.2.2. Stable carbon isotope signatures

Signatures of stable carbon isotopes of hopanoids and *n*-alkyl lipids from the *Desulfovibrio* strains are shown in Table 5.2. Biomass and the presumptive substrate, lactate, are in the same range, indicative of a predominantly heterotrophic mode of carbon assimilation. However, *n*-alkyl lipids and hopanoids differ greatly in their $\delta^{13}C$ values. In the three strains hexadec-9-enoic acid is depleted in ^{13}C by 15–20‰ relative to biomass, whereas $\delta^{13}C$ values of hopanoids are only slightly depleted by less than 3‰. Most commonly, isotope fractionation occurs during the formation of

acetyl-CoA, which is the biosynthetic precursor for *n*-alkyl lipids (i.e. fatty acids; Hayes, 2001).

Table 7.2. $\delta^{13}C$ values of hopanoid and *n*-alkyl lipids from all three *Desulfovibrio* strains

Strain	Biomass	Diploptene	Diplopterol	β,β-*bis*-homo-hopanol[a]	FA16:1Δ9	$\Delta\delta^{13}C_{Dipl\text{-}FA16:1\Delta9}$
Desulfovibrio BSS2	−25.1	−26.4	−26.2	−27.2	−45.0	+18.8
Desulfovibrio BSS3	−27.2	−30.6	−30.9	−27.4	−45.3	+14.4
Desulfovibrio BSS6	−26.6	−26.2	−27.7	−28.7	−41.9	+14.2

a. After side-chain cleavage (periodate treatment). $\Delta\delta^{13}C_{Dipl\text{-}FA16:1\Delta9}$ gives the difference in $\delta^{13}C$ between diplopterol and hexadec-9-enoic acid.
$\delta^{13}C$ values are given in per mill versus VPDB. $\delta^{13}C$ values of the substrates were for the lactate −27.6‰ and for the CO_2 in the gas phase −35.2‰. $\delta^{13}C$ values of FA16:1Δ9 were corrected for the addition of carbon atoms during derivatization.

However, different sources for acetyl-CoA may be used even in one organism and may result in considerably different stable carbon isotope signatures of the respective products. For instance, $\delta^{13}C$ analyses of an iron-reducing bacterium grown under different levels of oxygen exposure showed that fatty acids synthesized under anaerobic conditions are much more depleted in ^{13}C relative to biomass than if synthesized under aerobic conditions (Teece *et al.*, 1999). In fact, the primary route of acetyl-CoA production in this bacterium grown with oxygen involves the pyruvate dehydrogenase enzyme complex, leading to only slightly ^{13}C-depleted fatty acids (relative to the substrate; Teece *et al.*, 1999). In contrast, under anoxic conditions acetyl-CoA is mainly formed via the serine cycle, leading to strong differences in $\delta^{13}C$ values between fatty acids and biomass by up to 15‰ (Teece *et al.*, 1999; Zhang *et al.*, 2003). Consequently, the strong depletions in ^{13}C of the fatty acids relative to substrate and biomass we observed suggest that the serine cycle plays the key role in the acetyl-CoA production in our *Desulfovibrio* strains.

However, in contrast to fatty acids, the hopanoids in the *Desulfovibrio* species are only slightly depleted in ^{13}C compared with biomass (Table 7.2). These results suggest that, compared with fatty acids a different carbon source is used for isoprenoid biosynthesis. Two major pathways for isoprenoid biosynthesis are known, the classical mevalonate pathway (MVA route) and the recently described methylerythritol pathway (MEP route; Flesch and Rohmer, 1988; Rohmer *et al.*, 1993; Rohmer, 1999). Isoprenoid biosynthesis via the MVA route bases, alike within fatty acid synthesis, on acetyl-CoA. Assuming biosynthesis of the hopanoids by *Desulfovibrio* using that pathway, the respective acetyl-CoA must derive from a

carbon source different from that of the large acetyl-CoA pool used for fatty acid synthesis. In contrast, the MEP route is independent of acetyl-CoA. Considering the high difference in $\delta^{13}C$ between hopanoids and fatty acids, the MEP route is therefore more likely as enzymatic pathway of hopanoid biosynthesis in our *Desulfovibrio* strains. Support for this assumption comes from findings of MEP-specific enzymes in phylogenetically related bacteria (Rohmer, 1999; Heidelberg *et al.*, 2004). Nevertheless, in view of organic geochemical interpretations our data show that different lipid classes synthesized by the same organism can differ considerably in stable carbon isotope signatures.

7.2.3. Extended hopanoids in environments showing sulphate reduction

In addition to the hopanoids in the *Desulfovibrio* cultures, extended hopanoids were also found in a microbial mat that oxidized methane anaerobically and a deep-sea sediment obtained from the Black Sea, which demonstrates the presence of hopanoid-producing bacteria in this strictly anoxic environment. In both samples, BHT and aminotriol were most dominant, followed by aminotetrol (Table 7.3). Total BHP accounted for 0.1% of the lipids extracted from the mat and 0.2% from the sediment respectively. Based on the amounts of hopanoids found in the lipid extracts of the *Desulfovibrio* strains, hopanoid-producing bacteria are only minor constituents of the microbial community in the mat and the sediment. Provided that Desulfovibrionaceae are the main sources for the hopanoids in these samples, this would be in line with minor occurrences of members of this group in the Black Sea and other AOM (anaerobic oxidation of methane) environments demonstrated by molecular microbiological investigations (Knittel *et al.*, 2003; Treude, 2004). However, we also found a BHT cyclitol ether (**VI**) in the microbial mat and the sediment (Table 7.3). This compound was absent in the *Desulfovibrio* strains in pure culture. Therefore, additional, still-unknown, anaerobic hopanoid-producing bacteria are likely present in the Black Sea samples.

Table 7.3. Concentrations of bacteriohopanepolyols (BHPs) in a microbial mat and a sediment from anoxic parts of the Black Sea

	BHT[a]	Aminotriol[a]	Aminotetrol[a]	Cyclitolether[a]	Total BHPs[b]
Microbial mat (No. 757)	25	43	8	25	0.004
Sediment (No. 30)	62	8	Tr.	30	0.010

a. Amounts are given in percentage of total hopanoids.
b. Total hopanoid concentrations in mg g^{-1} calculated on dry weight. For structures see Fig. 6.2. Compounds were identified and quantified using LC-MS; Tr. indicates trace amounts.

Hopanoids have also been found at anaerobic sites that are not primarily influenced by AOM. These include upwelling areas such the Benguela system off the coast of Namibia (Bednarczyk *et al.*, 2005) or off the coast of Peru (Farrimond *et al.*, 2000). Upwelling areas are characterized by high primary production and intermittent or constant anoxic conditions in the sediment. Sulphate reduction plays a key role in the respective sediments (Brüchert *et al.*, 2003) and, interestingly, tetrafunctionalized BHPs prevail within the hopanoid fraction (Farrimond *et al.*, 2000; Bednarczyk *et al.*, 2005). This dominance is apparently characteristic of marine deposits (Farrimond *et al.*, 2000). In contrast, the hopanoid pool of freshwater environments is often enriched in penta- and hexafunctionalized hopanoids (Farrimond *et al.*, 2000) originating from type I and type II methanotrophic bacteria and/or cyanobacteria (Bisseret *et al.*, 1985; Talbot *et al.*, 2003a). As in marine settings, sediments of several anoxic Spanish lakes with high concentrations of sulphate also contain mostly tetrafunctionalized hopanoids with BHT and aminotriol predominating (Talbot *et al.*, 2003a). Our data and the distribution of hopanoids in sulphate-containing environments indicate that members of the Desulfovibrionaceae or yet-uninvestigated hopanoid-producing SRB are possible contributors of tetrafunctionalized BHPs at recent marine and specific freshwater environments.

