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Liberation of low molecular weight organic acids from sedimentary organic matter and their role on microbial activity

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I, Patrick Sauer, hereby state that either myself or any other person at either the Mathematisch-Naturwissenschaftliche Fakultät at the University of Potsdam or at any other institution has not previously submitted this thesis for assesment, either in whole or part.

To the best of my knowledge and belief, this thesis contains no material which has been previously published or written by another person except where due references is made.

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List of publications

Publications:

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Green-Saxena, A., Feyzullayev, A., Hubert, C. R. J., Kallmeyer, J., Krueger, M., **Sauer, P**., Schulz, H.-M. and Orphan, V. J.: “Active sulfur cycling by diverse mesophilic and thermophilic microorganisms in terrestrial mud volcanoes of Azerbaijan”, Environmental Microbiology, 2012, Volume 14 (12), pp. 3271 - 3286

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Sauer, P., Kallmeyer, J.: "HiPP - High Partial Pressure" at the GeoEn - Meeting at the Geoforschungszentrum Potsdam (German Research Center for Geosciences) 2010, Potsdam

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Abbreviations

ANME	anaerobic methanotroph
AOM	anaerobic oxidation of methane
aprA	adenosine-5-phosphate reductase gene
CARD-FISH	catalysed reporter deposition – fluorescence *in-situ* hybridisation
CCS	carbon capture and storage
DEBITS	Deep Biosphere in terrestrial Systems
ECBM	enhanced recovery of coalbed methane
EGR	enhanced gas recovery
EOR	enhanced oil recovery
FEP	fluorinated ethylene propylene
FFKM	perfluoro-elastomers
HPLC	high-performance liquid chromatography
IC	ion chromatography
ICCP	International Commission for Coal Petrology
IMV	Isis mud volcano
LMWOA	low molecular weight organic acid
MBq	Megabecquerel
MPa	Megapascal
NDSF	Nile deep-sea fan
PCR	polymerase chain reaction
PDB	PeeDee belemnite
PTFE	polytetrafluorethylene
PVDF	polyvinylidene-fluorite
RFLP	restriction fragment length polymorphism
SCFA	short chain fatty acid
SMOW	standard mean ocean water
SOB	sulphur oxidising bacteria
SRB	sulphate reducing bacteria
SRR	sulphate reduction rate
TGB	thermal gradient block
TMV	terrestrial mud volcano
TOC	total organic carbon
VFA	volatile fatty acid

Abstract

Low molecular weight organic acids (LMWOAs) are important nutrients for microbes. However, most LMWOAs do not exist freely in the environment but are bound to macromolecular organic matter, e.g. kerogen, lignite and coal. During burial and geological maturation of sedimentary macromolecular organic matter biological and abiological processes promote the liberation of LMWOAs into the surrounding sediment. Through this process, microbes in sedimentary subsurface environments are supplied with essential nutrients.

To estimate the feedstock potential of buried macromolecular organic matter to many environments it is important to determine the amount of LMWOAs that are bound to such a matrix. However, high-pressure and high temperature are a key feature of deep subsurface environments, and these physical parameters have a profound influence on chemical reaction kinetics. Therefore it is essential for the estimation of the feedstock potential to generate high-pressure and high temperature for the liberation of LMWOAs to recreate true *in-situ* conditions.

This work presents a newly developed, inexpensive incubation system for biological and geological samples. It allows the application of high-pressure and high temperature as well as a subsampling of the liquid phase without loss of pressure, thereby not disturbing the on-going processes.

When simulating the liberation of LMWOAs from sedimentary organic matter, the newly developed incubation system produces more realistic results than other extraction systems like Soxhlet. The extraction products remain in the extraction medium throughout the extraction, influencing the chemical conditions of the extraction medium. Sub-bituminous coal samples from New Zealand as well as lignite samples from Germany were extracted at elevated temperature (90˚C) and pressure (5 MPa). The main LMWOAs released from these low rank coals were formate, acetate and oxalate. Extraction efficiency was increased by two to four times for formate, acetate and oxalate in comparison to existing extraction methods without pressurisation and with demineralised water. This shows the importance of pressure for the simulation of true *in-situ* conditions and suggests that the amount of bioavailable LMWOAs is higher than previously thought.

With the increase in carbon capture and storage (CCS) and the enhanced recovery of oil and gas (EOR/EGR), more and more CO_2 becomes injected into the underground. However, the effects of elevated concentrations of carbon dioxide on sedimentary organic matter are rarely investigated. As the incuabtion system allows the manipulation of the composition and partial pressure of dissolved gasses, the effect of highly gas-enriched (CO_2, CO_2/SO_2, CO_2/NO_2; to simulate flue gas conditions) waters on the extraction yield of LMWOAs from macromolecular organic matter was evaluated. For sub-bituminous coal the concentrations of all LMWAOs decreased upon the addition of gas, irrespective of its composition, whereas for lignite formate always and acetate mostly increased, while oxalate decreased. This suggests an positive effect on the nutrient supply for the subsurface microbiota of lignite layers, as formate and acetate are the most common LMWOAs used for microbial metabolism.

In terrestrial mud volcanoes (TMVs), sedimentary material is rapidly ascending from great depth to the surface. Therefore LMWOAs that were produced from buried macromolecular organic matter at depth are also brought up to the surface, and fuel heterotrophic microbial ecosystems at the surface. TMVs represent geochemically and microbiologically diverse habitats, which are supplied with organic substrates and electron acceptors from deep-seated hydrocarbon-generating systems and intersected shallow aquifers, respectively. The main electron donor in TMVs in Azerbaijan is sulphate, and microbial sulphate reduction leads to the production of a wide range of reduced sulphur species that are key players in several biological processes. In our study we estimated the effect of LMWOAs on the sulphur metabolising activity of microorganims in TMVs from Azerbaijan. The addition of a mixture of volatile fatty acids containing acetate and other LMWOAs showed significant positive response to the sulphate reduction rate (SRR) of samples of several mud volcanoes. Further investigations on the temperature dependency of the SRR and the characterisation of thermophilic sulphate-reducing bacteria (SRB) showed a connection between the deep hot subsurface and the surface.

Zusammenfassung

Niedermolekulare organische Säuren (nachfolgend als LMWOAs - low molecular weight organic acids - bezeichnet) stellen wichtige mikrobielle Substrate dar. Jedoch liegen die meisten LMWOAs nicht in freier, bioverfügbarer Form vor, sondern sind vielmehr an hochmolekulare organische Substanzen gebunden, z.B. Kerogen, Lignit und Kohle. Während der geologischen Verbringung in tiefe Erdschichten und der geologischen Reifung von sedimentären hochmolekularen organischen Substanzen, führen biologische und abiologische Prozesse zu einer Freisetzung von LMWOAs in die umgebenden Sedimente. Durch diesen Prozess werden Mikroorganismen in unterirdischen sedimentären Ökosystemen mit essentiellen Nährstoffen versorgt.

Um das Nährstoffpotential tief liegender hochmolekularer organischer Substanzen für diverse Ökosystemen abschätzen zu können, ist es notwendig, die Menge an LMWOAs, die an solch eine hochmolekulare Matrix gebunden ist, zu bestimmen. Dabei stellen hoher Druck sowie hohe Temperatur entscheidende Faktoren in tiefen unterirdischen Ökosystemen dar, welche einen signifikanten Einfluss auf chemische Reaktionen haben. Daher ist es für die Abschätzung des Nährstoffpotentials entscheidend, hohen Druck und hohe Temperatur bei der Freisetzung von LMWOAs zu erzeugen, um wahre *in-situ* Bedingungen zu schaffen.

In der vorliegenden Arbeit wird ein neu entwickeltes, preiswertes Inkubationssystem für biologische und geologische Proben präsentiert. Es erlaubt die Verwendung von hohem Druck als auch hoher Temperatur sowie eine Unterprobennahme der flüssigen Phase ohne Druckverlust, um den fortlaufende Prozess nicht zu unterbrechen.

Bei der Simulierung der Freisetzung von LMWOAs aus sedimentären organischen Substanzen erhält man mit dem neu entwickelten Inkubationssystem realistischere Resultate als mit anderen Extraktionssystemen, wie z.B. eine Soxhlet-Apparatur. Die Extraktionsprodukte verbleiben während der Extraktion im Extraktionsmedium, wodurch die chemischen Bedingungen verändert werden. Proben subbituminöser Kohle aus Neuseeland sowie Lignit aus Deutschland wurden mittels erhöhter Temperatur (90°C) und Druck (5 MPa) extrahiert. Die wichtigsten LMWOAs, die aus diesen Kohlen freigesetzt wurden, waren Formiat, Acetat und Oxalat. Die Extraktionseffizienz für diese LMWOAs konnte im Vergleich zu existierenden Extraktionsmethoden ohne Druck und mit entmineralisiertem Wasser um den Faktor 2 bis 4 gesteigert werden. Dies zeigt die

Bedeutung von Druck bei der Simulation von *in-situ* Bedingungen und legt nahe, dass die Menge an bioverfügbaren LMWOAs größer ist als bisher angenommen.

Durch die Zunahme der CO_2-Speicherung im Untergrund (carbon capture and storage, CCS) sowie der erweiterten Förderung von Öl und Gas (enhanced recovery of oil and gas, EOR/EGR) wird immer mehr CO_2 in den Untergrund gepresst. Jedoch sind die Auswirkungen von erhöhten CO_2-Konzentrationen auf sedimentäre organische Substanzen noch unerforscht. Da mit dem Inkubationssystem die Veränderung der Zusammensetzung und des Partialdruckes von gelösten Gasen möglich ist, wurde der Effekt von hoch mit Gasen (CO_2, CO_2/SO_2, CO_2/NO_2; um Kraftwerksabgase zu simulieren) angereicherten Wässern auf die Extraktionsausbeute von LMWOAs untersucht. Bei der subbituminösen Kohle zeigte sich eine Abnahme aller LMWOAs-Konzentrationen durch die Lösung von Gas im Extraktionsmedium, wobei die Art des Gases keine Rolle spielte. Bei Lignit konnte hingegen festgestellt werden, dass die Extraktionsausbeute an Formiat immer und an Acetat meistens erhöht wurde, während sie sich bei Oxalat verringerte. Dies deutet auf einen positiven Effekt für die Nährstoffversorgung von Mikroorganismen um Lignit-Lagerstätten an, da Formiat und Acetat die am häufigsten verwendeten LMWOAs im mikrobiellen Stoffwechsel darstellen.

In terrestrischen Schlammvulkanen (terrestrial mud volcanoes, TMVs) steigt sedimentäres Material rapide aus großen Tiefen an die Erdoberfläche. Somit werden auch LMWOAs, welche aus hochmolekularen organischen Substanzen freigesetzt werden, an die Oberfläche verbracht, und ermöglichen dort heterotrophe Ökosysteme. TMVs stellen dabei geochemisch und mikrobiell unterschiedliche Habitate dar, welche mit organischen Substraten und Elektronenakzeptoren aus tief liegenden, Kohlenwasserstoffe erzeugenden Systemen versorgt werden. In TMVs in Aserbaidschan stellt Sulfat den Hauptelektronenakzeptor dar, wobei mikrobielle Sulfatreduktion zu einer Vielzahl an reduzierten Schwefelspezies führt, welche zu den wichtigsten Akteuren in biologischen Prozessen zählen. In der vorliegenden Arbeit wurde der Effekt von LMWOAs auf die Aktivität von Mikroorganismen bei der Umsetzung von Schwefel in TMVs in Aserbaidschan untersucht. Die Zugabe einer Mischung verschiedener kurzkettiger Fettsäuren (welche Acetat und andere LMWOAs enthielt) zu Schlammproben verschiedener TMVs erzeugte eine signifikant positive Reaktion in Bezug auf die Sulfat-Reduktionsraten. Weiterführende Untersuchungen zur Temperaturabhängigkeit der Sulfat-Reduktionsraten und die Charakterisierung thermophiler, Sulfat-Reduzierender Bakterien zeigte eine Verbindung zwischen der tiefen, heißen Biospäre und der Erdoberfläche auf.

1. Introduction

1.1 Introduction to the deep biosphere

Lourens Baas-Becking wrote in 1934: "Everything is everywhere; the environment selects" (Baas-Becking et al., 1934), describing the notion that every microbe can be found everyhere on Earth. Even though this statement is no longer accepted (Whitfield, 2005), microbes are present any habitable environment on Earth. They are found in the atmosphere, hydrosphere and lithosphere. Though findings of microbes in sediments were reported quite early (Fischer, 1894; Waksman, 1934; Rittenberg, 1940), little was known about microbes in the deep subsurface and there was no general idea about the existence of a biosphere until the 1980s (review in Parkes et al., 1994), as previous findings of microbes were dismissed as contamination.

Morita and ZoBell (1955) provided the first definition of the depth limit of the subsurface biosphere, when they found microorganisms in pelagic Pacific Ocean sediments. However, they defined the lower limit of life at a sediment depth of about 7 meters due to their inability to cultivate any microbes from deeper samples. With the ongoing research the depth limit of live was set deeper and deeper. Bacteria were discovered in the deep subsurface of terrestrial coastal plain sediments (Chapelle et al., 1987) as well as in several hundred of meters depth of sediments of the Pacific Ocean (Parkes et al., 1994). Furthermore microbes were found 1.5 km beneath the land surface (Stevens and McKinley, 1995), 1.6 km below the seafloor (Roussel et al., 2008) and in Late Cretaceous rocks in depths of 2000 m (Colwell et al., 1997). Together with findings of metabolic activity at 1000 MPa (Sharma et al., 2002), it is clear that pressure does not play such an important role as a selective criterion for subsurface colonisation. Moreover microbes are able to adapt to elevated pressure (Abe, 2007).

In contrast to pressure, temperature appears to be a much more important factor for microbial colonisation of the subsurface. The present temperature limit was set to 113°C by Blöchl et al. (1997). A controversial discussion about higher maximum temperatures up to 121°C (Kashefi and Lovley, 2003) and even higher (Takai et al., 2008) is going on (Cowan, 2004; Kashefi, 2004).

The number of microbes in marine and terrestrial subsurface was estimated to be up to 90 % of total microbial numbers on Earth (Whitman et al., 1998). Even with new calculations (Kallmeyer et al., 2012) subsurface biomass is enormous (Jørgensen, 2012)

and it can be summarised that abundant microbial life can be found everywhere in the subsurface, as long as temperature allows life to persist.

Apart from pressure and temperature there are other factors influencing the subsurface microbial communities. The availability of water, pore space and carbon as well as energy sources are essential for habitability. So one of the fundamental questions is: What are the substrates that provide energy to the microbial communities? In the deep subsurface there is no sunlight by which phototrophs could generate energy and synthesize biomass. There is no newly produced organic carbon to generate cellular matter. And there is no exchange with the productive surface. So how does the deep biosphere survive?

1.2 Metabolism in the deep biosphere - buried organic matter as feedstock for subsurface microbial life

Several studies have shown that deep subsurface microbes have different survival strategies. As photosynthesis is absent, chemical redox reactions are the only possibility to generate energy for cellular processes. One option is the use of hydrogen as an energy source, which can be abiotically produced by weathering of volcanic rocks (Pedersen, 2000; Stevens and McKinley, 1995) or radiolysis of water. Dissolved carbon dioxide is used by lithoautotrophic microbial ecosystems (SLiME / HyperSLiME; Takai et al., 2004) to produce biomass. Nevertheless most subsurface microbes are heterotrophs, living on buried macromolecular organic matter. Reduced organic carbon is usually oxidised by transferring the electrons onto the oxygen. In most parts of the deep subsurface oxygen is not available, therefore anaerobic processes are used by microbes to transfer electrons and to gain energy (see table 1.1).

Sedimentary organic matter is usually finely dispersed (Horsfield et al., 2006). However, in some locations organic matter may be locally enriched in the form of oil and coal deposits. In these settings carbon appears to be abundant (Stetter et al., 1993). However, this sedimentary organic carbon is present mainly as macromolecules, which are not immediately biologically available. To be bioavailable, macromolecules have to be cracked into smaller molecules such as fatty acids or low molecular weight organic acids (e.g. formate and acetate). Therefore macromolecular organic matter (polymeric organic matter Horsfield and coal) undergoes transformation and maturation processes, which are running over geological timescales. The maturation pathway of the most

common coals (and kerogen type III) follows the path of humic coals, which is shown in figure 1.1, leading from lignite over bituminous coal to anthracite. An excellent overview about the maturation of buried organic matter is given in Glombitza (2011) and is briefly discussed here for coal, as coal is a central theme of this thesis.

Table 1.1: Gibbs free energy of selected redox reactions, taken from McKinley, 2001

reactions	ΔG (kJ/mol)
aerobic respiration	-475
denitrification	-448
Mn(IV) reduction	-349
methanogenesis (hydrogenotrophic)	-130
Fe(III) reduction	-114
sulfate reduction	-77
methanogenesis (acetoclastic)	-36

Maturation of higher plant material into coal starts with peatification of this material, which was deposited under anoxic conditions in swamps, thereby preserved from oxidative processes. This process is dominated by biological alteration and depolymerisation (Catcheside and Ralph, 1999), causing a degradation of cellulose, leaving lignin and lipids behind.

The following early diagenetic coalification is a mixture of biochemical and geochemical processes, causing a decrease of the atomic O/C ratio and leading to the formation of lignite (see figure 1.1). Diagenetic coalification is followed by catagenesis, which is characterised by an increase in pressure and temperature (ranging from 60 to 100°C, Killops and Killops, 2005), causing a stop of biological processes (Wilhelms et al., 2001). Maturation due to increased pressure and temperature leads to a decrease of the atomic O/C ratio as well as – in the last stages – H/C ratio, forming anthracite as the final product. During catagenesis the atomic H/C ratio is also decreasing, indicating a release of hydrocarbons. Volatile fatty acids (VFA) and low molecular weight organic acids (LMWOAs), which are suitable substrates for almost every microbe (Thauer et al., 1977), are formed from sedimentary organic matter at this stage (Horsfield et al., 2006; Killops and Killops, 2005).

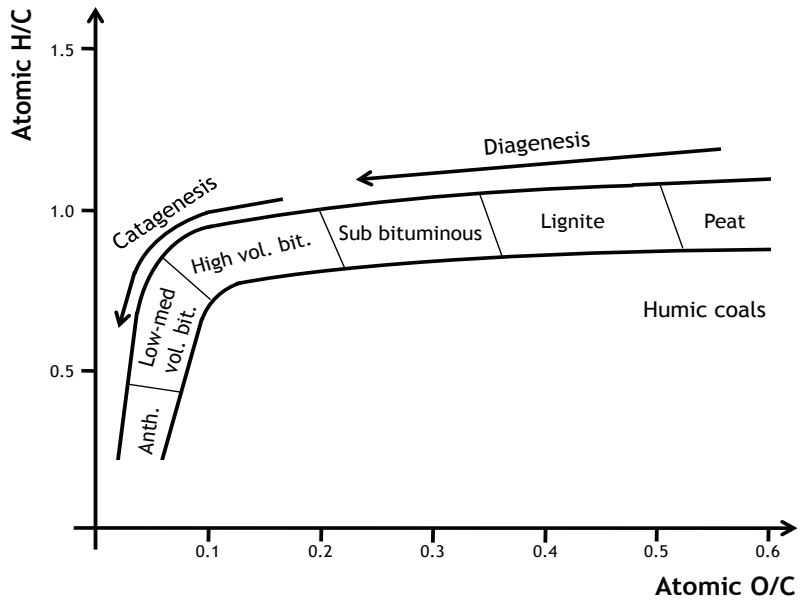

Figure 1.1: Maturation stages of coals plotted in a van Krevelen type diagram. Redrawn after Glombitza, 2011.

Besides the thermal release of VFAs and LMWOAs, there is another process leading to the liberation of small organic molecules from macromolecular organic matter: hydrolysis. This reaction requires water, which is provided by the pore water. The rate of reaction can be increased by an increase in temperature, e.g. during burial to greater depths (Wellsbury et al., 1997) as well as by an acidification of the porewater due to the liberated organic acids. So the natural acidification will lead to an increase in the acidic hydrolysis and an increase of the liberation of VFA and LMWOAs.

In low rank coal deposits acidification of porewater can also be caused by the oxidation of pyrite, leading to the formation of sulphuric acid, which causes an acidification. The decrease in pH causes an increase in hydrolysis, thereby further liberating organic acids.

However, not only geochemical maturation will lead to a breakdown of macromolecular organic matter. During diagenesis and the early stages of catagenesis, also a biological degradation through microbial activity takes place. During the stage of peatification microbial activity causes depolymerisation, fragmentation of complex organic matter and macromolecules into aromatics, polyaromatics, ketones, carboxylic acids and long chain fatty acids. In the later stages of maturation (diagenesis, early catagenesis) anaerobic oxidation and fermentation takes place. Strapoc et al. (2008) proposed a mechanism for the conversion of macromolecular organic matter (here sub-bituminous coal) into carbon dioxide and methane as end products (figure 1.2). As e.g.

methanogens are not able to use fragmented aromatics and fatty acids with more than two carbon atoms, fermentation and anaerobic oxidation processes are required for the conversion into compounds that are usable for methanogens (Lovley and Chapelle, 1995; Strapoc et al., 2008; figure 1.2). It has to be assumed that processes similar to the so-called "intermediary ecosystem metabolism" of soils, by which extracellular hydrolysis, fermentation of monomers and carbon mineralisation are linked together (Drake et al., 2009), are involved in the degradation of macromolecular organic matter, which leads to an increase in the bioavailability of carbon for the deep biosphere.

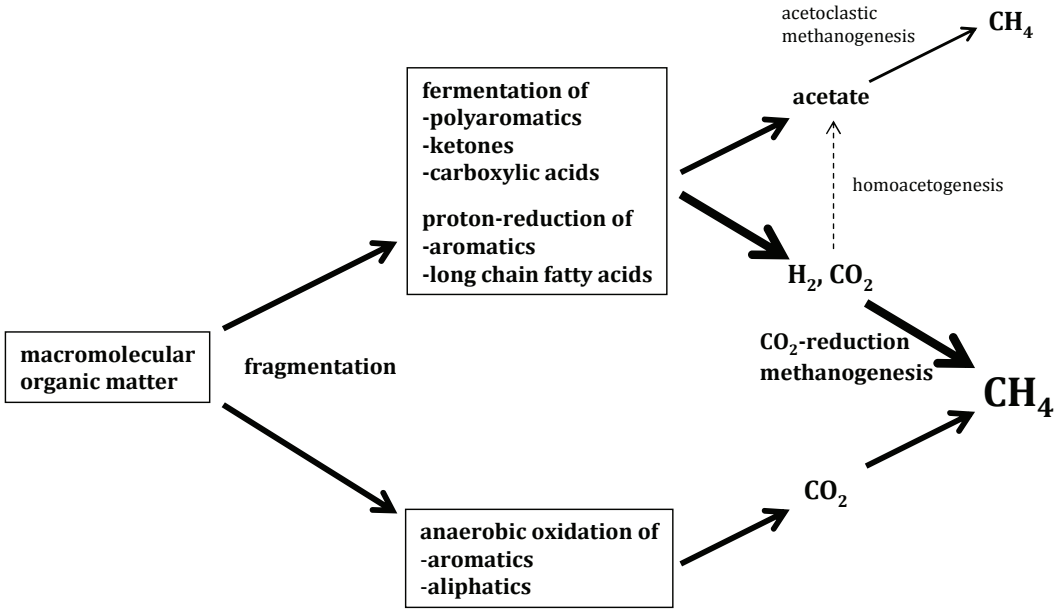

Figure 1.2: Conversion of macromolecular organic matter into methane. Redrawn after Strapoc et al., 2008. For structures of diverse macromolecular organic matter of different coal ranks see Mathews and Chaffee, 2012

It has been shown that methane from sub-bituminous coal of the Illinois Basin, USA, is of biogenic origin (Strapoc et al., 2007), having a stable isotope ratio $\delta^{13}C_{methane}$ of -58 to –62 ‰ (see figure 1.3). Also, methane from hard coal deposits showed isotopic compositions that are characteristic for microbial methane formation ($\delta^{13}C_{methane}$ -40 to –57 ‰ in Thielemann et al., 2004; $\delta^{13}C_{methane}$ -47 to -56 ‰ in Krüger et al., 2008). Biological isotopic fractionation is caused due to the slight enzymatic preference for the isotopically lighter ^{12}C, causing an enrichment in the lighter isotope upon formation of methane (Killops and Killops, 2005). Thermogenic formation of methane does not lead to an isotopic fractionation, causing no enrichment in $\delta^{12}C$.

Biological methanogenesis can operate at a broad range of temperatures; from sub-zero temperatures (Wagner et al., 2007) up to 75°C (Killops and Killops, 2005). So methanogenesis is possible over a wide range of temperatures. By utilisation of buried organic matter (dispersed or compact) methanogenesis is feasible over geologic time periods.

The only known organisms capable to produce methane all belong to the domain archaea. These microbes are strictly anaerobic. Three pathways are known for the synthesis of methane, while the microbes are capable of only one of these three pathways. In the first pathway hydrogenotrophic methanogens synthesise methane from carbon dioxide and hydrogen (figure 1.4 A). In the second pathway acetoclastic methanogens convert acetate into methane and carbon dioxide (figure 1.4 B). The third pathway is called methylotrophy, which utilises one carbon compounds such as formic acid or methanol (exemplarily shown for methanol in figure 1.4 C). However, this pathway is not known for methane production from macromolecular organic matter (Moore, 2012).

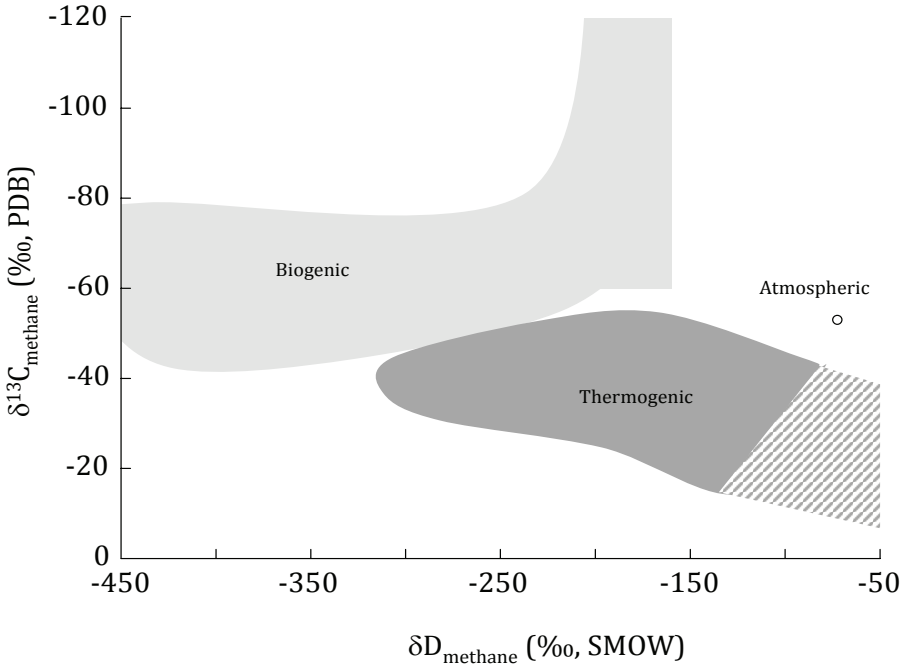

Figure 1.3: Diagramm for classification of biogenic and thermogenic methane; redrawn after Whiticar, 1999

It has been shown for sub-bituminous coal of the Illinois Basin, USA, that the methanogenic archaea *Methanocorpusculum parvum* is the most abundant microbe in this coal bed (Strapoc et al., 2008). Furthermore, methanogens have been found in sub-

bituminous coal from the DEBITS-1 well of the Waikato Basin of North Island, NZ (Fry et al., 2009). Also from coals of Northern Russia (Vorgashorskaya and Vorkutinskaya coal), a methanogenic microbial consortium was isolated (Shumkov et al., 1999) as well as from production water of coal bed methane from the San Juan Basin, USA (Wawrik et al., 2012). As mentioned above biogenic methane formation has been shown also for hard coal (Thielemann et al., 2004; Krüger et al., 2008). However, no methanogens had been detected for such hard coals so far. Up to now no methanogens had also been found for other low rank coals, e.g. in lignite (Catcheside & Ralph, 1999).

A $CO_2 + 4\ H_2 \longrightarrow CH_4 + 2\ H_2O$ $\Delta G = -130.4$ kJ / mol CH_4

B $2\ CH_3COO^- + H_2 \longrightarrow 2\ CH_4 + 2\ CO_2$ $\Delta G = -36$ kJ / mol CH_4

C $4\ CH_3OH \longrightarrow 3\ CH_4 + CO_2 + H_2O$ $\Delta G = -106$ kJ / mol CH_4

$CH_3OH + H_2 \longrightarrow CH_4 + H_2O$ $\Delta G = -112.5$ kJ / mol CH_4

Figure 1.4: Pathways for microbial methanogenesis; (A) hydrogenotophic, (B) acetoclastic, (C) methylotrophic

Jaschof and Schwartz (1969) reported bacteria and funghi in lignite from the Eastern Germany brown coal district. However, their cell density data were obtained from culture-dependent techniques, and much lower than counts with DNA-specific dyes for epifluorescence microscopy (Collins and Kipling, 1957). So far, no further counts of were published for microbial abundance of low-rank coals.

The methane derived from the utilisation of VFAs and LMWOAs as a degradation product of sedimentary macromolecular organic matter (figure 1.2) is either trapped inside the Earth, utilised by microbes or escapes into the atmosphere. Thereby methane acts as a carbon and energy sources for microbes, which are capable in the utilisation of methane.

There are two ways for microbes to use methane as carbon and energy source: Aerobic oxidation and the anaerobic oxidation of methane. During aerobic oxidation the electrons of the reaction of methane to carbon dioxide are transferred onto atmospheric oxygen forming water and CO_2 as the end products.

70 to 300 Tg of the annually emitted methane is consumed by anaerobic oxidation of methane, which is a significant fraction of the total annual methane output of 600 Tg (Reeburgh, 2007). This process is carried out by a consortium of methanogenic archaea

and bacteria capable of the reduction of oxidised inorganic compounds. The associated bacterium is usually a sulphate-reducing bacterium, which is a member of the delta-proteobacteria. However, it has been reported recently that anaerobic oxidation of methane was performed by using iron, manganese, nitrate and nitrite (Raghoebarsing et al., 2006; Beal et al., 2009; Ettwig et al., 2010). Surprisingly, the process using the electron acceptor with the lowest energy yield was discovered first (Boetius et al., 2000; Orphan et al., 2001; table 1.2).

Table 1.2: Gibbs free energy for all AOM electron acceptors

electron acceptor	ΔG (kJ/mol)
nitrite (NO_2)	-928
nitrate (NO_3)	-765
manganese (MnO_2)	-556
ferrihydrite ($Fe(OH)_3$)	-270
sulphate (SO_4^{2-})	-21

It is remarkably that the ANME microbes are not methanotrophic but methanogenic archaea, usually responsible for methane formation. In a still unknown process the pathway for methanogenesis is turned around to oxidise methane to carbon dioxide. The electrons, which are resulting in this conversion, were transferred onto the associated chemolithotrophic bacteria, which are reducing the inorganic electron acceptors. Microbes are therefore responsible not only for the formation of methane but also for a reduction of the amount of methane that is reaching the atmosphere.