Moreover, fossil samples dating back to the Archean show high amounts of diagenetically resistant hopanoid hydrocarbons. Both A-ring-methylated and, quantitatively dominant, non-methylated hopanes occur in 2.77-Gya-old Western Australian bitumens (Brocks *et al.*, 1999). Based on the presence of 2α-methylhopanes, oxygenic photosynthetic cyanobacteria are suggested as the sources. Sources of the accompanying non-methylated hopanes have not been discussed for these and also younger, hopanoid-containing samples. Knowing now that at least some SRB are able to produce hopanoids under strictly anoxic conditions makes them a likely source for these compounds in fossil sediments. The interpretation of hopanoid occurrences in sediments covering nearly 3 Gya of Earth history might thus need to be reconsidered. In any case, the presence of hopanoids in sediments can no longer be used as an indicator for oxygen-respiring microorganisms.

7.3. Experimental procedures

7.3.1. Sampling

For bacterial isolation a microbial mat was obtained using the manned submersible '*Jago*' aboard the Russian RV '*Professor Logachev*' on the north-western shelf near the Ukrainian Crimean Peninsula in the summer of 2001 (230 m water depth; Michaelis *et al.*, 2002). The sediment was sampled during the same cruise from heavy-box coring at a water depth of 2000 m (43°32′N, 33°07′E; sample code 30), while the mat for lipid investigations was also obtained from the anoxic part of the Black Sea at 180 m water depth (43°47′N, 31°59′E; sample code 757).

7.3.2. Bacterial isolation and cultivation

Direct dilution series were prepared from a homogenized mat sample. The samples were incubated at 12°C in glass tubes (20 ml) with butyl-rubber stoppers and screw caps and contained 14 ml of artificial seawater medium (Widdel and Bak, 1992) adjusted to the salinity and sulphate content of the Black Sea. Bicarbonate-CO_2 was used as a buffer (pH 7.5) under a headspace of N_2/CO_2 [90/10 (v/v)] pre-reduced with 0.5 mM sulfide. Lactate was added from sterile stock solutions. Growth was detected by measuring sulfide and subsequent microscopic examination of positive tubes. Pure cultures were obtained after two agar dilution series of the highest positive dilution. Mass cultures of the strains BSS2, BSS3 and BSS6 for further analysis were grown in 5 l glass bottles. Cells were harvested in an anoxic chamber, centrifuged and stored at -80°C.

7.3.3. Sequencing and phylogenetic analysis

DNA was extracted from approximately 10 ml of freshly grown cultures with the DNeasy tissue kit (Qiagen, Hilden, Germany). The purified polymerase chain reaction product of the GM3/G4 primer pair (Murray *et al.*, 1996) for the 16S-rRNA gene was sequenced with the 3100 Genetic Analyser Abi Prism (Applied Biosystems, Hitachi) using dye-labelled dideoxyoligonucleotides. Sequence data were analysed with the sequencher program (Gene Codes Cooperation, MI, USA). Phylogenetic affiliations were checked by Blast search in the recent GenBank Data Library (http://www.ncbi.nlm.nih.gov/blast/). Subsequently, the sequences were checked for nearest relatives and aligned and phylogenetically analysed with the ARB software package (Ludwig *et al.*, 2004).

7.3.4. Lipid extraction and derivatization

About 200 mg of dry cells was extracted using a modified method (Summons *et al.*, 1994) based on Bligh and Dyer (1959) leading to total lipid extracts of 88 mg g^{-1} dw for *Desulfovibrio* BSS2, 67 mg g^{-1} dw for BSS3 and 78 mg g^{-1} dw for BSS6. Total hopanoids accounted for 16% of lipids from *Desulfovibrio* strain BSS2, 23% from strain BSS3 and 13% from strain BSS6. Extraction of the mat (1 g_{dw}) and the sediment (2 g_{dw}) yielded 7 mg g^{-1} dw and 8 mg g^{-1} dw total-lipid extracts respectively. Total hopanoids accounted for 0.1% for the mat and 0.2% for the sediment of the total lipid extracts respectively.

Alcohols in the combined, total-organic extracts were acetylated and/or silylated and analysed by gas chromatography (GC), GC-MS, GC-MS-MS, and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Alcohols were acetylated using a mixture of acetic acid anhydride and pyridine (1:1, v:v, 50°C for 1 h and overnight at room temperature). The pyridine/acetic acid anhydride mixture was dried under vacuum. Additionally, alcohols were silylated using BSTFA (N,O-*bis*[trimethylsilyl]-trifluoroacetamide) for 1 h at 80°C.

The presence of BHPs was examined by *cis*-1,2-diol cleavage induced by H_5IO_6 oxidation, and subsequent $NaBH_4$ reduction after a method previously described (Rohmer *et al.*, 1984). Alcohols formed during this procedure were analysed as their acetates or BSTFA derivatives. Cleavage of the hopanoid side-chain by treating extracts of the isolates *Desulfovibrio* strain BSS2, BSS3 and BSS6 resulted in the release of high amounts of 17β(H),21β(H)-*bis*-homohopanol (C_{32}-hopanol). This demonstrated the presence of BHPs with hydroxy groups at C-32 and C-33, because hopanoid side-chains are cleaved at vicinal hydroxy groups. Minor amounts of 17β(H),21β(H)-homohopanol (C_{31}-hopanol) were also found.

Fatty acids for $\delta^{13}C$ analyses were obtained using a method described elsewhere (Thiel *et al.*, 2002).

7.3.5. Gas chromatography mass spectrometry (GC-MS)

Gas chromatography-amenable hopanoids were analysed by GC-MS-MS using a Micromass Quattro II spectrometer (EI, 70 eV) interfaced to a HP6890 GC with a DB5-HT [15 m length, 0.32 mm ID, 0.1 µm film thickness; temperature programme 1 min at 50°C; from 50°C to 200°C at 15°C min^{-1} (held for 1 min), from 200°C to 250°C at 10°C min^{-1} (held for 1 min), and from 250 to 350°C min^{-1} at 5°C min^{-1} (held for 8 min)]. The GC-MS interface was held at 350°C (source temperature 230°C).

Under these conditions, diploptene (**I**), diplopterol (**II**), BHT (**III**) and aminotriol (**IV**) are GC-amenable (Fig. 7.3). Hopanoids were quantified using the GC-MS total ion current relative to two internal standards [5α(H)-cholestane for the non-extended hopanoids and pregnane-3β,20β-diol acetate for the BHPs].

Hopanoids more polar than aminotriol were identified and quantified using an LC-MS^n system (see below).