However, the annual global methane output is about 600 Tg (IPCC, 2001) Thereof, the global geological non-anthropogenic methane emission to the atmosphere is estimated to be 45 Tg (teragrams; 10^{12} g) per year (Kvenvolden and Rogers, 2005). Thereby mud volcanoes were estimated to emit 7 Tg CH_4/yr.

1.3 Impact of anthropogenic derived carbon dioxide on geochemical and biological processes in the deep subsurface

Although the amount of expelled carbon dioxide is at least 40 times higher in comparison to the output of methane (see table 1.3), methane has a 3.7 times higher global warming potential than carbon dioxide and contributes therefore with a strong proportion to the green house effect (Lashof and Ahuja, 1990).

Despite recent technical advantages, renewable energy sources (wind, solar, hydrothermal, water) can only supply a fraction of the current total energy requirement. Due to the associated risks like reactor failures and long-term issues of waste storage, nuclear energy generation does not appear to be an alternative for the future.

Therefore power plants for burning fossil fuels, which provide 35% of the worldwide electric energy (Notz et al., 2011) and at least 49% in the European Union and 50% in Germany (data for the latter from 1999, Stamatelopoulos and Scheffknecht, 2005), are currently the only way so far to provide sufficient electric power. So fossil fuelled power plants are one of the main sources of global carbon dioxide emissions. Even new and highly efficient power plants emit large amounts of CO_2. In perspective of the increasing demand of electric energy in the next years, 74 percent of new added power generation is expected to be fossil-fuelled power plants (McKee, 2002). This means that the worldwide output of carbon dioxide will increase over the next years.

To reduce the emission of carbon dioxide from power plants three major options are being discussed. First the modernisation of existing power plants, second the increase in efficiency of power plants and third the capture of carbon dioxide from flue gas and storage in underground reservoirs (Notz et al., 2011). While the modernisation and the increase in efficiency will not stop the emission of carbon dioxide but only reduce them, capture and storage is the only way to decrease the emission of carbon dioxide from fossil fuelled power plants.

Two technologies have been developed to capture the carbon dioxide and to separate it from the flue gas: the pre-combustion capture and post-combustion capture (Notz et al., 2011). The different technologies will not be explained here because it would be beyond the scope of this thesis.

Table 1.3: Sources of CO_2 emissions from fossil fuel combustion (data for 2001; redrawn after IPCC, 2005)

sources of CO_2	emissions ($MtCO_2\ yr^{-1}$)	
public electricity and heat production	8,236	
other energy industries	1,228	
transport	5,656	
of which: road		4,208
Manufacturing and construction	4,294	
autoproducers	963	
other sectors	3,307	
of which: residential		1,902

The captured carbon dioxide has to be stored in order to be permanently removed from the environment. Several possible storage options have been discussed. An overview of possible storage facilities is given in Orr (2009) and Schrag (2009). Apart from the ”hiding” of CO_2 in streams of seawater for hundreds of years (Arlt, 2003) the storage of liquid carbon dioxide in the depths of the oceans was mentioned as well (Fujioka et al., 1995). A further option was mentioned in the use of deep saline aquifers (van Engelenburg and Blok, 1993), permeably filled with saline water and isolated by a cap rock, which makes such aquifers suitable for the storage of carbon dioxide. Further storage facilities are depleted oil and gas reservoirs in which gas and oil has been stored over hundred of thousands of years, proving that they are suitable to store carbon dioxide gas. The total storage capacity is estimated from 750 to more than 11.000 Gt_{CO2} (Notz et al., 2011).

Although the carbon capture and storage (CCS) technology is not ready to be used widely on an industrial scale (Haszeldine, 2009) carbon dioxide is already being injected into underground reservoirs, namely in smaller scale research operations (e.g. at the Ketzin research site close to Potsdam, Germany), but also on an industrial scale (e.g. Sleipner Gas Field off Norway). Furthermore enhanced oil recovery (EOR) is a proven and applied technology and commonly used in many oil fields. Carbon dioxide or other gases and liquids are injected into the oil reservoirs in order to maintain formation pressure and to help in the exploitation of the remaining oil and to generate pressure for the production of oil (Alvarado and Manrique, 2010). A further application, which is currently under intense research, is the enhanced recovery of coal bad methane (ECBM, White et al., 2005), using the injected carbon dioxide to maintain pressure and to displace the coal bed methane for production (for a review of coalbed methane see Moore et al., 2012).

Microbes do not live directly in the coal itself but in porous rocks around. The lack of data regarding microbial distribution and community composition in low-rank coals makes it difficult to estimate the effect of injected CO_2 on the microbial communities in coal seams. Therefore it is nessecary to investigate possible effects of injected carbon dioxide on the habitatability as well as on the nutrient supply of the microbes. This thesis aims primarily on the latter question.

1.4 Mud volcanoes and the emission of methane

Not all methane that is coming from deep inside the Earth is trapped as a natural gas deposit or methane hydrate. A massive part of this methane escapes storage or oxidation by methanotrophic microorganisms and diffuses into the atmosphere (Reeburgh, 2007). There are natural sources of atmospheric methane gas worldwide, adding to the green house effect and contributing to global warming (see chapter 1.3). Such phenomenons are e.g. gas seepages and mud volcanoes. An overview of the worldwide potential methane emmission sites is given in Etiope and Klusman (2002).

Mud volcanoes occur worldwide onshore (Azerbaijan, Romania, Pakistan) as well as offshore (Kopf, 2002; Dimitrov, 2002). An overview of the formation of mud volcanoes is given in Kopf (2002). In brief, mud volcanoes occur at subduction zones, where thick marine sediments were buried rapidly. This fast compaction results in a lack in dewatering of the sediment, also called undercompaction, leading to an overpressurisation of the sediment. There are several secondary factors that could generate overpressure in the setting, e.g. tectonic activity, hydrocarbon formation or lateral influx of low-density fluids, but there may be other fluid sources than just the pore fluid from buried marine sediments (Kopf et al., 2002). The overpressured water, sediment and fragments of surrounding rocks form a mud that has a strong positive buoyancy. This mud is then forced upwards along cracks and faults towards areas of lower pressure (surface), forming a mud diapir by deposition of the transported sediment. The shape of the mud volcano depends on the viscosity of the mud, leading to either pie shaped (low viscosity) or dome shaped extrusions (high viscosity).

Rapid subduction of the buried marine sediments causes a maturation of relatively fresh organic matter and a generation of hydrocarbons (Dimitrov, 2002), which are sometimes responsible for the mud volcanism. Therefore mud volcanoes often emit large amounts of natural gas and even small amounts of oil (Kopf, 2002). Mud volcanoes emit 6 to 9 Mt y^{-1} methane (Etiope and Milkov, 2004) and may contribute up to 9% of the natural global emmision of methane (Milkov et al., 2003) and are the major geological source of methane to the atmosphere.

This methane however is mostly not of biogenic origin like coal bed methane, but rather a thermogenic product of buried organic matter (Schoell, 1980). In 76% of the known mud volcanoes, the emitted methane gas is of thermogenic origin (Etiope et al., 2009 a). Only 4% is of microbial origin, the remainder is mixed. The isotopic composition of methane emitted from the mud volcanoes of the Gobustan area and the

Aspheron Peninsula in Azerbaijan, which are one subject of this work, range between -44‰ and -37‰ PDB (Dadashev and Guliyev, 1989; see figure 1.3) and are considered to be of mainly thermogenic origin.

Almost every mud volcano in Azerbaijan is linked to deep hydrocarbon pools (Etiope et al., 2009 a), expelling thermogenic methane. The source of these pools are gas-generating organic rich rocks of relatively high thermal maturity (Etiope et al., 2009 b), which are located predominantly below the oil window (Feyzullayev and Ismaylova, 2007). These rocks were deposited during the Cenozoic at high sedimentation rates (Etiope et al., 2009 a). However, biogenic methane of mud volcanoes is the result of a secondary methanogenesis, via the hydrogenotrophic pathway (Etiope et al., 2009 b). These mud volcanoes are a product of a rapid subduction of Pliocene-Quarternary basins with very recent or neo-tectonic compressional stress and faulting (Etiope et al., 2009 a).

1.5 Microbial substrates, sulphate reduction and sulphate reducing bacteria in mud volcanoes

The transport of marine mud, water and rocks to the surface results not only in the emission of methane, but also a wide range of other hydrocarbons (Dimitrov, 2002), which act as a carbon as well as an energy source for microbes. On samples from mud volcanoes from Azerbaijan Green-Saxena et al. (2012) showed that microbial activity strongly increases by the addition of VFAs. These compounds are used as electron donors by the microbes, which oxidise them with electron acceptors such as nitrate, sulphate and iron, which are also present in the mud (Planke et al., 2003; Mazzini et al., 2009). It is therefore not surprising that microbes other than methane oxidisers are inhabiting the mud of mud volcanoes, which is exemplarily discussed here for sulphate reducing bacteria (SRB).

The anaerobic respiration of iron, manganese and nitrate may provide a higher reduction potential (see table 1.1) and will therefore provide more energy for microbes. However, in seawater the concentration of sulphate is – with few exceptions (see Chang et al., 2012) – much higher than those of all other electron acceptors combined. Seawater is a major constituent of the mud of Azerbaijan MV (for the chemical composition of mud volcanoes of Azerbaijan see Planke et al., 2003). Depending on the location and individual hydrology of a mud volcano, the concentration of sulphate in the

mud can be in the range of 0.03 to 0.09 mM (Taiwan, Chang et al., 2012), over 0.16 to 1.65 mM for some mud volcanoes of Azerbaijan (Green-Saxena et al., 2012) to 14 mM for a mud volcano in China (Yang et al., 2012). Therefore sulphate is common as an electron acceptor among the microbial communities in mud volcanoes (Alain et al., 2006; Chang et al., 2012) and large numbers of sulphate-reducing bacteria have been found in mud volcanoes (Alain et al., 2006; Heller et al., 2012; Yang et al., 2012).

Sulphate reduction is limited to anoxic conditions. Although there are studies showing short-time (maximum 3 hours) survival at oxygen exposure (e.g. Cypionka et al., 1985), in general viability and cell motility decreases with exposure time (Marschall et al., 1993). Sulphate reduction is inhibited within certain SRB at oxygen levels of only 1% (Krekeler et al., 1998). Even though there are strategies for SRB to defend against oxygen stress (Dolla et al., 2006) they cannot compete against atmospheric oxygen concentrations. In pools with sticky and viscous mud and little to no internal turnover, the uppermost layer may be oxidised but the conditions in deeper parts still allow sulphate reduction.

Because SRB are quickly dying upon oxygen contact, any transport via oxic surface waters can be ruled out. The question is if there are special strategies like the formation of extremely resistant spores. It is not out of scope that microbes may originate from deep inside Earth and are transported together with mud and hydrocarbons from buried sediments to the surface.

Studies about spore-forming SRB (Castro et al., 2000) showed that it is possible to be transported via wind and air into the mud pools of mud volcanoes. Supporting the alternative model of a flow of microbes with the mud from deep inside Earth are the findings of Hubert et al. (2009), revealing thermophilic species of SRB in permanently cold sediments of the Svalbard region in the arctic North Atlantic. So where do these thermophilic SRB come from in a cold ocean?

Moussard et al. (2004) extracted thermophilic SRB from the Central Indian Ridge, giving rise to the hypothesis of a dispersal of thermophilic SRB through the ocean. This hypothesis stated that thermophilic microorganisms are transported by gas and oil fluxes from deeper, hotter sediments into the cold Arctic Ocean. Also in deep gold mines of South Africa (Baker et al., 2003; Moser et al., 2005) and in deep gas-bearing formations (Onstott et al., 1998) thermophilic SRB have been found.

Therefore it is possible that the SRB in mud volcanoes come from buried sediments and are lifted to the surface by the upwards-flowing mud.

1.6 Objectives

This thesis was focused on the utilisation of small organic molecules (volatile fatty acids, VFAs; low molecular weight organic acids, LMWOAs; short chain fatty acids, SCFAs) by deep subsurface microbes. The question was if buried macromolecular organic matter such as coals could provide nutrients and substrates for microbial metabolism. Furthermore, with the increase in carbon dioxide sequestration and the use of deep coal beds as reservoir it is of great interest, how the injected CO_2 would influence a possible nutrient release and the microbial communities. Nutrient in form of small organic molecules are also released from buried organic matter at subduction zones and emitted by mud volcanoes. Therefore these molecules can act as substrates for the microbial mud volcano community. It was the aim to determine what kind of sulphur species are present in those mud volcanoes and how the VFAs are utilised by sulphate reducing microbes.

Within the scope of this thesis, three different objectives were considered:

- To evaluate the effect of true in-situ pressure on the extractable amount of LMWOAs from coals, first of all a high pressure high-temperature incubation system for microbes and geological samples had to be developed, built and tested. The requirements for this incubation system were (I) to generate and manipulate a high partial pressure of different gases and gas compositions, (II) to separate the pressure generating fluid and the sample to avoid contaminations of the sample, (III) to take subsamples without loss of pressure to avoid disruption of microbes or degassing of the incubation fluid. The system should allow for the incubation of geological as well as biological samples to demonstrate the proof of concept. To determine the amount of LMWOAs as a microbial feedstock in coals, the incubation system had to be tested and compared with existing extraction systems. Therefore sub-bituminous coal samples from the DEBITS-1 well (North Island, New Zealand) were extracted.
- In the context of carbon storage in subsurface coal seams, the effect of pure and impure carbon dioxide on the extraction yield for LMWOAs from coals was determined. Sub-bituminous coal from the DEBITS-1 well as well as lignite from an open-cast lignite mine (Welzow-Süd, Niederlausitz, Germany) were taken as samples. The aim was to investigate the effect of non-supercritical carbon dioxide on the supply of the indigenous microbial community ith substrates, i.e.

LMOAs. This will give an indication on the possibility of an enhanced recovery or the biogenic formation of methane in coal seams.

- Also microbial communities in mud volcanoes are based on LMWOAs liberated from buried macromolecular organic matter. As microbial processes are poorly investigated in terrestrial mud volcanoes, the cycle of sulphate as electron acceptor in dependency on the presence of LMWOAs should be evaluated. Furthermore the temperature profile of the sulphate reduction rates should be determined. The phylogenetic characterisation of associated microbes should show the relationship to other SRB worldwide.

1.7 Outline of the Thesis

This cumulative thesis consists of 6 chapters, from which 3 chapters are manuscripts from peer review journals.

Chapter 1: Introduction

This chapter provides an introduction to the topic of this thesis. It presents brief informations about the deep biosphere and live on buried organic matter, the impact of anthropogenic derived carbon dioxide on the deep biosphere and mud volcanoes as a vent and habitat for processed buried macromolecular organic matter.

Chapter 2: A system for incubations at high gas partial pressure

P. Sauer, C. Glombitza and J. Kallmeyer

Frontiers in Microbiology (2012), Volume 3, Article 25

Chapter 2 describes the construction and operation of the incubation system and shows its applicability for geological and biological samples. Results from the extraction of LMWOAs from sub-bituminous coal from the DEBITS-1 well of New Zealand to determine the feeding possibilities for deep subsurface microbes are given. Furthermore comparing results for the extraction by the addition of subcritical carbon dioxide to the extraction medium are presented.

As a first author I developed and constructed the incubation system, performed the extractions and incubations and wrote the manuscript with the assistance of my PhD supervisor.

Chapter 3: Liberation of low molecular weight organic acids from lignite by highly CO_2-saturated water and their role as potential microbial substrates

P. Sauer, C. Glombitza and J. Kallmeyer

submitted to *Environmental Science and Technology (2013)*

In chapter 3 we describe the extraction of lignite from the Niederlausitz brown coal district in Eastern Germany. Furthermore the effect of carbon dioxide on the extraction

yield of LMWOAs was pointed out. Additionally the effect of a more complex extraction medium due to impurities (NO_2, SO_2) in the added carbon dioxide was demonstrated.

I performed the extractions of the lignite coals including the applications of carbon dioxide (including impurities) with the high-pressure incubation system and wrote the manuscript.

Chapter 4: Active sulfur cycling by diverse mesophilic and thermophilic microorganisms in terrestrial mud volcanoes of Azerbaijan

A. Green-Saxena, A. Feyzullayev, C. R. J. Hubert, J. Kallmeyer, M. Krüger, P. Sauer, H.-M. Schulz and V. J. Orphan

Environmental Microbiology (2012), 14 (12), 3271 - 3286

The link of the liberation of LMWOAs in buried, organic rich sediments with the sulphate reduction and SRB in mud volcanoes of Azerbaijan was prospected in chapter 4. Reduced sulphur species were determined as well as sulphate reduction rates of the inhabiting microbial community in dependency of the temperature profile and the presence of LMWOAs. Furthermore microbes were genetically and phylogenetically classified to clarify the origin of the microbial community responsible for the sulphate reduction.

I contributed the experimental and written parts of fractionated distillation of the reduced sulphur species as well as the temperature and LMWOAs depending sulphate reduction rates to this publication.

Chapter 5: Conclusion

This chapter summerises the conclusions of this thesis. Furthermore an outlook on possible future work is given.

Chapter 6: References

This chapter lists all references of this thesis that are used for the introduction and conclusion of this thesis.

2. "A system for incubations at high gas partial pressure"

Patrick Sauer, Clemens Glombitza and Jens Kallmeyer

Frontiers in Microbiology 2012, Volume 3, Article 25

High-pressure is a key feature of deep subsurface environments. High partial pressure of dissolved gasses plays an important role in microbial metabolism, because thermodynamic feasibility of many reactions depends on the concentration of reactants. For gases, this is controlled by their partial pressure, which can exceed 1 MPa at *in situ* conditions. Therefore, high hydrostatic pressure alone is not sufficient to recreate true deep subsurface *in situ,* conditions, but the partial pressure of dissolved gasses has to be controlled as well. We developed an incubation system that allows for incubations at hydrostatic pressure up to 60 MPa, temperatures up to 120°C, and at high gas partial pressure. The composition and partial pressure of gasses can be manipulated during the experiment. To keep costs low, the system is mainly made from off-the-shelf components with only very few custom-made parts. A flexible and inert PVDF (polyvinylidene fluoride) incubator sleeve, which is almost impermeable for gases, holds the sample and separates it from the pressure fluid. The flexibility of the incubator sleeve allows for sub-sampling of the medium without loss of pressure. Experiments can be run in both static and flow-through mode. The incubation system described here is usable for versatile purposes, not only the incubation of microorganisms and determination of growth rates, but also for chemical degradation or extraction experiments under high gas saturation, e.g., fluid–gas–rock-interactions in relation to carbon dioxide sequestration. As an application of the system we extracted organic compounds from sub-bituminous coal using H_2O as well as a H_2O–CO_2 mixture at elevated temperature (90°C) and pressure (5 MPa). Subsamples were taken at different time points during the incubation and analyzed by ion chromatography. Furthermore we demonstrated the applicability of the system for studies of microbial activity, using samples from the Isis mud volcano. We could detect an increase in sulfate reduction rate upon the addition of methane to the sample.

2.1. Introduction

The incubation of deep subsurface microorganisms under high-pressure conditions is necessary because under non-*in* sit*u* conditions (especially low pressure) metabolic processes and survival of microorganisms adapted to high hydrostatic pressure are negatively impacted (Yayanos and Dietz, 1983; Fang et al., 2010). Since the first isolation of a pressure-adapted bacterium by Yayanos et al. (1979) numerous studies on the effect of elevated pressure on genetic, metabolic, and physiological aspects of microorganisms were carried out. Multiple biological effects of pressure on organisms were observed: shifts in metabolic activity (Abe et al., 1999; Bothun et al., 2004), transcription profiles (e.g., Boonyaratanakornkit et al., 2007), and the dissociation of ribosomes (e.g., Schulz et al., 1976), changes in growth rates (Yayanos, 1986; Boonyaratanakornkit et al., 2006; Takai et al., 2009), gene regulation (Bartlett et al., 1989), stabilization of proteins (Hei and Clark, 1994; Sun and Clark, 2001), and the composition of membrane lipids (Delong and Yayanos, 1985; Kaneshiro and Clark, 1995). For reviews of pressure effects on biological processes see Jaenicke (1983) and Bartlett (2002). Biochemical processes are also influenced by physical implications of high hydrostatic pressure, because the thermal expansion coefficient (Frank, 1970) as well as viscosity and fluidity of water (Horne and Courant, 1965) affect chemical reactions and cellular processes.

The idea of constructing and using a high-pressure vessel for studying deep-sea life is quite old. Zobell and Oppenheimer (1950) described a simple pressure vessel for the application of high hydrostatic pressure on microorganisms. Pressure was applied to a culture tube with a neoprene stopper working as piston for transmitting pressure to the sample. This type of pressure application is still being used today (Orcutt et al., 2008). Yayanos (1969) and later Taylor and Jannasch (1976) presented techniques for sub-sampling of media and bacteria and the determination of reaction rates without decompression, thereby eliminating the repetitive and time-consuming decompression. The use of glass syringes or a flexible Teflon container instead of a sealed culture tube (Schmid et al., 1978) had the benefit of an inert reaction chamber. However, the leakage of gases from the media into the pressure liquid or vice versa required a gas-tight incubation chamber. Bernhardt et al. (1987) used flexible nickel tubes for incubations of methanogenic microorganisms with hydrogen. Also flexible cells made of gold (Seyfried, 1979) or titanium (Seyfried and Janecky, 1985) were used as high-pressure reaction chamber. However, such devices were designed for studies of hydrothermal alteration

of basalt and therefore made for much higher temperatures than what is necessary for biological incubations. All described techniques are still in use. Recently Parkes et al. (2009) presented a high-pressure system that can accept drill cores, taken with a high-pressure corer without decompression. The system also allows for sub-sampling without decompression.

Temperature also has an effect on growth rates and other physiological characteristics of all microorganisms. Thermophilic and thermotolerant microorganisms can be found at hydrothermal vents, terrestrial hot springs, and intraterrestrial habitats (Pedersen, 2000) like salt mines (Vreeland et al., 1998), groundwater deep within Earth (Lin et al., 2006; Chivian et al., 2008), or oil reservoirs (e.g. L'Haridon et al., 1995).

Several techniques for the incubation of these thermophilic microorganisms are used: thermistors (e.g., Bernhardt et al., 1987), drying ovens (Miller et al., 1988; Takai et al., 2008), and water baths (e.g. Jannasch et al., 1996). Incubators and water baths became the most commonly used techniques for keeping pressure vessels at the desired temperature.

The application of elevated gas concentration in high-pressure incubations started about 25 years ago. Gases were applied to high-pressure vessels to maintain anaerobic conditions in incubations of hyperthermophilic archaea (e.g., Raven et al., 1992), to obtain higher cell densities during incubation (e.g., Mukhopadhyay et al., 1999) or as substrate for methanogenic microorganisms (Bernhardt et al., 1987; Takai et al., 2008). Nauhaus et al. (2002) incubated sediment samples from a methane hydrate field at different partial pressures of methane and showed a strong correlation between microbial activity and methane partial pressure. None of these incubation systems allowed manipulation of the gas partial pressure during the incubation or sub-sampling without decompression.

Here, we present an inexpensive high-pressure high-temperature incubation system that allows the incubation of a sample at high hydrostatic pressure as well as the manipulation of the composition and concentration of the dissolved gasses in the medium during incubation. It is designed for both static and flow-through experiments and allows for sub-sampling the liquid phase including the dissolved gases without decompression. The key objective was to build a moderately priced incubation system that can easily be constructed and operated. To keep costs low we used standardized off-the-shelf items and only a few custom-made parts.

With this system not only microbiological experiments under high hydrostatic and

gas partial pressure can be performed. Geochemical experiments, for example the extraction of organic and inorganic compounds from rock samples under specific pressure and temperature conditions or mineral alteration studies are also possible.

Initial tests of the system included applications for geochemical and microbiological experiments. The effect of high concentrations of CO_2 dissolved in water on the release of low molecular weight organic acids from sub-bituminous coal from the Waikato Basin (NZ) was studied, as well as the effect of high methane partial pressure on microbial activity in samples from the Isis mud volcano (IMV), off the Mediterranean coast of Egypt.

2.2. Materials and methods

The high-pressure incubation system (Figure 1) is composed of a reservoir vessel and a reaction vessel for the application of hydrostatic pressure on an incubator sleeve, which hangs inside the pressure vessel and holds the sample. A sub-sampling system allows the retrieval of liquid subsamples during the experiment without decompression. Temperature is maintained by a heating/cooling bath (Julabo Labortechnik GmbH, Seelbach, Germany) that pumps the liquid through heating jackets around the reservoir and the reaction vessel. Medium is circulated in a closed loop and HPLC pumps maintain hydrostatic pressure. A photograph of the entire unit is shown in Figure 2.

For microbiological experiments all parts of the high-pressure incubation system can be sterilized by autoclaving.

2.2.1. Reservoir vessel

The task of the temperature-controlled reservoir vessel is to saturate the medium with gas to the desired level and to hold a reservoir of medium that is pumped through the system.

The reservoir vessel is stainless steel cylinder (Dunze GmbH, Hamburg, Germany), with a volume of 255 cm^3 (inner dimensions: 3.4 cm diameter, 28.15 cm high; Figure 3A). Top and bottom are closed with plugs with bores for 1/16'' HPLC lines to allow for transfer of gas and medium in and out of the vessel.

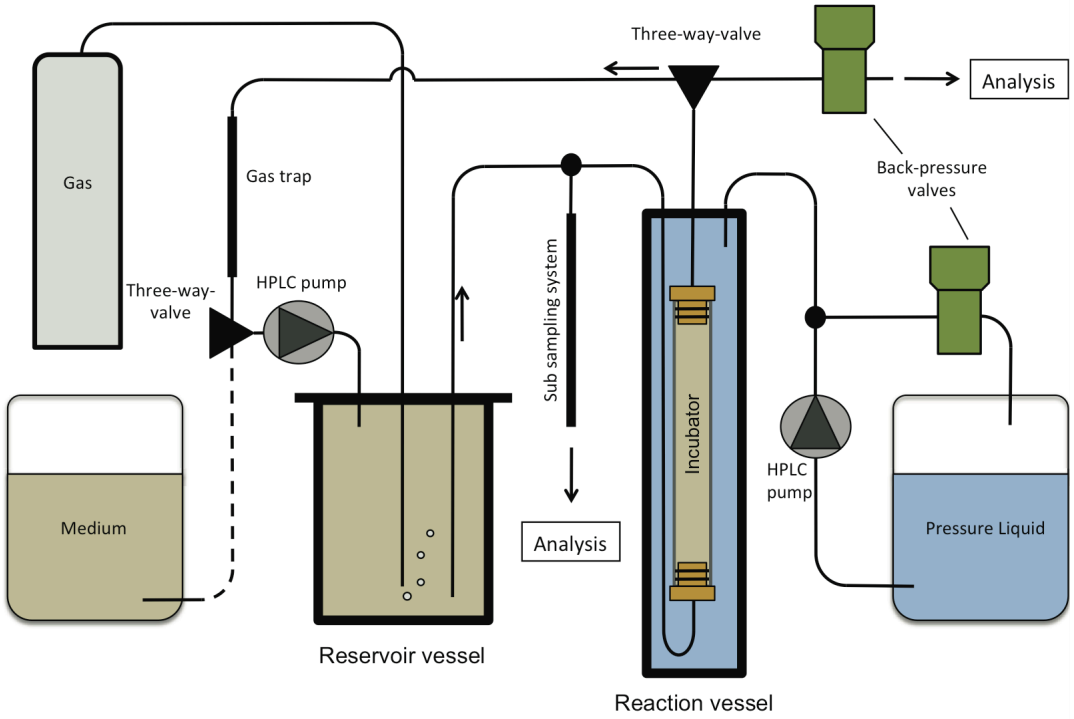

Figure 1: Schematic drawing of the high-pressure incubation system. The medium is pressurized and enriched with gas in the reservoir vessel prior to flowing through the incubator sleeve that hangs inside the reaction vessel. Pressure in the reaction vessel is set and kept constant by a HPLC pump and a backpressure valve.

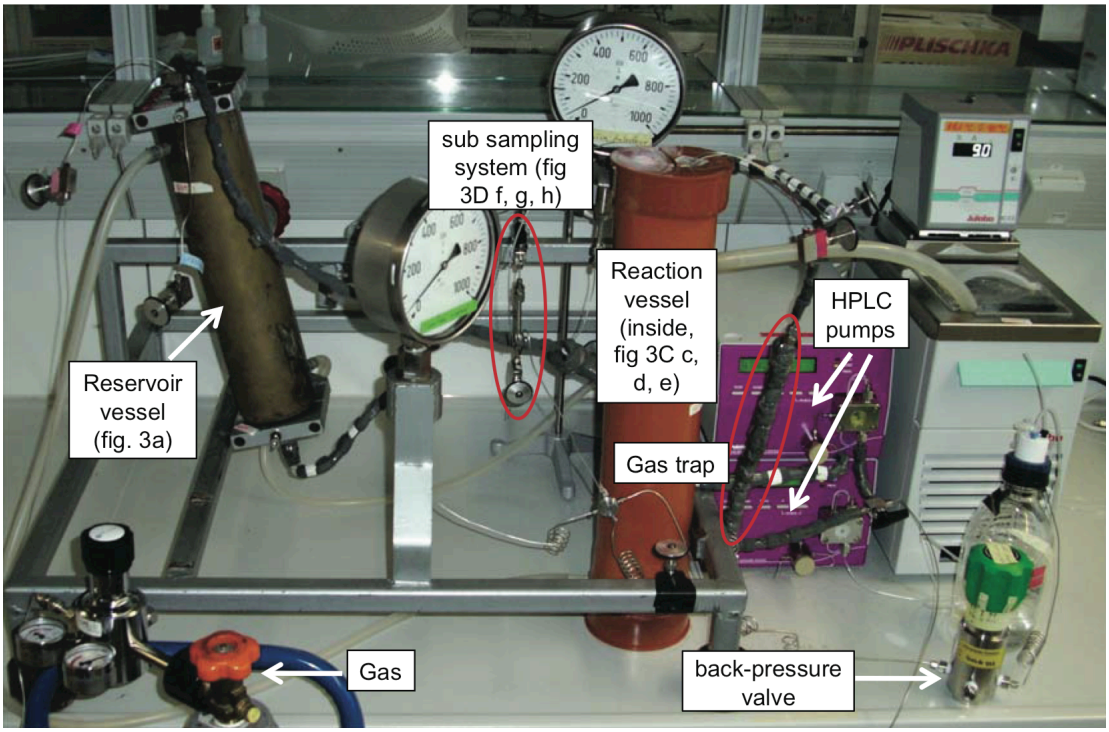

Figure 2: Photograph of the high-pressure incubation system as seen in Figure 1. Reservoir vessel, reaction vessel, incubator and sub-sampling device are shown in detail in Figure 3.

2.2.2. Reaction vessel

The reaction vessel (Figure 3B) is a stainless steel cylinder (Dunze GmbH, Hamburg, Germany). The cylinder has an inner diameter of 3.5 and 27.0 cm in length (volume of 259.7 cm^3). Top and bottom are closed with plugs, each with bores for four 1/16'' HPLC lines each. The vessel is sealed with banjo screws that push the plugs into their seals.