7.3.6. Liquid chromatography mass spectrometry (LC-MS)

LC-MS^n analyses were performed using a Surveyor HPLC system (ThermoElectron, Hemel Hempstead, UK) interfaced with a ThermoElectron Finnigan™ LCQ ion trap mass spectrometer. HPLC separation was achieved using a Phenomenex Gemini C_{18} 5 µm HPLC column (150 mm × 3 mm ID) and a Security Guard™ of the same material. The column oven temperature was maintained at 30°C. The HPLC flow rate was 0.5 ml min^{-1} with the following solvent gradient profile: 90% A and 10% B (0 min); 59% A, 1% B and 40% C (at 25 min) then isocratic to 45 min [where A = MeOH, B = water and C = propane-2-ol; all HPLC grade, purchased from Fisher (Loughborough, UK)]. The mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode. Atmospheric pressure chemical ionization source settings were: capillary temperature 160°C, vaporizer temperature 490°C, corona discharge current 8 µA, sheath gas flow 60 and auxiliary gas 10 (arbitrary units). The instrument was tuned as described previously (Talbot *et al.*, 2003b). Detection was achieved at an isolation width of *m/z* 3.0 and fragmentation with normalized collisional dissociation energy of 30% and a *Q*-value of 0.15. Bacteriohopanepolyols were identified by comparison of their APCI MS^2 spectra with those of authentic peracetylated standards provided by Michel Rohmer (Université Louis Pasteur, Strasbourg, France). Quantification was achieved relative to the acetylated internal standard (pregnane-3β,20β-diol) allowing for response factors as follows under the conditions used: BHPs which do not contain nitrogen give a response eight times that of the standard; BHPs containing one or more nitrogen atoms give a response 12 times that of the standard.

7.3.7. Peak identification

Structural determinations of triterpenoids base on GC and LC co-injections with authentic standard components (e.g., diploptene, diplopterol, peracetylated BHT, aminotriol and aminotetrol) and mass spectral fragmentations of BSTFA derivatives, acetylated alcohols (see Talbot *et al.*, 2003b) and the non-derivatized compounds.

7.3.8. $\delta^{13}C$ analysis of biomass, lactate, CO_2 and lipids

$\delta^{13}C$ values of biomass and the substrate (lactate) were analysed using an elemental analyser coupled to a Finnigan MAT 252 IRMS (EA-*C*-IRMS). CO_2 from the gas phase was analysed – after cryogenic separation from N_2– using a dual-inlet system linked to the IRMS. For compound-specific stable carbon isotope analyses (GC-*C*-IRMS), samples were injected splitless (1 min) onto a 30 m fused silica column (DB5-MS, 0.32 mm ID, 0.25 µm film thickness; Carrier gas: He). Combustion of the components to CO_2 was performed with a CuO/Ni/Pt-furnace operated at 940°C. Gas chromatography temperature programme was the same as described above. Standard deviations for replicate injections were less than 0.5‰. Gas chromatography-combustion-isotope ratio mass spectrometry precision was checked using a standard alkane mix (*n*-C15 to *n*-C29) with known isotopic composition. The stable carbon isotope compositions are reported in the delta notation ($\delta^{13}C$) versus the VPDB standard.

Hopanoid-production by *Desulfovibrio bastinii* isolated from oilfield formation water

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Abstract

Hopanoids are important lipid components of many bacterial groups and are therefore ubiquitous in soils, sediments, and rocks. Until recently it was thought that the synthesis of hopanoids is restricted to at least microaerophilic bacteria and consequently geological findings of hopanoids were used as an indication for oxygenated settings. Recent studies, however, demonstrated the biosynthesis of hopanoids under strictly anoxic conditions by a few bacterial groups, though their relevance is still unclear. We therefore extended our previous work studying hopanoid production among members of the genus *Desulfovibrio,* a group of sulphate reducing bacteria (SRB) widely distributed in marine sediments, water logged soils, and oil reservoirs. We found three species (*D. halophilus, D. vulgaris* Hildenborough, and *D. africanus*) to be absent of hopanoids. In contrast, *D. bastinii* contains high amounts of non-extended hopanoids and bacteriohopanepolyols (BHPs) with diploptene, 17β(H),21β(H)-bacteriohopane-32,33,34,35-tetrol (BHT) and 17β(H),21β(H)-35-aminobacteriohopane-32,33,34-triol being the major compounds. Since the moderately halophilic *D. bastinii* was isolated from a deep subsurface oil formation water, a contribution of hopanoids by SRB to the intrinsic oil hopanoid inventory is feasible which would influence hopanoidal compositions often used for organic-geochemical characterization purposes. Nevertheless, our data indicate that hopanoid production might be common but not obligate in the genus *Desulfovibrio*.

8.1. Introduction

The wide distribution of polyfunctionalised pentacyclic triterpenoids (biohopanoids) in the domain bacteria (Rohmer *et al.*, 1984, Talbot & Farrimond, 2007) is thought to account for the ubiquitous presence of the so called sedimentary geohopanoids, which have even been preserved in rocks as old as 2.77 billion years (Brocks *et al.*, 1999). The majority of biohopanoids consists of bacteriohopanepolyols (BHPs) which include, in addition to the pentacyclic triterpenoidal ring system, a side chain exhibiting different functionalities (hydroxy-, amino-, sugar-groups etc.). Based on the study of almost 300 bacterial isolates it was suggested that about 60% of bacteria produce hopanoids (Talbot & Farrimond, 2007). However, these data may be biased towards the selection of bacteria. Indeed, contrary to these reports, a recent genetic study revealed that only less than 10% out of 600 bacteria are potentially capable of producing hopanoids (Pearson *et al.*, 2007). The respective bacteria possess the squalene-hopene-cyclase enzyme, which is necessary for hopanoid biosynthesis. Hopanoids are proposed as essential bacterial membrane constituents, analogue to sterols in eukaryotes (Ourisson & Rohmer, 1992). However, the relatively low number of potential hopanoid producing bacteria demonstrated by Pearson *et al.* (2007) suggests a different, yet unexplained but vital role in the bacterial domain.

Although hopanoids biosynthesis is apparently independent of molecular dioxygen (Ourisson & Rohmer, 1991), laboratory studies indicated that hopanoid production among bacteria is restricted to aerobic species with only a small number of facultative anaerobic bacteria as exceptions (e.g., Neunlist *et al.*, 1985; Neunlist *et al.*, 1988). However, a few recent studies revealed the capability of BHP-synthesis by strictly anaerobic bacteria such as Planctomycetes performing the anaerobic oxidation of ammonium (Sinninghe Damsté *et al.*, 2004), iron-oxidising *Geobacter* species (Fischer *et al.*, 2005, Härtner *et al.*, 2005), and sulphate-reducing *Desulfovibrio* species isolated from very special Black Sea microbial mats (Blumenberg *et al.*, 2006).

Extending our previous work on hopanoid production by SRB, we here present data of four additional members of the genus *Desulfovibrio.* Therewith we demonstrate that hopanoid production by sulphate-reducers is not restricted to the already known SRB isolated from a Black Sea microbial mat but also occurs within bacterial strains phylogenetically closely clustering together among the genus *Desulfovibrio*.