2.2.3. Incubator sleeve

The incubator (Figure 3C) is a sleeve of polyvinylidene fluoride (PVDF, Novoplast, Halberstadt, Germany), a polymer that is inert to almost all chemicals. Although this material is not as flexible as polytetrafluoroethylene (PTFE) or fluorinated ethylene propylene (FEP), it was chosen due to its very low permeability for gases (Table 1). The sleeve is closed with two gold-coated stainless steel plugs (3 µm gold thickness; Schempp and Decker, Berlin, Germany) with two gutters, each holding a perfluoro-elastomer O-ring (FFKM, Parker Hannifin, Pleidelsheim, Germany). FFKM was used for its chemical resistance. Both stoppers have a central threaded bore for the connection with a 1/16'' HPLC line: one for inflow of medium at the bottom of the incubator and one for outflow at the top of the incubator. The incubator was designed to avoid corrosion, therefore only inert materials (PVDF, FFKM, and gold) were used. The sleeve has a diameter of 25 mm and can have a maximum length of 22 cm, which leads to a maximum volume of 68 cm^3. Inside the incubator the medium first has to pass through a 1.5 cm thick layer of 2 mm diameter glass beads, followed by a 1.5 cm thick layer of quartz wool (organic free by annealing in a muffle furnace) so that the stream of medium passes evenly through the sample material over the entire cross section of the incubator sleeve. On top of the sample, the described layers follow in opposite order; first quartz wool for holding back most of the fine particles that could clog the lines and valves, followed by glass beads until the incubator is full and the sample well packed.

Additional to the chemical resistance and the almost complete impermeability for gases, further advantages of using a PVDF sleeve is its relatively low price, allowing for the possibility of using it as a disposable article. Thereby, cross contamination between samples can be excluded. Furthermore, mechanical stress leading to a weakening of the material and, therefore, a possible leakage of medium or inflow of pressure fluid will be prevented. The incubator sleeve hangs inside the reaction vessel and is connected to the top plug of the incubation vessel via the 1/16'' HPLC lines.

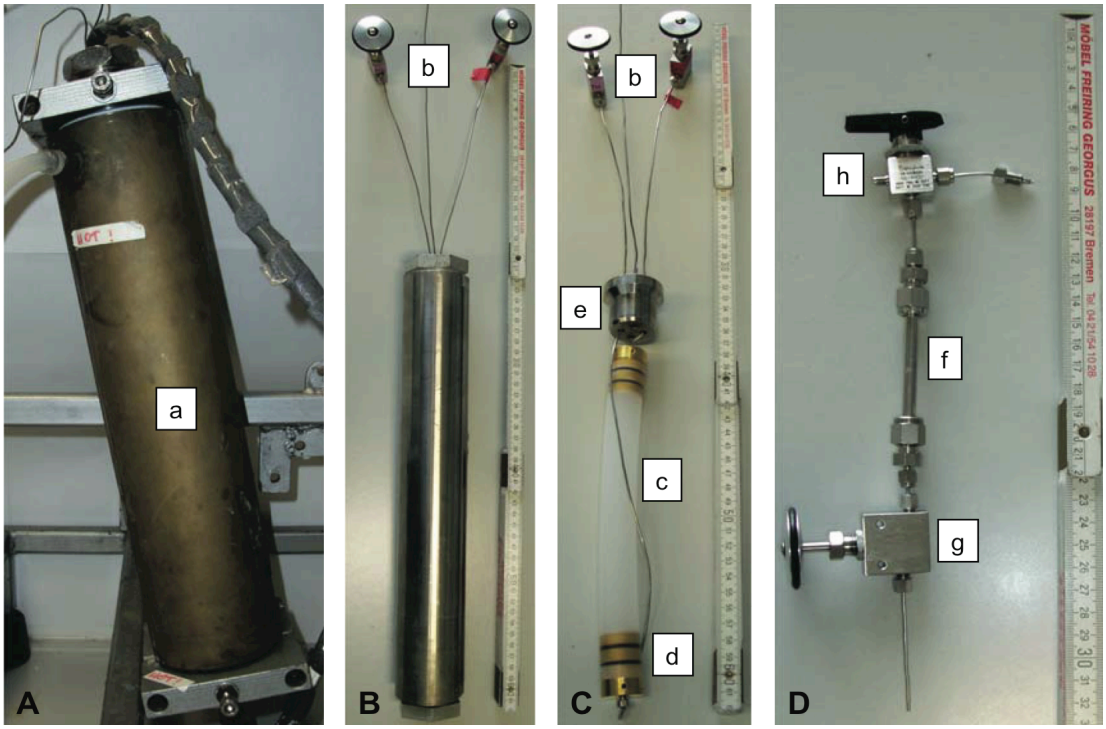

Figure 3: (A) Reservoir vessel with a total volume of 255cm^3; (a) pressure cylinder (invisible) with heating/cooling jacket; **(B)** high-pressure reaction vessel including connections with (b) valves for in-and out-flow of medium via the incubator; the third line (without a valve) is for the application of pressure to the reaction vessel; **(C)** PVDF-incubator with (b) valves, (c) PVDF incubation sleeve; maximum volume 60cm^3, connected to in-and out-flow lines via the plug of the pressure vessel (e), (d) gold-coated stainless steel plugs with FFKM O-rings; **(D)** sub-sampling device; (f) 1/4" high-pressure line, three-way-valve (h), shut-off valve (g).

2.2.4. Sub-sampling system

A sub-sampling device allows taking fluid samples during the experiment without decompression (Figure 3D). It is attached to the high-pressure line between the reservoir vessel and the reaction vessel. The sub-sampler is made from 1/4" stainless steel tubing (7.9 cm in length, inner diameter 0.225 cm, total volume 0.513 cm^3) and has a three-way-valve (Swagelok Limited, Tromode, UK) at the top and a shut-off valve (Supelco) at the bottom. The three-way-valve connects the sub-sampler to the incubation system. The third connection of the valve is used to either apply vacuum to the sub-sampler prior to sampling to avoid oxidation of the sample or to apply overpressure (nitrogen gas) to push out the remainder of the sample. After the sub-sampler is evacuated, the three-way-valve is turned and the sample enters the sub-sampler. Then the three-way-valve is closed and the shut-off valve at the bottom of the system is opened and the sample transferred into a sampling vial. Nitrogen gas is added through the three-way-valve to push out the remaining sample.

Table 1: Comparison of mechanical parameters and gas permeability of PTFE, FEP and PVDF. Data supplied by Bohlender GmbH, Grünsfeld, Germany.

Mechanical parameters	**Units**	**PTFE**	**FEP**	**PVDF**
Shore durometer D	Durometer	55–72	55–60	73–85
Flexural strength	N/mm^2	600–800	660–680	1200–1400
Gas permeability in cm^3/m^2 or d/bar		**PTFE**	**FEP**	**PVDF**
Nitrogen		0.7	3.8	0.06
Oxygen		2.05	30	0.05
Carbon dioxide		5.7	60	0.2

2.2.5. Gages and pumps, other hardware

Pressure is generated by a modified HPLC pressure pump (SYKAM S 1122, Sykam GmbH, Fuerstenfeldbruck, Germany, modifications according to Kallmeyer et al., 2003). A second identical pump is used to circulate the medium through the reservoir vessel and the incubator sleeve. Pressure is kept constant through a backpressure valve (pressure regulator series KHB, Swagelok Limited, Tromode, UK).

All pressure vessels (reservoir and reaction vessel) are connected to 100 MPa pressure gages (WIKA Alexander Wiegand SE and Co. KG, Klingenberg, Germany). All pumps and vessels are connected with 1/16'' HPLC lines (CS Chromatographie, Langerwehe, Germany). If not mentioned otherwise all valves were obtained from Supelco, Bellefonte, PA, USA.

2.2.6. Gas trap

To avoid the possible entry of gas bubbles into the pump, an empty HPLC column (25.1 cm in length, inner diameter 0.45 cm, total volume 4 cm^3; Sykam GmbH, Fuerstenfeldbruck, Germany) is used as a gas trap, mounted vertically between the reaction vessel and the pump to collect any gas bubbles that may form. The medium flows from top to bottom, so the gas bubbles are trapped at the top.

2.3. Application of the system

We used the system for the extraction of low molecular weight organic acids, which are a potential microbial energy source, from a coal sample using water and water–carbon dioxide mixture at 90°C and 5 MPa. In a second application we incubated sediment from a mud volcano that is known to exhibit high rates of anaerobic methane oxidation at 23°C and 10 MPa total pressure and 4 MPa methane partial pressure (96 mmol/l) and measured an increase in sulfide concentration.

2.3.1. Coal sample

We selected a sub-bituminous coal sample [vitrinite reflectance (R_0): 0.29%] from the Whangamarino formation (latest Miocene to late Pliocene), which is part of the Tauranga group. The sample was from a depth of 64.69 m below surface, taken from the DEBITS-1 well, which was drilled in 2004 within the scope of the Deep Biosphere in Terrestrial Systems (DEBITS) project at Ohinewai in the Waikare Coal Field of the Waikato Basin on the North Island of New Zealand.

The well had a total depth of 148 m and penetrated interbedded layers of organic-rich carbon (lignites and sub-bituminous coals) as well as mudstones, siltstones, and sandstones. The total organic carbon (TOC) content in the sample is approximately 30%. For further information about the sample material and the geology of the Waikato Basin see Glombitza et al. (2009).

Low molecular weight organic acids (LMWOAs) such as formate, acetate, and oxalate are known to be the main organic compounds obtained from aqueous extractions of lignites and coals (Vieth et al., 2008). The LMWOAs are also components of the macromolecular organic material of the coal (Glombitza et al., 2009) and are released from the coal matrix during ongoing maturation into the surrounding pore water.

2.3.2. Mud volcano sediment

Isis mud volcano lies on the Egyptian continental margin in the Nile deep-sea fan (NDSF) in a water depth of ~991 m and covers an area of approximately 10 km^2. The NDSF is a sedimentary wedge that is deposited since the late Miocene by the Nile river (Loncke et al., 2004) with an assumed thickness of up to 10 km. Deeper sediments

become strongly overpressured by the thick sedimentary overburden, resulting in an upward migration of fluids and gases (Loncke et al., 2004). Among other mud volcanoes in the area, IMV is emitting large volumes of gas (Dupré et al., 2008), including methane, ethane, and propane (Mastalerz et al., 2009). These gases are probably a mixture from different sources, because their isotopic composition is rather inconclusive with regards to a thermogenic or microbial origin (Mastalerz et al., 2007).

The emitted gases – mostly methane – are substrates for microorganisms. In sediment samples of the IMV Omoregie et al. (2009) found several genera of sulfate-reducing bacteria (*Desulfosarcina, Desulfococcus, Desulfocapsa, Desulfobulbus*) as well as *Methanococcoides*, a methanogenic *Archea*, and the anaerobic methane oxidizers ANME-1, ANME-2, and ANME-3.

The sample was taken during the NAUTINIL expedition in 2003 at 32°22'N; 31°23'E in 1020 m water depth and stored in a glass bottle at 4°C with a nitrogen headspace. About a week prior to the experiments, the headspace was flushed with methane.

Immediately prior to the incubation experiments, concentration of hydrogen sulfide and methane in the pore water was around 6 and 4.8 mmol/l, respectively.

2.3.3. Experimental procedure

We extracted the coal sample at elevated temperature (90°C) and pressure (5 MPa) using deionized water in the first experiment and a water–carbon dioxide mixture in the second. Five grams (approx. 9.4 cm^3) of the freeze-dried and powdered coal sample were placed in the incubator sleeve. For the water extraction, the experiment was started after reservoir and reaction vessel had reached 5 MPa and 90°C. In the second experiment with the H_2O–CO_2 mixture, gas was added to the reservoir vessel after heating to 90°C and left overnight for equilibration. The experiment and the circulation of the gas-saturated medium started after equilibration. Pressure was generated by adding CO_2 until 5 MPa were reached and pressure had stabilized as maximum gas saturation had been reached (approximately 106 g/l or 2.4 mol/l of dissolved CO_2).

Extractions were carried out for a total of 48 h. Subsamples (0.513 cm^3) of the medium were taken after 6, 22, 30, and 48 h and flushed into 513 µl of a 3.6/3.4 mmol/l solution of Na_2CO_3/$NaHCO_3$ containing 2% isopropanol to reduce the volatility of the LMWOAs thereby avoiding a loss of these compounds. The subsamples were immediately frozen until analysis (within 1 week). Sample analysis was performed by

ion chromatography (IC) without further dilution.

The incubation of sediment samples from IMV was performed at a pressure of 10 MPa and a temperature of 23˚C, using an artificial seawater medium (Widdel and Bak, 1992) with a sulfate concentration of 27 mmol/l.

In the first experiment the medium contains just 0.1 mmol/l of methane (from the dilution of the sediment sample pore water methane), whereas in the second experiment the medium contained 96 mmol/l methane. In order to be able to detect even small amounts of hydrogen sulfide, the volume of the reservoir vessel was reduced to 145 ml by adding glass beads (5 mm diameter). Inside an anaerobic glove box 10 cm^3 of IMV sediment were loaded into the incubator sleeve. The experiment with only artificial anoxic seawater medium was started after reservoir and reaction vessel were equilibrated to 10 MPa and 23˚C. In the second experiment the medium was first pressurized with methane to 4 MPa, leading to a methane concentration of approximately 1.6 g/l or 96 mmol/l of dissolved methane and left overnight for equilibration. After methane saturation was complete, pressure was increased hydrostatically to 10 MPa with anoxic artificial seawater medium. The sample was loaded into the incubation vessel and pressurized prior to the experiment; circulation of the gas-saturated medium started after equilibration.

Incubations were carried out for a total of 432 h (9 days). Sub-samples (0.513 cm^3) of the medium were taken every 2 days. The subsamples were mixed with equal volumes of a 5% (w/v) zinc acetate solution to fix the volatile sulfide as zinc sulfide. The fixed subsamples were immediately frozen until photometric analysis.

2.3.4. Sample analysis by ion chromatography

The samples were analyzed in replicates using IC. The IC system (Sykam GmbH, Fuerstenfeldbruck, Germany) was equipped with an LCA A 20 column, a suppressor (SAMS, SeQuant, Sweden) and a SYKAM S3115 conductivity detector. The mobile phase was a 1.8/1.7 mmol/l Na_2CO_3/$NaHCO_3$ mixture. Elution was performed at isocratic conditions. The eluent flow was set to 0.8 ml/min. A blank sample (deionized water) and a multi-compound standard containing each 50 mg/l formate, acetate, and oxalate (for the extraction of coal) were measured prior to each sample. Standard deviation of sample and standard quantification was below 10% (determined from replicate analysis).

2.3.5. Hydrogen sulfide quantification

Hydrogen sulfide concentration was quantified according to Cline (1969). In brief, 5 ml of deionized water and 400 µl of Cline-reagent (1.6 g of *N*,*N* -dimethyl-*p*-phenylenediamine sulfate plus 2.4 g of $FeCl_3 \cdot 6H_2O$ in 100 ml 50% HCl) are added to the sample. Adsorption is measured in a photometer at 680 nm after 20 min. The minimum detection limit with a 1 cm cell is around 50 µmol/l.

2.4. Results

The main organic compounds extracted with water at 5 MPa and 90°C are formate, acetate, and oxalate. Vieth et al. (2008) reported comparable results from aqueous Soxhlet extraction of similar sample material from the DEBITS-1 well. The amounts of extracted LMWOAs increase over the course of the experiment (Figure 4, blue circles). The strongest increase in concentration of LMWOAs was observed during the first 22 h of extraction. In the following 26 h a slow but steady increase of extracted LMWOA was observed, suggesting that the experiment did not run to completion after 48 h. Nevertheless, the yields of the extracted LMWOAs are approaching steady state. Total amounts of extracted LMWOAs after 48 h were 2.3 mg/g TOC formate, 3.8 mg/g TOC acetate, and 5.2 mg/g TOC oxalate.

In the second coal extraction experiment we used an H_2O–CO_2 mixture under the same pressure and temperature conditions (90°C, 5 MPa) but with 2.4 mol/l CO_2. Under these conditions, the same organic compounds (formate, acetate, and oxalate) were extracted (Figure 4, green circles). Like in the experiment with deionized water, the amounts of extracted LMWOAs increase with increasing extraction time, with the main increase during the first 22 h. The total amounts of extracted LMWOAs after 48 h were 2.4 mg/g TOC for formate, 2.7 mg/g TOC for acetate, and 4.5 mg/g TOC for oxalate. These numbers are somewhat lower than in the pure water extraction experiment (Figure 4).

During the first 48 h of both IMV sediment incubation experiment, hydrogen sulfide concentration increased to about 0.3 mmol/l due to the mixing of the sediment's pore water hydrogen sulfide with the medium. Over the course of the first experiment the concentration remained around 0.3 mmol/l for the remainder of the experiment (Figure 5, red circles).

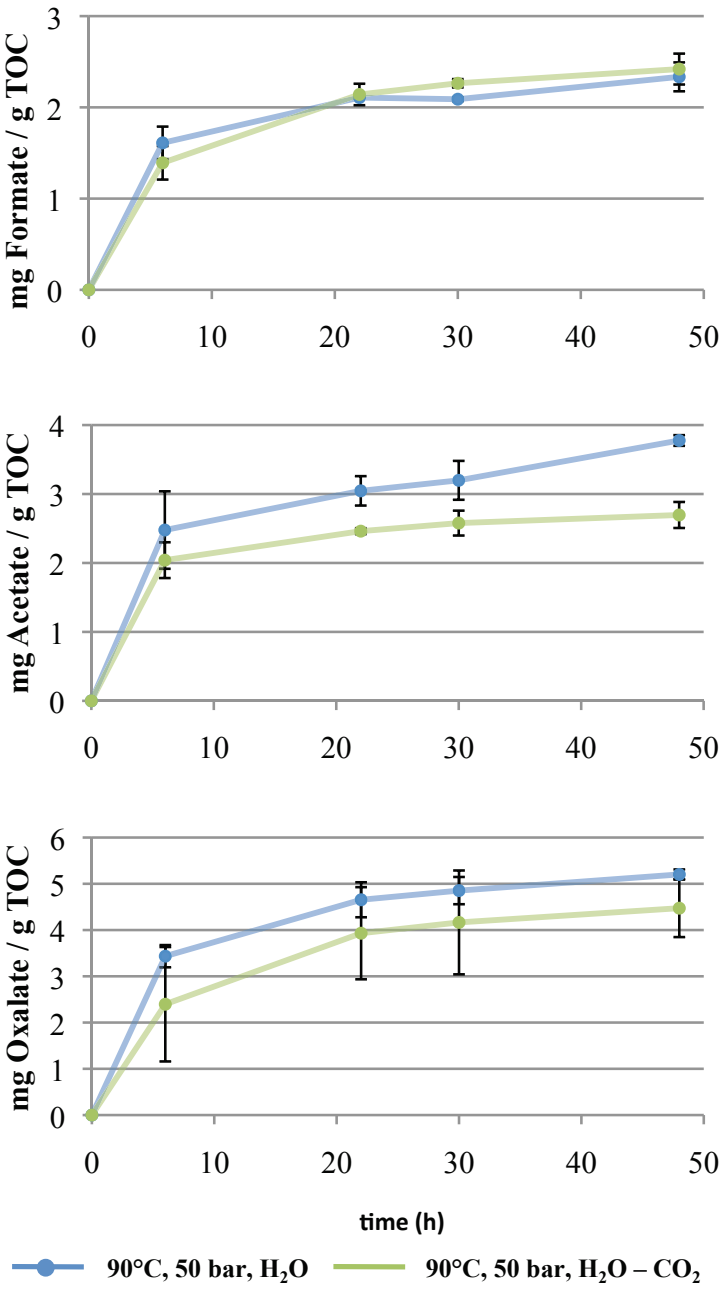

Figure 4: Extraction of (A) formate, (B) acetate, and (C) oxalate from sub-bituminous coal sample taken from the DEBITS-1 well at Waikato coal area, North Island, New Zealand. Five grams of coal (approximately 30% TOC) were extracted for 48 h at 5 MPa and 90°C. Extractions were performed with water (blue circles) and water-carbon dioxide mixture (green triangles). Sample analysis was performed by ion chromatography.

In the second experiment with the added methane (4 MPa, 96 mmol/l), sulfide concentration remained around 0.3 mmol/l until ca. 144 h, before increasing almost exponentially (Figure 5, blue circles). The experiment was stopped after 432 h at a hydrogen sulfide concentration of 0.93 mmol/l.

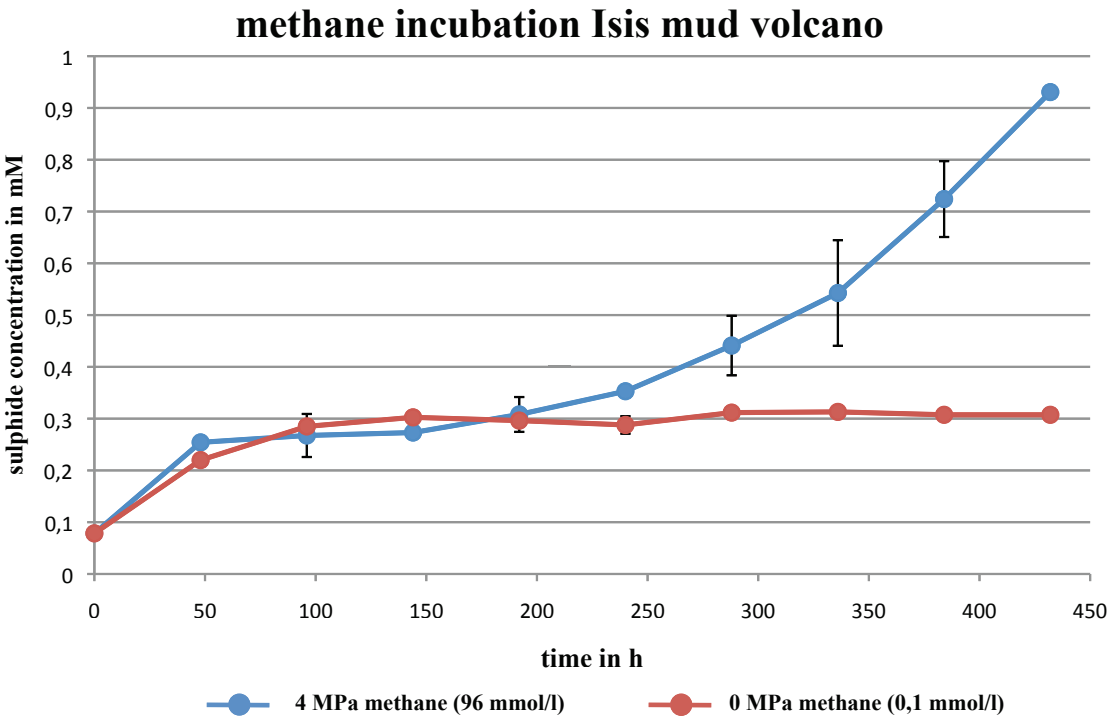

Figure 5: Incubation of sediment samples from Isis Mud Volcano (IMV). Ten cubic centimetres of sediment were incubated for 432 h at 10 MPa and 23°C. Incubations were performed with 4 MPa methane (blue circles) and without adtion of methane (red circles).

2.5. Discussion

The extracted LMWOAs (formate, acetate, and oxalate) have also been found to be the main organic acids obtained from water extraction of other low mature coals (Bou-Raad et al., 2000; Vieth et al., 2008; Glombitza, 2011).

High-pressure (5 MPa) extraction with pure water in our system resulted in significantly higher yields (2.4–4.5 mg organic acids/g TOC) than what was reported from Soxhlet extraction of coal samples from a similar depth interval of the DEBITS-1 well, which yielded between 0.7 and 1.4 mg organic acid/g TOC for individual LMWOAs (Vieth et al., 2008). The extraction of LMWOAs resulted in a decrease of pH in the extraction medium. The pH in the reservoir of a Soxhlet apparatus decreases to approximately pH 4 after 48 h of extraction with deionized water (data not shown). However, the sample only gets into contact with freshly distilled water (pH 7) that drips over the sample. In the high-pressure system the extraction medium containing the extracted organic acids circulates through the system, thereby extracting the coal with a low-pH medium. The lower pH is supposed to enhance the release of LMWOAs from macromolecular organic matter in the coal by hydrolysis (Glombitza, 2011). This might explain the higher extraction yields of LMWOAs in our reactor system as compared to Soxhlet extraction.

Under in situ conditions, the extracted LMWOAs will remain in the pore water and thereby cause a drop in pH before they are eventually removed by diffusion or fluid flow. As Fry et al. (2009) reported from samples from the DEBITS-1 well, the majority of microbial activity and abundance is not found in the coals but rather in the surrounding and more porous sandstones. So the consumption of the produced LMWOA does not take place inside the coal seams but above or below them and removal of these substances from the coals is controlled by diffusion or fluid flow, not by microbial activity. It is therefore reasonable to assume that our high-pressure system provides reaction conditions that are much more realistic than Soxhlet extraction because the extracted compounds are not removed from the reaction.

When comparing the results of the first extraction with deionized water and the second extraction with an H_2O–CO_2 mixture, it becomes obvious that CO_2 reduces the amount of extracted acetate by a factor of 1.39 and oxalate by a factor of 1.16. For formate no clear influence of CO_2 on the extraction efficiency could be observed. At first sight, this result is surprising because carbon dioxide dissolved in water lowers the pH due to formation of carbonic acid (Meyassami et al., 1992). The lower pH was expected to enhance hydrolysis and, therefore, increase the yield of extractable LMWOAs. However, we observed a suppressing effect of CO_2.

The LMWOAs found in the extraction fluid may not just result from the actual extraction of the coal but also from different secondary reactions. It was suggested that oxalate in aqueous extracts of coals is a result of the decomposition of 1,2-dihydroxy-carboxylic acids (Bou-Raad et al., 2000). Therefore it has to be assumed that at least for oxalate (and maybe for other LMWOAs as well) the extraction yield is not only affected by hydrolysis but also by secondary reactions, which may be inhibited or suppressed in the presence of CO_2 in the extraction medium.

The incubation experiment with sediment samples from the Isis mud volcano clearly showed the positive effect of elevated methane concentration on the rate of sulfide production, which is a direct result of sulfate reduction. Omoregie et al. (2009) conducted whole core $^{35}SO_4^{2-}$ radiotracer incubations of samples from the same mud volcano at atmospheric pressure (0.1 MPa) and with a maximum methane concentration of >10 µmol l^{-1}. They measured sulfate reduction rates of 7–240 nmol cm^{-3} day^{-1}. We conducted our experiment at much higher methane concentration (96 mmol l^{-1}) and in situ pressure (10 MPa), and measured a significantly higher sulfate reduction rate of ca. 2000 nmol cm^{-3} day^{-1}, which we attribute to the elevated methane concentration, as already shown by Nauhaus et al. (2002).

The aim of this paper is to present a new high-pressure incubation system and experiments to demonstrate the application of elevated temperature and pressure as well the use of elevated gas saturation and their effects on geochemical and microbiological processes. Therefore, we can only speculate about the reasons for the observed suppressing effect of the CO_2. This effect still remains puzzling and will be the topic of future investigations.

2.6. Conclusions

The high-pressure high-temperature incubation system is a moderately priced alternative to existing systems. Furthermore, it is easy to construct and to handle. Initial experiments demonstrate that the system is suitable for a wide range of applications in geo- and bio-sciences. The system allows the incubations at elevated pressure and temperature conditions (up to 120˚C and 60 MPa) as well as manipulating the dissolved gases throughout the experiment. The system also allows sub-sampling of the fluid phase during the course of the experiment without decompression. Extraction of a sub-bituminous coal samples under high-pressure and temperature conditions showed a higher yield in LMWOAs from macromolecular organic matter as compared to an extraction with a Soxhlet apparatus. The high-pressure extraction of the coal sample with CO_2-saturated water revealed a suppressing effect of the CO_2 on the extraction yield or secondary formations of LMWOAs. Possible reasons for this effect are not identified yet and will be in the focus of future investigations. The incubation of sediment samples from a mud volcano harboring sulfate-reducing methanotrophs showed a clear positive response of methane addition on the sulfate reduction rate. Our high-pressure high-temperature incubation system has proven its suitability for a broad range of scientific applications.

2.7. Acknowledgments

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3. „Liberation of low molecular weight organic acids from brown coal by highly CO_2-enriched water and their role as potential microbial substrates“

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The sequestration of the green house gas carbon dioxide and the enhanced recovery of oil and gas by carbon dioxide injection has become more and more common in recent years. However, the effects of elevated concentrations of carbon dioxide on sedimentary organic matter are rarely investigated.

The injected supercritical CO_2 will eventually dissolve in the pore water and become subcritical, forming various acids, which enhance acidic hydrolytic extraction of low molecular weight organic acids (LMWOAs) from the macromolecular organic matter. In subsurface environments, where carbon and energy sources are limited, LMWOAs play an important role as feedstock for microbial communities. However, research on CO_2-extraction of coals has only been carried out with supercritical CO_2 and no attention has been paid to the liberation of LMWOAs from coal by the use of CO_2.

This study was performed in order to evaluate the effect of highly CO_2-enriched waters on the nutrient supply for subsurface microbes. In this study we present results from extractions of LMWOAs from different coal horizons of the open-cast lignite mine Welzow-Süd in the Lower Lusatian brown coal district, Germany. Various types of gas - food grade CO_2 as well as mixes containing NO_2 and SO_2 - were used to mimic actual flue gas composition. With our high-pressure incubation system we mainly extracted formate, acetate and oxalate.

Keywords: high-pressure, extraction, low-rank coal, carbon dioxide, low molecular weight organic acids, flue gas

3.1. Introduction

In 1955 Morita and ZoBell defined the lower limit of life of the biosphere at about 4 meters below the seafloor due to the inability to culture any microbes from deeper samples (morita and ZoBell, 1955). Since then, much research has been carried out to extend this limit.

Since the discovery of microorganisms in deep marine sediments and different terrestrial subsurface environments such as oil fields or deep basalt aquifers it is commonly agreed that there is abundant microbial life in the subsurface (Stetter et al., 1993; Parkes et al., 1994; L'Haridon et al., 1995; Stevens and McKinley, 1995; Onstott et al., 1998). Due to the extreme environmental conditions in the subsurface such as high pressure and temperature as well as low nutrient concentrations, questions about survival strategies of the indigenous microbes in these great depths arose. When dealing with the question of metabolic activity, the main question is that of the carbon and energy sources for microorganisms in these subsurface environments.

In sedimentary settings with high carbon content e.g. coal seams, the question about the potential carbon and energy sources seems easy to answer, as carbon is abundant (Stetter et al., 1993). However, this carbon has a macromolecular structure and is not available for microbial utilization (Mathews and Chaffee, 2012). It has therefore to undergo maturation processes. However, it is still not fully understood if microbially available carbon (low molecular weight organic acids, LMWOAs) is the product of maturation of buried organic matter (catagenesis, diagenesis) or a degradation product of microorganisms (Killops and Killops, 2005). Horsfield et al. (2006) showed the abiotic formation of LMWOAs (e.g. formate, acetate, propionate, oxalate) from sedimentary organic matter during catagenesis (liberation of hydrocarbons from buried unoxidised organic matter – kerogen). This indicates the influence of thermal maturation on macromolecular degradation.

A further process for degradation of buried macromolecular organic matter is aqueous hydolysis. Chemical bonds between the macromolecules are cleaved and small organic molecules like LMWOAs are liberated. This hydrolysis requires the porewater as reactant, and increases with rising temperature, e.g. during burial to greater depth (Wellsbury et al., 1997). Furthermore the rate of hydrolysis depends on the pH and will be increased by the acidification of the porewater, which may be caused by the liberation of LMWOAs.