8.2. Materials and Methods

8.2.1. Bacterial isolates and phylogeny

Three members of the genus *Desulfovibrio* (*D. halophilus* DSMZ5663^T, *D. bastinii* 16055^T, and *D. africanus,* DSMZ2603^T) were grown anaerobically on two different media. For *D. halophilus* and *D. bastinii* the medium contained the following compounds per litre of deionised water: NaCl, 70 g; $MgCl_2$ • 6 H_2O, 3 g; KCl, 0,5 g; $CaCl_2$ • 2H_2O, 0.15 g; NH_4Cl, 0.03 g; KH_2PO_4, 0.2 g; Na_2SO_4, 3 g; $NaHCO_3$, 2.5 g; Na-(L)-lactate 2,25 g; Na_2S • 9 H_2O, 0.1 g; vitamin solution V7 (Pfennig & Trüper, 1992), 1 ml; trace element solution SL 12 (Overmann *et al.*, 1992), 1 ml and selenite-tungstate solution, 1 ml. The medium for *D. africanus* contained: $MgCl_2$ • 6 H_2O, 0,5 g; KCl, 0,5 g; $CaCl_2$ • 2 H_2O, 0.1 g; NH_4Cl, 0.5 g; KH_2PO_4, 0,2 g; Na_2SO_4, 2.84 g; $NaHCO_3$, 2.5 g; Na_2S • 9 H_2O, 0,1 g; Na-(L)-lactate 2 g; Na-acetate, 0,82 g; vitamin solution, 1 ml; trace element solution (SL 12), 1 ml; selenite-tungstate solution, 1 ml and yeast-extract 0,2 g per litre of deionised water. The media were prepared according to Widdel & Bak (1992). The cultures were incubated in glass bottles (2 l), closed with butyl-rubber stoppers under a headspace of N_2/CO_2 [90/10 (v/v)]. They were kept in the dark at a constant temperature of 30°C and manually shook three times a day. The pH was adjusted prior to inoculation and during growth with sterile NaOH and HCl (6.8-7.0 for *D. halophilus* and *D. bastinii* and 7.8 for *D. africanus*). Growth phase was monitored with optical density measurements at 450 nm (Spectronic 401; Spectronic Instruments, USA). The cells were then harvested during the early stationary growth phase of the culture, centrifuged, and the biomass was stored in organic free glass tubes (heated to 500°C for 4h) at -80°C until further analysis. Cell material of anaerobically grown *D. vulgaris* Hildenborough (DSMZ 644^T) was kindly provided by Florin Musat and Friedrich Widdel (Max-Planck-Institute for Marine Microbiology Bremen, Germany).

Sequences from the NCBI database as well as from Blumenberg *et al.* (2006) were aligned and phylogenetically analysed with the freely available MEGA 4.0 software (Tamura *et al.*, 2007).

8.2.2. Extraction, derivatisation, cleavage of hopanoid side chain, and hopanoid analyses (gas- and liquid chromatography-mass spectrometry, GC-MS; LC-MS)

Analytical details for the analysis of non-extended and extended hopanoids were previously published (Blumenberg *et al.*, 2006). Briefly, about 200 mg of dry cells were extensively extracted with dichloromethane/methanol (1/1; v/v). An aliquot of the combined extract was acetylated and analysed for BHPs by LC-MS (see below). Another aliquot was separated by column chromatography into a hydrocarbon-, an alcohol and keto-, and a polar fraction. The latter was subjected to a cleavage reaction of vicinal diols induced by H_5IO_6 oxidation, and subsequent $NaBH_4$ reduction after a method previously described (derivatization according to procedure 2 in Rohmer *et al.*, 1984). Hopanols were silylated using BSTFA (N,O-*bis*[trimethylsilyl]-trifluoroacetamide) for 1h at 80°C. Hopanoid hydrocarbons and derivatised non-extended hopanols were analysed by gas chromatography-mass spectrometry and quantified using an internal standard with known concentration (5α(H)-cholestane). For further experimental details see Blumenberg *et al.* (2006).

For the analyses of peracetylated BHPs an LC-MS system using positive atmospheric pressure chemical ionisation (APCI) was used (for further details see Blumenberg *et al.* (2007b). Please note that mass ions given represent nominal masses, ignoring the mass defect of isotopes, and not exact masses of the molecules and fragments. Concentrations of 17β(H),21β(H)-bacteriohopane-32,33,34,35-tetrol (BHT) and 17β(H),21β(H)-35-aminobacteriohopane-32,33,34-triol (35-aminobacteriohopanetriol) were gained from comparisons of acetylated BHP peaks in the sample with that of authentic standards (acetylated BHT and 35-aminobacteriohopanetriol) with known concentrations (from the total ion current) and were corrected for the addition of acetate groups.

8.3. Results and Discussion

Recent work showed that the commonly held assumption of pentacyclic triterpenoids (hopanoids) being almost exclusively produced by aerobic bacteria is incorrect (Sinninghe Damsté *et al.*, 2004, Fischer *et al.*, 2005, Härtner *et al.*, 2005, Blumenberg *et al.*, 2006). Those anaerobes include sulphate reducing bacteria belonging to the widely distributed genus *Desulfovibrio* (Blumenberg *et al.*, 2006), which prompted us to extend the search for hopanoid production to additional *Desulfovibrio* species. Our work demonstrated that three of four were absent of hopanoids in their lipids under the growth conditions applied (*D. vulgaris* Hildenbourough, *D. africanus*, *D. halophilus*; Table 8.1). The lack of hopanoids in *D. vulgaris* Hildenborough is in accordance with the absence of the squalene-hopene-cyclase in this fully sequenced member of the genus *Desulfovibrio* (Heidelberg *et al.*, 2004; standard tblastn search for the shc-gene (GI: 2851526) according to Pearson *et al.*, 2007). High amounts of BHPs, however, accompanied by diploptene (hop-22(29)-ene) and diplopterol (hopan-22-ol; Table 8.1), were present in *Desulfovibrio bastinii* (strain DSMZ 16055^T) grown under strictly anoxic conditions. By using liquid chromatography-mass spectrometry (LC-MS) we identified 17β(H),21β(H)-bacteriohopane-32,33,34,35-tetrol (bacteriohopanetetrol; BHT) and 17β(H),21β(H)-

Table 8.1. Concentrations of hopanoids in *Desulfovibrio* species studied

	GC-MS					LC-MS		
	Hop-22(29)-ene[§]	Diplopterol[§]	17β(H),21β(H)-Homohopanol[†]	17β(H),21β(H)-bishomohopenol[†]	17β(H),21β(H)-bishomohopenol[†]	35-Aminobacterio-hopanetetrol[‡]	35-Aminobacterio-hopanetriol[‡]	BHT[‡]
Desulfovibrio bastinii	55 (134)	86 (201)	131 (296)	85 (187)	630 (1382)	Trace	190 (349)	430 (788)
Desulfovibrio vulgaris Hildenborough	-	-	-	-	-	-	-	-
Desulfovibrio africanus	-	-	-	-	-	-	-	-
Desulfovibrio halophilus	-	-	-	-	-	-	-	-

[§]Free GC-amenable compounds.
[†]After cleavage of vicinal diols using periodic acid
[‡]Intact bacteriohopanepolyols [± 20%][#];
Concentrations are given in µg g^{-1} dry cells (in brackets in nmol g^{-1} dry cells).

35-aminobacteriohopane-32,33,34-triol as major BHPs (Fig. 8.1), both also predominating in the previously published *Desulfovibrio* species isolated from a Black Sea microbial mat (Blumenberg *et al.*, 2006).

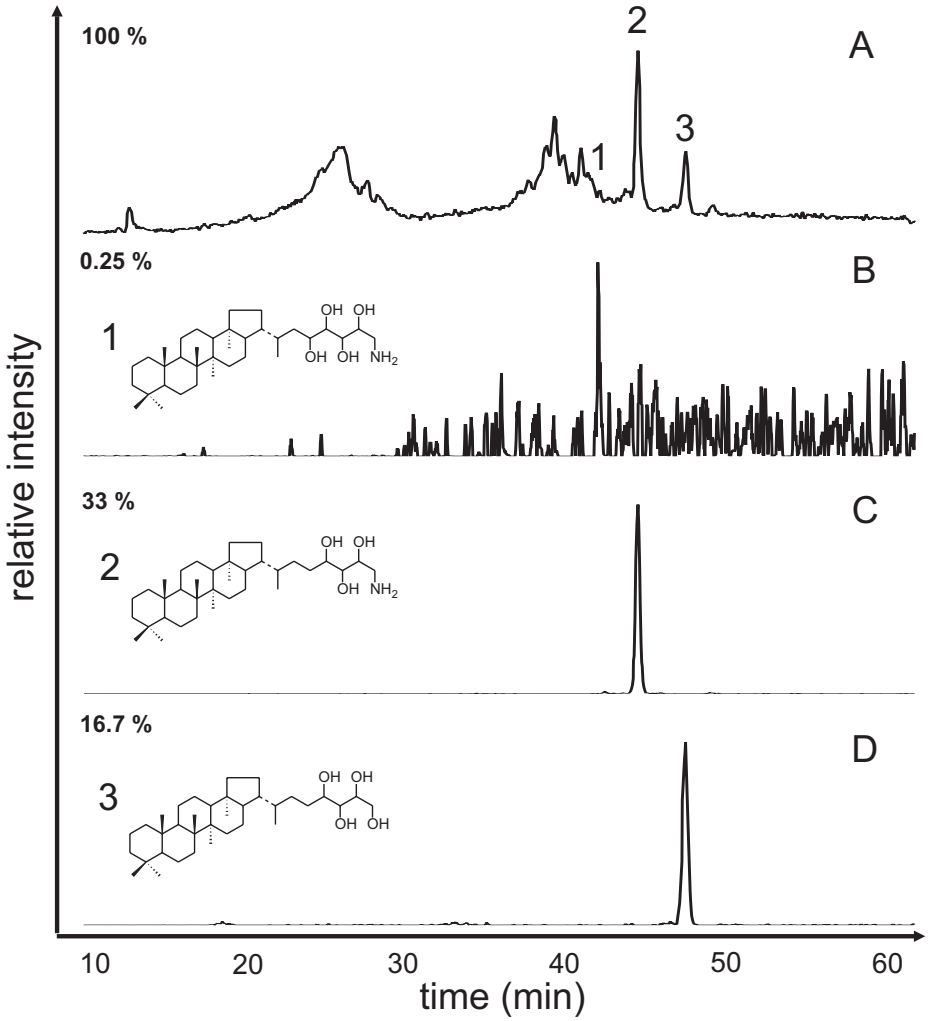

Fig. 8.1. A. LC-MS total ion chromatogram (TIC) of the acetylated extract of *Desulfovibrio bastinii* and ion traces indicative of bacteriohopane-polyols (B to D).
B. Ion trace of compound 1 in A (17β(H),21β(H)-35-aminobacterio-hopanetetrol; *m/z* 772).
C. Ion trace of compound 2 in A (17β(H),21β(H)-35-aminobacterio-hopanetriol; *m/z* 714).
D. Ion trace of compound 3 in A (17β(H),21β(H)-bacteriohopane-32,33,34,35-tetrol; *m/z* 655).

Trace amounts of the pentafunctionalised 17β(H),21β(H)-35-aminobacteriohopane-31,32,33,34-tetrol were also found. However, the vast majority of BHPs in *D. bastinii* consists of tetrafunctionalised compounds (about ~30% 35-aminobacteriohopanetriol and ~70% BHT of the sum of BHPs identified). The predominance of tetrafunctionalised BHPs in *D. bastinii* was also confirmed by performing a cleavage of vicinal diols, releasing mainly 17β(H),21β(H)-*bis*homohopanol (see Table 8.1). However, relatively high amounts of 17β(H),21β(H)-homohopanol were also found which can not be explained by cleaving the relatively low concentrations of 35-aminobacteriohopanetetrol found by LC-MS (35-aminobacteriohopanetetrol bears

OH-groups at positions C_{31} and C_{32}, resulting in the release of homohopanol after cleavage of vicinal diols). Consequently, additional BHPs with hydroxy groups at C_{31} and C_{32} must be present in *D. bastinii* which were not recognised by the LC-MS method used. In addition, high amounts of a monounsaturated 17β(H),21β(H)-*bis*homohopanol were also observed after cleavage of the hopanoid side-chain. The introduction of a double bond may represent an initial step of a degradation sequence leading to highly unsaturated and/or aromatic pentacyclic ring containing compounds often found in sediments (Ourisson & Albrecht, 1992). However, several bacterial isolates as well as natural samples were also demonstrated to contain considerable amounts of ring-unsaturated BHPs (Cvejic *et al.*, 2000a, 2000b, Fischer *et al.*, 2005, Talbot *et al.*, 2007, Wakeham *et al.*, 2007). We therefore rather believe the *bis*homohopenol to originate from a yet unknown unsaturated BHP although we cannot exclude an artificial formation induced by the cleavage of vicinal diols.

The hopanoid containing *D. bastinii* was first described by Magot *et al.* (2004) who isolated this slightly halophilic bacterium from production-water samples of the Emeraude Oilfield (Congo). The authors concluded this mesophilic SRB to be indigenous to the oil reservoir, originating from a deep subsurface environment. Mesophilic and thermophilic sulphate-reducing bacteria are common inhabitants of oil fields and formation waters (Tardy-Jacquenot *et al.*, 1996) and may penetrate into oil reservoirs together with the injection water, thus contaminating the producing well (e.g., Magot *et al.*, 2000). Interestingly, the most frequently isolated mesophilic SRB from oil field formation waters belong to the genus *Desulfovibrio* (Magot *et al.*, 2000, Miranda-Tello *et al.*, 2003). Therefore, *D. bastinii* as well as yet unknown SRB may thus be contributors of hopanoids to the formation waters of oil reservoirs. Although the relevance of these processes still needs further investigation, the use of hopanoid distributions for oil-source rock correlation or other organic-geochemical purposes from formation waters which are strongly affected by SRB needs caution.

In contrast to earlier conclusions based on negative findings in *Desulfovibrio desulfuricans* (NCIB 8310) (Rohmer *et al.*, 1984), which was obviously erroneously classified and is now named *Desulfomicrobium norvegicum* (Genthner *et al.*, 1997), our observations of hopanoids in *D. bastinii* and other *Desulfovibrio* species (Blumenberg *et al.*, 2006) clearly show the general capability of hopanoid-production by members of this genus (Fig. 8.2). Future studies have to show if additional, phylogenetically closely related *Desulfovibrio* (e.g., *D. zosterae*, *D. salexigens*, *D.*

hydrothermalis; Fig. 8.2) are also sources of hopanoids and may thus potentially contribute to the hopanoid pool in their natural anoxic habitats.