LMWOAs act as the main microbial substrates in nearly every environment (Thauer et al., 1977). Even in very oligotrophic environments like the North Pacific Gyre

microbes are utilizing sedimentary organic matter (Røy et al., 2012). Only basaltic environments, which are free of organic carbon, are dominated by lithoautotrophic microorganisms. These microbes are generating small organic molecules from hydrogen gas (produced by reactions of basalt and anoxic water) and carbon dioxide (Stevens and McKinley, 1995).

The rise of the underground storage of carbon dioxide to reduce green house gas emissions or the enhanced recovery of oil and gas (EOR/EGR) may lead to an enhanced acidification of sedimentary porewater. The injection of carbon dioxide in underground reservoirs leads to a formation of carbonic acids and a decrease of the pH, causing an acidification of the pore water (Meyssami et al., 1992; Adamczyk et al., 2009). However, it is quite possible that both mechanisms – biological and geological degradation of organic macromolecules – will lead to an acidification and an increased hydrolysis. It is therefore of high interest to evaluate a possible enhancement effect of injected carbon dioxide on the hydrolysis and the resulting liberation of LMWOAs from buried macromolecular organic matter.

Carbon dioxide that is used for CCS (carbon capture and storage) and EGR/EOR originates from power plants (also called flue gas) and is contaminated with traces of O_2, SO_X, NO_X. When dissolving this flue gas in water, these impurities lead to lower pH values than carbon dioxide alone due to the formation of nitric acid and sulphuric acid (Johnstone and Leppla, 1934; Hales and Sutter, 1973; Sakamaki et al., 1983; Pitts et al., 1984). The expected acidification of the pore water should therefore cause an increase in the rate of acidic hydrolysis, which is controlled by the pH. This would lead to an increase in the liberation of LMWOAs.

Glombitza et al. (2009) showed on low maturity coals from New Zealand that released organic acids act as an important feedstock for indigenous subsurface microbes. Therefore it can be assumed that LMWOAs act as nutrients for the subsurface microbiota also in other coal seams as well. We hypothesize that the injection of carbon dioxide into coal beds enhances the nutrient supply for subsurface microbes.

In this study we present data from the extraction of low molecular weight organic acids from different lignites under elevated hydrostatic and gas partial pressure. These experiments were performed in order to evaluate the effect of porewater, which is highly saturated with CO_2 and different impurities, on the liberation of LMWOAs from coals. We simulated the effects of enhanced CO_2 levels on the amount of extracted LMWOAs from macromolecular organic matter. To do so we saturated water with carbon dioxide to a high level (5 MPa) but remained below supercritical conditions. The

aim of this study was to evaluate the possible effect of highly CO_2-enriched porewater on the liberation of LMWOAs as nutrient supply for subsurface microbes.

3.2. Materials and methods

3.2.1. Sample material

Coal samples (vitrinite reflectance (R_0): 0.3 %) were collected in the open-cast lignite mine Welzow-Süd, Niederlausitz, Brandenburg, which belongs to the 2. Lusatian seam (2nd Miocene Seam Horizon, Middle Miocene, Niederlausitz brown coal district, Standtke et al., 1993). This mine is operated by Vattenfall Europe AG, Berlin, Germany. The coal is ranked as brown coal within the International Commission for Coal Petrology (ICCP).

Samples from three different horizons were used: horizon 2, 6 and 9 (H2, H6, H9). The chemical composition of all three horizons is given in table 1.

Table 1: Analysis of coal from Welzow-Süd, Germany (in mass percent of dry coal)

parameter	horizon 2	horizon 6	horizon 9
water content (wet coal)	58.3	55.4	56.6
carbon	26.6	29.1	28.0
fixed carbon	18.3	20.2	19.0
total organic carbon (TOC)	58.33	57.82	56.12
volatile compounds	21.2	22.0	22.1
total sulfur	1.33	0.19	0.50
sulfate	0.02	<0.01	0.01
organic sulfur	1.13	0.15	0.46

3.2.2. Methods

The extraction of LMWOAs from the sample material was performed with a high-pressure incubation system that allows for flow-through incubation with highly gas-saturated medium. It also allows for subsampling of the liquid phase without

depressurization. A sketch of the incubation system as well as pictures is given in Sauer et al. (2012).

The reservoir vessel holds the medium that is used for the extraction. In this vessel the medium can be saturated with gas to the desired level. The medium is pumped into the reaction vessel and through the incubator sleeve, which hangs inside the reaction vessel and holds the sample. Both vessels can be heated and pressurized. The pressure liquid is separated from the extraction fluid by the flexible walls of the incubator sleeve, made from PVDF (polyvinyledene fluoride; Novoplast Schlauchtechnik GmbH, Halberstadt, Germany), an inert and almost gas-impermeable plastic. The pressure of the system is kept constant by a constant flow of pressure fluid and a back-pressure valve. The incubator sleeve is closed with two gold-plated plugs equipped with two FFKM (perfluoro elastomer; Parker Hannifin GmbH, Bielefeld, Germany) O-rings each.

3.2.3. Experimental procedure

The coal samples were extracted at elevated temperature (90° C) and pressure (5 MPa) with different extraction media: (I) deionised water, (II) water saturated with carbon dioxide (food grade 99,9 %; Air Liquide, Berlin, Germany), (III) water saturated with carbon dioxide containing 1% nitrogen dioxide or (IV) water saturated with carbon dioxide containing 1% sulphur dioxide (both Linde Ag, Berlin, Germany). The exact conditions of the experiments are given in table 2. For the extraction with deionized water, the experiment was started after reservoir and reaction vessel had reached 5 MPa and 90°C. In the experiments with gas-saturated water the reservoir vessel was first heated to 90°C, then gas was added and left overnight for equilibration.

Table 2: Experimental conditions and setup for the extraction of lignite samples

	pure water	water saturated with CO_2	water saturated with CO_2/NO_2	water saturated with CO_2/SO_2
pressure	5 MPa	5 MPa	5 MPa	5 MPa
concentration		106 g / l	105 g / l CO_2 1 g / l NO_2	105 g / l CO_2 1 g / l SO_2
molar concentration		2.4 mol/l CO_2	2.28 mol/l CO_2 0.02 mol/l NO_2	2.28 mol/lCO_2 0.016 mol/l SO_2

The coal samples were freeze-dried and ground with a mortar to a fine powder. Five grams (approx. 9.4 cm^3) of the freeze-dried and powdered coal sample were placed in the incubator sleeve. The experiment and the circulation of the gas-saturated medium started after equilibration.

Extractions were carried out for a total of 72 hours. Subsamples (each 0.513 µl; volume predefined by the size of the subsampling device) of the medium were taken at the start of the experiment and after 6h, 22h, 30h, 48h and 72h. Each subsample was flushed into the same volume of a 3.6/3.4 mmol/l Na_2CO_3/$NaHCO_3$ solution containing 2% isopropanol to reduce the volatility of the LMWOAs, thereby avoiding a loss of these compounds. The mixing of the subsample with the Na_2CO_3/$NaHCO_3$ solution resulted in a solution with the same Na_2CO_3/$NaHCO_3$ concentration as the eluent used for ion chromatography. Subsamples were immediately frozen until analysis (within one week). Sample analysis was performed by ion chromatography without further dilution.

Due to the lack of a pH electrode inside the incubation system we could not measure the in situ pH. The subsamples were depressurised upon flushing them into the 3.6/3.4 mmol/l Na_2CO_3/$NaHCO_3$ solution and some of the dissolved gases where lost. Therefore we measured pH values from the reservoir after depressurisation at the end of each experiment. These values do not represent the true in situ pH but rather an upper limit.

3.2.4. Analysis

Samples analysis was performed in duplicates using an ion chromatography system (Sykam GmbH, Fuerstenfeldbruck, Germany) equipped with a LCA A 20 column, a suppressor (SAMS, SeQuant, Sweden) and a SYKAM S3115 conductivity detector. Elution was performed at isocratic conditions with a 1.8/1.7 mmol/l Na_2CO_3/$NaHCO_3$ solution as mobile phase. The flow rate was set to 0.8 ml / min. Prior to each measurement a blank sample (deionised water) as well as a multi-component standard (containing 50 mg/l each of formate, acetate and oxalate) were measured.

With this setup we encountered problems with the detection of acetate due to coelution of an unknown component. Therefore, for the separate quantification of acetate, we used a BP-100 H^+ column (Benson Polymeric Inc. NV, USA) with a 20 mm pre-column. Elution was performed at isocratic conditions with solution of 50 mM boric acid with 50 µL of 100% trifluoroacetic acid. The flow rate was set to 0.56 ml/min.

3.3. Results

Generally, the amounts of extracted LMWOAs increase over the course of the experiment throughout all horizons with the strongest increase during the first 22 hours. After this time the extraction yield slowly approaches steady state. All extraction yields after 72 hours including standard deviation and percentage change in comparison to the extraction with deionized water is given in table 3.

Table 3: Extraction yields (in mg acid / g of freeze-dried coal) of all three LMWOAs for all three horizons and all extraction media. Values in brackets are first the standard deviation and second the extraction yields in percent compared to the extraction with pure water.

Horizon 2

	pure water	water saturated with CO_2	water saturated with CO_2/NO_2	water saturated with CO_2/SO_2
formate	1.02 (±0.03)	1.20 (±0.08; +17%)	1.64 (±0.07; +61%)	1.58 (±0.10; +55%)
acetate	0.76 (±0.17)	0.85 (±0.18; +12%)	0.83 (±0.03; +9%)	0.79 (±0.01; +4%)
oxalate	0.78 (±0.01)	0.44 (±0.07; -44%)	1.01 (±0.21; +30%)	0.65 (±0.13; -17%)

Horizon 6

	pure water	water saturated with CO_2	water saturated with CO_2/NO_2	water saturated with CO_2/SO_2
formate	1.04 (±0.02)	1.36 (±0.04; +31%)	1.98 (±0.34; +92%)	1.89 (±0.02; +82%)
acetate	0.35 (±0.29)	0.88 (±0.05; +151%)	0.79 (±0.61; +126%)	0.64 (±0.37; +83%)
oxalate	1.12 (±0.02)	0.59 (±0.03; -47%)	0.53 (±0.07; -53%)	0.61 (±0.09; -45%)

Horizon 9

	pure water	water saturated with CO_2	water saturated with CO_2/NO_2	water saturated with CO_2/SO_2
formate	1.34 (±0.29)	1.70 (±0.03; +26%)	1.77 (±0.01; +32%)	1.80 (±0.20; +34%)
acetate	0.77 (±0.51)	0.51 (±0.17; -34%)	0.53 (±0.20; -31%)	0.78 (±0.27; +1%)
oxalate	1.04 (±0.25)	0.51 (±0.06; -51%)	0.94 (±0.42-10%)	0.70 (±0.20; -33%)

The final formate extraction yield for all three coal horizons – in comparison to the extraction with pure water, which is taken as reference – increased upon the addition of gases to the extraction medium (figure 1 A, B, C). The addition of carbon dioxide resulted in an increase of 17 to 31 %. The addition of carbon dioxide mixtures (CO_2/NO_2 and CO_2/SO_2) further increased the formate yield, but no significant difference in the

extraction yield between both gas mixtures could be observed. For horizon 9 there is no clear difference in the final formate extraction yield between all three additions of gas (CO_2 - CO_2/NO_2 - CO_2/SO_2) to the extraction medium (26 to 34 %, figure 1 C).

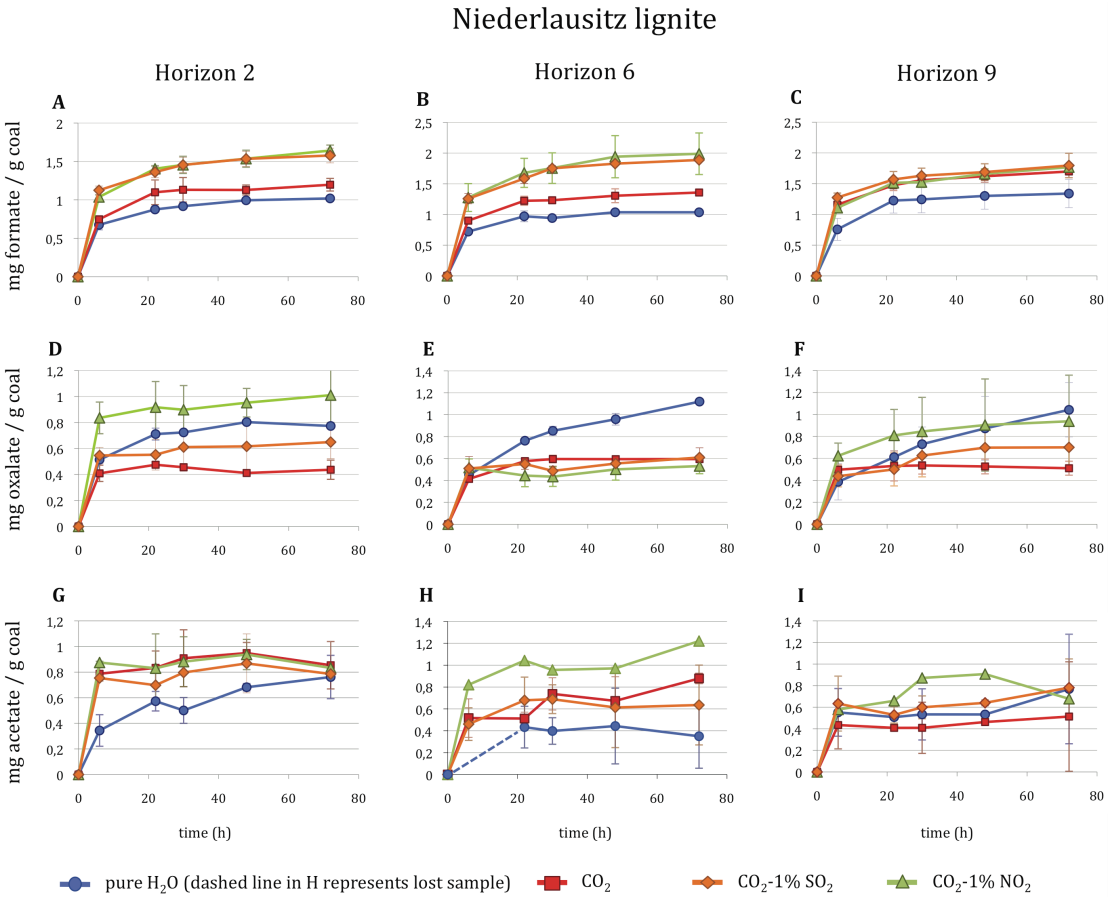

Figure 1: Extraction yield of formate, oxalate and acetate for all three horizons of lignite from Welzow-Süd, Niederlausitz Brown Coal District, Niederlausitz, Germany. Error bars are one standard deviation.

For acetate a significant change of the final extraction yield could only be observed for horizon 6 (figure 1 H). The addition of carbon dioxide to the extraction medium resulted in an increase of 150 %. The total amount increased by 126 % and by 83 % upon addition of CO_2/NO_2 and CO_2/SO_2, respectively. Unfortunately, the sample of the 6h time point of the water extraction was lost. A dashed line in the diagram represents the missing extraction value. For horizon 2 only minor changes in the final extraction yield upon the addition of gases were detected (figure 1 G). The overall change ranged between 4 to 12 %. So no significant effect on the extraction yield by addition of gases

could be observed. However, the form of the extraction yield curves is different for the addition of gases in comparison to the extraction with pure water.

After a rapid increase of the extraction yield in the first 6 hours upon the addition of gases to the extraction medium a maximum has reached. For horizon 9 (figure 1 I) the extraction yield decreased by 31 and 34% upon the addition of CO_2 and CO_2/NO_2 in comparison to the extraction with pure water, whereas the yield for CO_2/SO_2 remained unchanged. Furthermore the acetate extraction yields for all medium compositions and horizons already reached steady state after 6 hours (with the exception of the extraction with pure water at horizon 2, see figure 1). This was not observed for formate and oxalate.