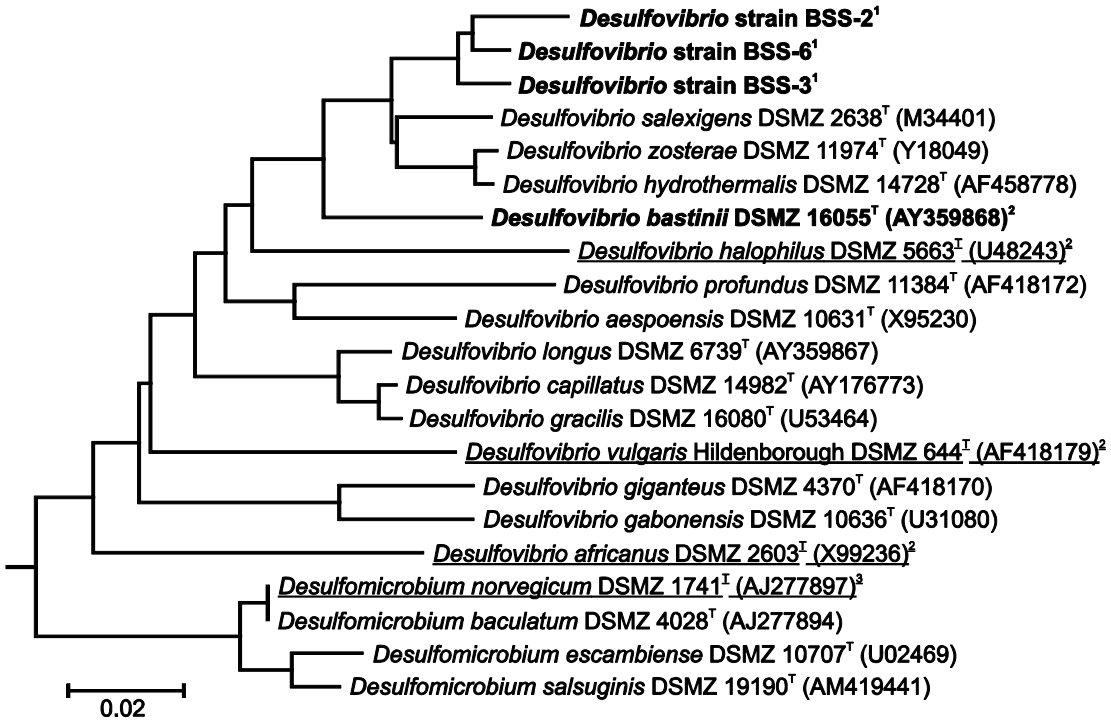

Fig. 8.2. Rooted phylogenetic tree showing the affiliation of *Desulfovibrio* species analysed here based on 16S rRNA Gene sequences. The tree was calculated using the Neighbour-Joining algorithm. Tree topology was evaluated by Maximum Likelihood analyses. Accession numbers are indicated. Underlined species were analysed and free of hopanoids whereas species in bold contain hopanoids. 1 = Blumenberg *et al.* (2006), 2 = this study, 3 = Rohmer *et al.* (1984).

Nevertheless, the role of hopanoids in *Desulfovibrio* as well as other bacteria is still enigmatic. This holds especially true since the recently demonstrated relatively low distribution of hopanoids in bacteria (Pearson *et al.*, 2007) clearly argues against the yet proposed essential role in bacteria. Feasible, however, is the protection of sensitive enzymes, such as the nitrogenase enzyme complex, against oxygen exposure (Rohmer, (1999) and refs. cited therein) or also other sensitive enzymes (e.g., the anammoxosomes of anaerobically ammonia oxidising Plantomycetes). For instance, although the identification of such a function needs further investigation there appears to be a wide, even though not complete, overlap of bacteria containing *nif* genes that encode the nitrogenase enzyme complex and the squalene-hopene cyclase, necessary for the production of hopanoids (Berry *et al.*, 1993, Auman *et al.*, 2001; Pearson *et al.*, 2007). Interestingly, also members of the genus *Desulfovibrio* contain the *nif* gene (Heidelberg *et al.*, 2004, Pearson *et al.*, 2007) necessary for the fixation of N_2 and, in fact, nitrogen fixation is common in SRB (Postgate *et al.*, 1988). However, sulphate-reducing bacteria commonly thrive in anoxic environments so that

their enzymes do not have to be protected from oxygen. On the other hand many members of the *Desulfovibrio* are known to cope with low amounts of oxygen while some are able to grow in the presence of nearly atmospheric oxygen levels (Cypionka, 2000, Lobo *et al.*, 2007). Among sulphate reducing bacteria various strategies against oxygen exposure have been developed, including aggregation or aerotaxis, and enzymatic systems dedicated to the reduction and the elimination of oxygen and its reactive species (Dolla *et al.*, 2006). Whether or not hopanoids may be one strategy in oxygen protection in facultative anaerobic bacteria is not known yet. Further respective studies have to be conducted but a possible explanation for the capability of some *Desulfovibrio* to survive in sub-oxygenated settings is that they contain hopanoids while other *Desulfovibrios* and SRB of other groups do not (*Desulfobacterium autotrophicum*, one *Desulfobacterium* and two *Desulfobacter* strains; own unpublished data).

Conclusion

In this thesis, two volcanic CO_2 vents were used as natural laboratories to assess the consequences of CO_2 leakage on a soil ecosystem. Significant, CO_2-induced changes in the soil community and microbial carbon cycle, were detected.

As study sites, CO_2 vents in the Caldera di Latera (Italy) and in the Laacher See Caldera (Germany), both located on pasture land, were chosen. Both CO_2 vents consisted of a 5 to 6 m wide area of maximal CO_2 concentration in the soil gas (>90% and >80%, respectively). In comparison the CO_2 vent in the Caldera di Latera showed higher CO_2 fluxes of 2000-3000 g m^{-2} d^{-1} than the one in the Laacher See Caldera with 400-580 g m^{-2} d^{-1}. Outside these vent areas the CO_2 concentrations in the soil gas rapidly returned to background values within a diameter of 2.5 meters.

The high CO_2 conditions led to a set of abiotic changes like anoxia, higher electrical conductivity, higher K^+, PO_4^{3-} SO_4^{2-} and NH_4^+ concentrations, lower NO_3^-, NO_2^-, Fe and Mn concentrations and a decrease of soil pH. These CO_2-induced changes, especially the soil anoxia and the low pH values, inhibited eukaryotic growth. As a consequence both sampling sites were characterised by a vegetation-free vent area. The similarities detected at both CO_2 vents suggest that the observed changes present general adaptations of highly CO_2 influenced environments.

Besides higher plants, also fungi were suppressed by high CO_2 conditions, as shown by a decrease of diagnostic fatty acids. Also aerobic microorganisms such as Crenarchaeota were diminished. Furthermore the conditions at both CO_2 vents led to enhanced production of more robust lipids like MAGEs, DAGEs and GDGTs by resident microorganisms. Interestingly, production of significant amounts of bacterial MAGEs and DAGEs were also detected at the reference site (>10 µg g^{-1} TOC). This finding presents the first report of bacterial MAGEs and DAGEs in a mesophilic terrestrial environment. SRB are possible source organisms of the bacterial MAGEs and DAGEs but further research is needed to identify the source organisms. Moreover, we were able to gain information on the still unknown source organisms of the bacterial GDGTs. As higher concentrations of bacterial GDGTs were detected at both permanently anaerobic CO_2 vents compared to the respective reference sites (7.5-times higher at the CO_2 vent/Latera and 1.7-times higher at the CO_2 vent/Laacher See), our studies can support the hypothesis of Weijers *et al.* (2007)

that the source organisms are anaerobic. Furthermore we showed by stable carbon isotope analyses that the respective source organisms are heterotrophic.

The CO_2 introduced large amounts of carbon into the studied soils. Moreover, this volcanic CO_2 is enriched in ^{13}C compared to other carbon sources in these terrestrial environments. At both study sites soil TOC showed significant ^{13}C enrichment, suggesting significant biological uptake of volcanic CO_2. Hence, due to the natural isotopic label, the uptake of CO_2 by microorganisms could be traced. We applied a complimentary set of lipid biomarker studies and stable carbon isotope analyses to determine the physiology of dominant microbial groups at the CO_2 vents. By this we were able to show that autotrophic carbon fixation was strongly enhanced at both CO_2 vents. Moreover, microbial key players of the autotrophic carbon fixation and the microbial recycling of CO_2 derived carbon were determined.

The carbon cycle and the microbial key players at the CO_2 vent in the Caldera di Latera were assessed in closer detail. Hereby, ^{13}C enriched GDGT derived biphytanes, biomarkers of methanogenic Archaea, were detected here. Additionally, the concentrations of the corresponding GDGTs increased by a factor of 90 compared to the reference soil. This illustrated, supported by microbiological data, that autotrophic methanogenic Archaea profit from the conditions at the CO_2 vent. The utilisation of CO_2 by methanogenic Archaea at the CO_2 vent enhanced the production of CH_4. Since CH_4 has a 25-time higher global warming potential then CO_2, this process needs special attention considering the purpose (the reduction of GHG emissions) of CCS. Further research should also assess the possibility of additional CH_4 production along the migration pathway of a potential CO_2 leakage. As storage sites for CCS will most likely be located 800 m below the Earth surface, the findings of significant archaeal activity in the deep biosphere by Biddle *et al.* (2006) and Lipp *et al.* (2008) need to be considered in future research.

At both CO_2 vents the CH_4 production was enhanced. At the CO_2 vent in the Caldera di Latera also enhanced CH_4 consumption was detected. A high capability of aerobic methanotrophy was detected, *in vitro*, at the anoxic CO_2 vent in the Caldera di Latera. The high rates of aerobic methanotrophy can derive either from aerobic microorganisms outliving anaerobic conditions or from facultative aerobic microorganisms, capable of using multiple electron acceptors for CH_4 oxidation. Potential microbial groups for the utilization of multiple electron acceptors for CH_4

oxidation were investigated by using biomarker analyses, $\delta^{13}C$-values of biomarkers and microbiological methods.

After the consumption of O_2, organic carbon is subsequently oxidised with nitrate, Mn(IV), Fe(III) and sulphate, following the maximum energy yields of these oxidation reactions. Although this succession was not shown for AOM yet, nitrate, Mn(IV), Fe(III) and sulphate were considered as electron acceptors for AOM.

At the CO_2 vent in the Caldera di Latera, nitrate coupled AOM (0.18 nmol CO_2 d^{-1}) was detected *in vitro*. Interestingly, lipid biomarkers, previously found in aerobic methanotrophic Bacteria but also in anaerobic, denitrifying methanotrophic Bacteria were found in high quantities at the CO_2 vent (e.g., CA 18:1Δ10 up to 157.6 µg g^{-1} TOC) compared to the reference site (e.g., CA 18:1Δ10 was absent). But as their concentration increased in the lower part of the soil profile, where O_2 supply is unlikely and maximum rates of nitrate coupled AOM were detected, they were possibly related to the AOM coupled to denitrification. Moreover, as the microorganisms responsible for AOM coupled to denitrification utilise the liberated oxygen from the nitrate reduction for CH_4 oxidation, they might also be capable to proceed with their metabolism under aerobic conditions. Nevertheless, nitrate concentrations were very low in the depth of maximum nitrate coupled AOM rates, hinting to a lower relevance of this process *in situ*.

At the CO_2 vent in the Caldera di Latera low concentrations of Mn and Fe were detected compared to the reference soil, especially in the lower soil horizons (e.g., Fe: 33.29 vs. 65.11 g kg_{dw}^{-1}, respectively). Since the original material was the same at the references site and the CO_2 vent, the decrease in Mn and Fe concentrations indicate mobilisation (either biotic or abiotic) and subsequent transport of these metals. Interestingly, low pH values as detected at the CO_2 vent are a precondition for the bioavailability of Mn and Fe. Hence, also the possibility for AOM coupled to reduction of Mn and Fe was assessed. The responsible organisms in the newly discovered AOM coupled to reduction of Mn and Fe are yet unknown. But among the currently debated candidates we only detected indications for the presence of Verrucomicrobia at the CO_2 vent in the Caldera di Latera (CA *i*-14:0, *ai*-15:0 and 3-OH-16:0). Interestingly, these lipids were significantly enriched in ^{12}C (-30.6‰ for CA *ai*-15:0 and -34.4‰ for CA *i*-14:0) relative to the soil TOC (-20.7‰) in the lower soil horizon. These $\delta^{13}C$ values indicate uptake of CH_4 by the source organisms.

Moreover, Verrucomicrobia are the only methanotrophic Bacteria known to survive in a low pH (pH <4) environment like the one at the studied CO_2 vent (pH 3.2-4.1).
As shown by lipid biomarkers and sulphate reduction rates, SRB were stimulated at the CO_2 vent in the Caldera di Latera. But both partners in the sulphate coupled AOM, SRB and methanotrophic Archaea, are very sensitive to oxygen. Correspondingly, lipid biomarkers of ANME Archaea were either absent (crocetan, pentamethylicosane and hydroxyarchaeol) or their $\delta^{13}C$-values (GDGT derived biphytanes) indicated methanogenic Archaea as source. Moreover, the $\delta^{13}C$-values of lipid biomarkers for SRB were enriched in ^{13}C, indicating activity of autotrophic SRB. As confirmed by data of M. Krüger (BGR), SRB are actually, together with methanogenic Archaea, the dominating autotrophic group in the CO_2 vent. But no indications for the involvement of SRB in AOM, in the studied CO_2 vents, were found.
Further research is necessary to assess the possibility of methanotrophic microorganisms utilising multiple electron acceptors and the relevance *in situ* of different methanotrophic microorganisms. Moreover, the capability of '*Candidatus* Methylomirabilis oxyfera' to oxidise CH_4 with O_2 should be tested. The relevance of Verrucomicrobia or closely related species in these anaerobic soils should also be checked. Lipid biomarker inventories of incubation experiments with microorganisms performing Mn and Fe coupled AOM should be assessed. The methanotrophic community at the CO_2 vent in the Laacher See Caldera will be studied in closer detail to identify the responsible microorganisms in this environment, in order to reveal the causes of differences between the two environments and the reason for lower CH_4 consumption.
The absence of sulphate coupled AOM was unexpected. To confirm this finding an additional class of biomarkers was assessed. In the Black Sea, microbial consortia conducting sulphate coupled AOM form thick microbial mats. Studies by Thiel *et al.* (2003) showed that these mats contained significant amounts of hopanoids and the $\delta^{13}C$-values of these hopanoid suggested that they derived from SRB involved in AOM. This study motivated us to assess hopanoids production of SRB to clarify, if hopanoids could be used as biomarkers of SRB involved in AOM.
SRB isolated from an AOM performing microbial consortia, oil reservoirs, an anoxic benthic microbial mat and fresh water were examined for their hopanoid content.
In these surveys production of diploptene, diplopterol and tetrafunctionalised BHPs (BHT and aminotriol) by SRB strains isolated from an AOM performing microbial

consortia and an oil reservoir were shown. However, the BHT cyclitol ether, which was found in the microbial mat performing AOM, was absent in the studied *Desulfovibrio* strains. Hence, the isolated *Desulfovibrio* strains are only minor constituents of the AOM microbial community and are not the relevant SRB in AOM. Therefore, additional, still-unknown anaerobic hopanoid-producing Bacteria are likely present in the Black Sea samples.

Nevertheless, our results show that some heterotrophic *Desulfovibrio* produce significant amounts of BHT and aminotriol. Interestingly, tetrafunctionalised BHPs prevail within the hopanoid fraction of sediments where sulphate reduction plays a key role but which are not primarily influenced by AOM.

BHT and aminotriol were contained in higher concentrations at the CO_2 vent. But these hopanoids are not highly diagnostic for SRB and could also derive, at least in parts, from type II methanophic Bacteria.

However, our results show that at least some SRB are able to produce hopanoids. Hence, SRB have to be considered as possible source for these compounds in fossil sediments and some earlier interpretation of hopanoid in fossil sediments might thus need to be reconsidered. In any case, the presence of hopanoids in sediments can no longer be used as an indicator for oxygen-respiring microorganisms.

Future studies have to show if additional, phylogenetically closely related *Desulfovibrio* (e.g., *D. zosterae*, *D. salexigens*, *and D. hydrothermalis*) are also sources of hopanoids and may thus potentially contribute to the hopanoid pool in their natural anoxic habitats.

The natural isotope label, as well as the limited spatial extent, made the volcanic CO_2 vents excellent natural laboratories to assess the consequences of a potential CO_2 leakage from a CO_2 storage site. The microbial carbon cycle and the responsible microorganisms were analysed with the help of lipid biomarker studies and stable carbon isotope analyses. Our findings strongly suggest that the CH_4 cycle in terrestrial systems is much more complex than previously assumed. Consequently, more research is needed to investigate the importance and distribution of Verrucomicrobia and other methanotrophic groups in these soils.

This study contributes to a deeper understanding of microbial community composition in anaerobic CO_2 dominated environments. The gained information is valuable to assess the consequences of CO_2-leakage from a carbon storage site on a soil environment. The ecosystem appears to have adapted to the different conditions

through species substitution or adaptation, showing a shift towards anaerobic and acidotolerant to acidophilic species under elevated CO_2 concentrations. CO_2 vents located in other environments and climate zones should be assessed next to evaluate the findings of this study.

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Appendix

Presentation list

Oral presentations

Oppermann, B.I. and Krüger, M. BGR plans for microbiological impact studies. March, 2007. Maria Laach, Germany.

Oppermann, B.I. and Krüger, M. Effects of Elevated CO_2 Concentrations on Microbial Populations at a Terrestrial CO_2 Vents. September, 2007. Maria Laach, Germany.

Oppermann, B.I., Michaelis, W., Schulz, H.M., Krüger, M. Effects of Elevated CO_2 Concentrations on Microbial Populations at a Terrestrial CO_2 Vent at Latera, Italy. EGU 2008 Meeting, April, 2008. Vienna, Austria.

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Poster presentations

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Sampling campaign

Black Sea, FS Meteor, 07.07 – 20.02,2007

Laacher See, Germany, 25.03 - 28.03.2007

Laacher See, Germany, 24.09 - 28.09.2007

Latera, Italy, 02.06.09 - 05.06.2009

Other official journals

CO_2GeoNet – The European Network of Excellence on CO_2 Geological, Storage R&D Workshop on CO_2 Geological Storage. November 19-21, 2007. Hannover, Germany.

23rd International Meeting on Organic Geochemistry, September 9-14, 2007. Torquay, United Kingdom.

International Interdisciplinary Summer School on carbon capture and storage 2007, August 19-24, 2007. Kloster Seeon, Germany.

Additional publications (not peer reviewed)

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Ecosystem effects of elevated CO_2 concentrations on microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. *Energy Procedia* 1, 1933-1939.

Figure captions

Table captions

List of abbreviations

16S rRNA – Small subunit of ribosomal ribonucleic acid with a sedimentary unit of 16
acetyl-CoA – acetyl-Coenzyme A
a*i*- – *anteiso*-chain methyl branched
AOM – anaerobic oxidation of methane
APCI – atmospheric pressure chemical ionization
aminopentol – 35-aminobacteriohopanepentol
aminotriol – 35-aminobacteriohopanetriol
ANME – Anaerobic Methane Oxidizing Community
BHP – bacteriohopanepolyol
BHT – bacteriohopanetetrol
BSTFA – *N,O*-bis(trimethylsilyl)trifluoroacetamide
CA – carboxylic acid
CAM – crassulacean acid metabolism
CAME – carboxylic acid methyl esters
CBT – cyclisation ratio of branched tetraethers
CCS – carbon capture and storage
CPR – CO_2 production
DAGE – dialkyl glycerol diethers
DCM – dichloromethane
DIC – Dissolved inorganic carbon
DGGE – denaturing gradient gel electrophoresis
DMDS – dimethyl disulfide
DNA – deoxyribonucleic acid
dw – dry weight
ε – isotope fractionation factor
EA-MS – elemental analyser coupled to mass spectrometry
FID – flame ionisation detector
GC – gas chromatography
GC-*C*-IRMS –gas chromatography coupled to *combustion* isotope ratio mass spectrometry
GC-FID – gas chromatography equiped with a flame ionisation detector
GC-MS – gas chromatography coupled to mass spectrometry

GDGT – glycerol dialkyl glycerol tetraethers

GHG – greenhouse gases

HC – hydrocarbon

HPLC – high-performance liquid chromatography

i- – *iso*-chain methyl branched

IPCC – Intergovernmental Panel on Climate Change

LC-MS – liquid chromatography coupled to mass spectrometry

m- – mid chain methyl branched

MAGE – monoalkyl glycerol monoethers

MEP – methylerythritol pathway

MPR – methane production

MVA pathway – mevalonate pathway

Q-PCR (or qPCR) – quantitative polymerase chain reaction

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

RuMP pathway – ribulose monophosphate pathway

SDS – sodium dodecyl sulphate

SMOW – Standard mean ocean water

sn – stereospecific numbering for the identification of carbon atoms chiral glycerin derivates. The carbon atom, which is located above the secondary carbon atom, in the fischer projection, is labled *sn*-1.

SRB – sulphate-reducing Bacteria

SRR – sulphate-reduction rates

TC – total carbon

TCA cycle – tricarboxylic acid cycle

TCD – thermal conductivity detector

TLC – thin layer chromatography

TN – total nitrogen

TOC – total organic carbon

V-PDB (or PDB) – Vienna Pee Dee Belemnite

Curriculum Vitae

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