In contrast to formate and acetate (except horizon 9) the extraction yield of oxalate decreased by the addition of gas to the extraction medium (including one exception for horizon 2). Thereby, the addition of pure carbon dioxide resulted in the strongest decrease of the oxalate extraction yield. Saturation of the extraction medium with impure CO_2 does not lead to a further decrease in the oxalate extraction yield. The yields ranged in between the yields of pure water and that of water saturated with pure carbon dioxide with the exception of horizon 6 where all three yields lay in the same range (figure 1 E). However, for horizon 2 the extraction yield of the CO_2/SO_2-experiment was 30 % above the yield of the extraction with pure water (figure 1 D). For horizon 6 and 9 the extraction yield is not reaching steady state and still approaching to the maximum (figure 1 E, F).

To exclude temperature –induced degradation effects incubations of an aqueous oxalate solution (20 mg/l Na_2-Oxalate) without coal under the same conditions and gas mixtures as the coal samples were performed. As result no decrease of the oxalate concentration was observed. Furthermore to evaluate the effect of oxygen on the oxalate extraction yield an oxygen scrubber (0.1 mM H_2S) was used. As a result of an incubation of a coal sample with carbon dioxide and oxygen scrubber no increase in the oxalate extraction yield could be observed.

3.4. Discussion

The results from our extractions with pure water agree well with previously published reports about the aqueous extraction of LMWOAs from low-rank coals (Bou-Raad et al., 2000; Vieth et al., 2008; Glombitza, 2011). In these studies sub-bituminous

coals as well as lignite were extracted in a Soxhlet apparatus. This procedure does not allow the application of gas or pressurisation. Much research has been done on the effects of supercritical carbon dioxide on coal and shale (Jaffé et al., 1997; Jaffé et al., 2000; Lifshits et al., 2012). Mostly long chain n-alkanes and aromatic compounds were extracted.

However no study has investigated the effects of water with sub-critical concentrations of carbon dioxide on coal so far. Therefore this study evaluates the effect of sub-critical carbon dioxide concentrations on the feedstock potential of subsurface macromolecular organic matter for a possible production of biogenic methane.

To simulate more realistic conditions with regard to flue-gas composition, we did not use just food grade carbon dioxide, but also CO_2/SO_2 and CO_2/NO_2 mixtures.

For all horizons some general trends could be observed. The extraction with gas-saturated medium (irrespective of the gas composition) will lead to an increase in formate yield in comparison to the extraction with pure water. For oxalate however the trend is reversed; extractions with gas-saturated medium always resulted in a decrease of the extraction yield of oxalate (with the exception of a slight increase in the CO_2/SO_2 extraction of horizon 2). This phenomenon of extraction yields developing in opposite directions for different LMWOAs was previously reported for sub-bituminous coal from the DEBITS-1-well of New Zealand (Sauer et al., 2012). In this study the saturation of the extraction media with CO_2 also led to the same pattern; an increase in formate yield and a decrease in the oxalate yield. For the extraction of acetate no clear trend could be observed. Horizon 2 showed no changes in the extraction yield upon addition of gases, whereas horizon 6 showed a significant increase and horizon 9 an overall decrease (see figure 1 G, H, I). Remarkably, except for the extraction of horizon 2 with pure water, acetate concentration already reached a maximum after 6 hours and remained stable over the rest of the experiment.

The increase in the extraction yield of formate (and also acetate of horizon 6) with increasing acidification of the extracting medium by the injection of gases supports the expected increase of acidic hydrolysis due to the acidification of the extraction medium. This acidification is caused by the formation of carbonic, nitric and sulfuric acid upon the addition of CO_2, CO_2/NO_2 or CO_2/SO_2 respectively, to the extraction medium (Hales and Sutter, 1973; Pitts et al., 1984; Meyssami et al., 1992).

The decrease of the extraction yield for oxalate with increasing acidification of the extraction medium does not fit in the hypothesis of increased extraction yield by an enhanced acidification of the medium.

Due to the acidification of the extraction medium, LMWOAs that are bound to macromolecular organic matter will be liberated by acidic hydrolysis, which cleaves the ester bond of the bound acids (figure 2). Acidification of the extraction medium due to liberation of acids formed by the injection of gases would therefore enhance the acidic hydrolysis. However for oxalate we observed an opposite effect. So the question is if the pH is actually the only factor influencing the extraction yield of LMWOAs from the coal?

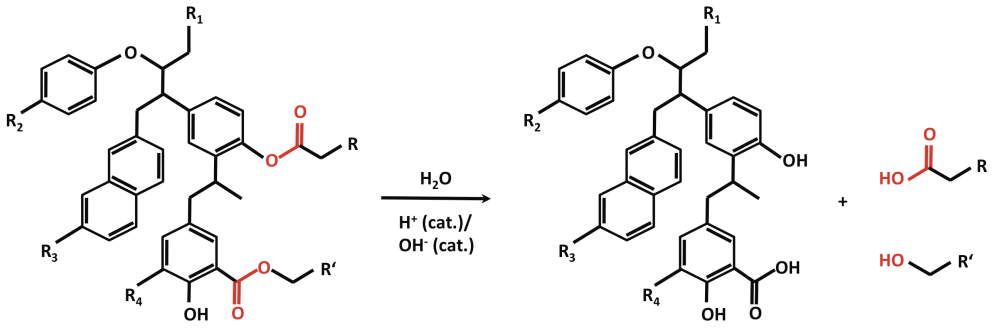

Figure 2: Liberation of terminally bound organic acids and alcohols from coals. Scheme redrawn after Glombitza (2011).

Bou-Raad et al.[24] speculated that oxalate might originate not only from the acidic cleavage of ester bonds of the coal matrix but also through the oxidative cleavage of diol functional groups. In the presence of catalysts, mildly oxidising agents such as metal acetates and metal oxides are able to cleave compounds like 1,2-dihydroxycarboxylic acids, which thereby release oxalate (fig. 3A). As we cannot completely exclude any trace amounts of oxygen in the extraction liquid, it seems possible that oxygen or released acetate may form compounds with metal ions. Metal ions may be provided by the steel cylinder or the coal (concentration of main metals in the coal is given in table 4). These metal oxides or metal acetates could then cleave diol functional groups, resulting in the formation of oxalate. However, the extraction of a coal sample with carbon dioxide and an oxygen scrubber (0.1 mM H_2S) did not increase the oxalate extraction yield. Either the scrubber had no effect because oxygen concentration was to low in the medium or there is no formation of metal oxides that can cause a cleavage of oxalate.

Furthermore it is possible that the decrease in pH will inhibit the cleavage of 1,2 dihydroxycarboxylic acids and similar groups or enhance alterations of molecules. The drop in pH could enhance the cleavage of an oxalate molecule into formate and

bicarbonate. Crossey (1991) showed that oxalate can be degraded to either formate and CO_2 or CO_2, CO and water in aqueous solution at temperatures above 160°C (fig. 3B). This reaction is pH controlled, as oxalate degradation increases with decreasing pH. The finding of decreasing concentrations of oxalate in deep subsurface waters at temperatures above 90°C supports the assumption that oxalate may also be degraded at temperatures lower than 160°C if the pH is low enough (Carothers and Kharaka, 1978; Fisher, 1987). To test the hypothesis that oxalate is degraded into two formate molecules we incubated an aqueous oxalate solution without coal at 90°C and 5 MPa, but could not detect any changes in oxalate or formate concentration. As we could not detect any degradation of oxalate the hypothesis of oxalate degradation at temperatures as low as 90°C may be wrong or the pH was not low enough.

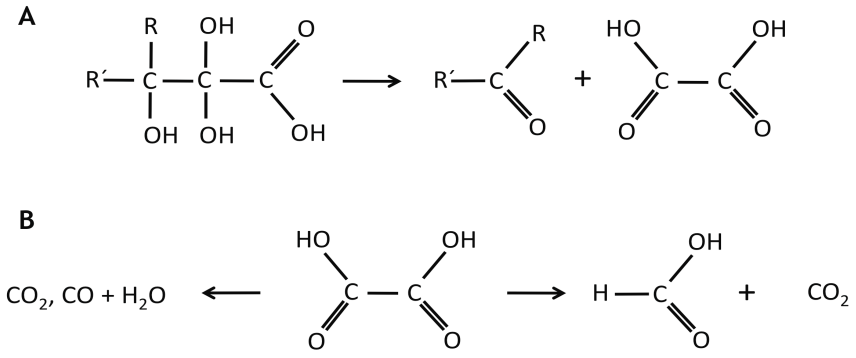

Figure 3: A: Proposed cleavage of 1,2 dihydroxycarboxylic acids and the release of oxalate. Redrawn after Bou-Raad et al. (2000) B: Possible cleavage of oxalate into either CO_2, CO and water or formate and CO_2.

It is also possible that oxalate and calcium form calcium oxalate, which is highly insoluble (solubility product $1.78 * 10^{-9}$ mol^2/l^2). There is calcium in the coal, as 19 to 27 percent of coal ash is calcium (table 4).

It was shown that the concentration of formate and acetate, which are the quantitatively most important LMWOAs for the microbial metabolism, increased upon the addition of pure and impure carbon dioxide (Thauer et al., 1977). Only for oxalate the extraction yield decreased upon the acidification of the extraction medium.

We therefore assume that the injection of carbon dioxide into coal seams will have an impact on the structure and composition of the coal. From a bio- and geochemical point of view, the liberation of LMWOAs will lead to an enhanced nutrient supply for subsurface microbial communities, which would result in an enhanced growth and metabolic activity of microbes.

Kawaguchi et al. (2010) noted the possibility to produce methane from sequestered carbon dioxide in geological reservoirs. They mentioned that in greater distance from the injection well (no actual distances given), the concentration of carbon dioxide has decreased to moderately low concentrations so that methanogens could produce methane. They showed that there is at least a theoretical possibility of biogenic *in-situ* methane production from CO_2 and hydrogen (Kawaguchi et al., 2010). Due to the acidification of the pore water, protons would be present around the injection sites. The storage of carbon dioxide in coal beds could therefore enhance *in-situ* methane production if methanogens are present. In water samples from coal beds of the Illinois Basin, methanogens have been found and there is indirect evidence for methanogens in coal from the Ruhr Basin (Thielemann et al., 2004; Strapoc et al., 2008; Krüger et al., 2008).

So far no methanogens have been found in lignite. However, there is abundent microbial life in such low-rank coals. In slightly higher ranked sub-bituminous coal from the DEBITS-1 well of New Zealand methanogens have been found (Fry et al., 2009). Also in the lignite from the Niederlausitz brown coal district, microorganisms have been found in the order of 10^1 cells / g coal (Jaschof and Schwartz, 1969). However, these counts were obtained with culture-based techniques, as at that time there were no culture-independent molecular techniques available. Due to the development of fluorescent DNA-specific dyes like SYBR Green, DAPI or Acridine Orange a robust quantification of total microbial abundance is possible (Noble and Fuhrmann, 1998; Weinbauer et al., 1998; Kallmeyer, 2011). However, no further studies on microbial cell counts in lignite have been published so far. Therefore it is reasonable to assume that with modern techniques a higher number of microbial cells could be detected in this coal.

It is possible that there are methanogens in lignite or sub bituminous coal, producing methane from either dissolved carbon dioxide and hydrogen or from acetate and formate. It is known that methanogens in coals produce methane from either formate plus hydrogen or by the conversion of acetate (Moore, 2012). As we found both acids in our extractions from lignite, substrates for an acetoclastic methane production are available. It is therefore reasonable to assume that the injection of carbon dioxide into coal beds could enhance microbial activity and provide the necessary substrates for acetoclastic methanogenesis.

3.5. Acknowlegments

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4. "Active sulfur cycling by diverse mesophilic and thermophilic microorganisms in terrestrial mud volcanoes of Azerbaijan"

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Terrestrial mud volcanoes (TMVs) represent geochemically diverse habitats with varying sulfur sources and yet sulfur cycling in these environments remains largely unexplored. Here we characterized the sulfur-metabolizing microorganisms and activity in four TMVs in Azerbaijan. A combination of geochemical analyses, biological rate measurements and molecular diversity surveys (targeting metabolic genes aprA and dsrA and SSU ribosomal RNA) supported the presence of active sulfur-oxidizing and sulfate-reducing guilds in all four TMVs across a range of physiochemical conditions, with diversity of these guilds being unique to each TMV. The TMVs varied in potential sulfate reduction rates (SRR) by up to four orders of magnitude with highest SRR observed insediments where in situ sulfate concentrations were highest. Maximum temperatures at which SRR were measured was 60°C in two TMVs. Corresponding with these trends in SRR, members of the potentially thermophilic, spore-forming, *Desulfotomaculum* were detected in these TMVs by targeted 16S rRNA analysis. Additional sulfate-reducing bacterial lineages included members of the *Desulfobacteraceae* and *Desulfobulbaceae* detected by aprA and dsrA analyses and likely contributing to the mesophilic SRR measured. Phylotypes affiliated with sulfide-oxidizing *Gamma*-and *Betaproteobacteria* were abundant in aprA libraries from low sulfate TMVs, while the highest sulfate TMV harboured 16S rRNA phylotypes associated with sulfur-oxidizing *Epsilonproteobacteria*. Altogether, the biogeochemical and microbiological data indicate these unique terrestrial habitats support diverse active sulfur-cycling microorganisms reflecting the in situ geochemical environment.

4.1. Introduction

Terrestrial mud volcanoes (TMVs) occur where high fluid pressure in the deep subsurface results in the transport of mud, water and gas to the surface (Feyzullayev and Movsumova, 2010; Niemann and Boetius, 2010). This process creates diverse morphological features rich in methane and other hydrocarbons (Dimitrov, 2002), and hosting a suite of electron acceptors including oxygen, nitrate, iron, manganese and sulfate (Planke et al., 2003; Alain et al., 2006; Mazzini et al., 2009; Chang et al., 2012). Mud volcanoes are a major source of methane flux to the atmosphere (6–9 Tg per year; Etiope and Milkov, 2004), and geochemical and microbiological studies thus far have primarily focused on microorganisms and processes involved in the oxidation of methane, including the detection of anaerobic methane-oxidizing archaea (ANME) in individual volcanoes (Alain et al., 2006; Niemann et al., 2006; Schulze-Makuch et al., 2011; Wrede et al., 2012; Yang et al., 2011; Chang et al., 2012). 16S rRNA surveys of TMVs in Romania, Taiwan and China contain a large number of putative sulfate-reducing deltaproteobacterial phylotypes, consistent with the potential for sulfate-dependent anaerobic methane oxidation (Alain et al., 2006; Yang et al., 2011; Chang et al., 2012); however, in some cases sulfate reduction rates (SRR) appear to be significantly greater than anaerobic methane oxidation rates (Alain et al., 2006).

The ecology of organisms involved in sulfur cycling remains largely unexplored in TMVs. Unlike marine MVs, very little is known about chemosynthetic communities such as sulfur-oxidizing bacterial (SOB) populations in TMVs (Niemann and Boetius, 2010) and while sulfate-reducing bacterial (SRB) phylotypes based on 16S rRNA have been reported from individual TMVs, their diversity in these habitats has not been examined by analysing sulfur-cycling functional genes. TMVs have the potential for supporting active sulfur cycling with typical sulfate concentrations of approximately 2 mM (Yakimov et al., 2002; Planke et al., 2003; Alain et al., 2006; Mazzini et al., 2009; Nakada et al., 2011), and these natural venting structures may also serve as a window into sulfur-cycling processes in the deep biosphere. While previous microbiological studies have characterized individual TMVs, regions hosting multiple TMVs also make it possible to focus on the same microbial guilds and processes across several sites.

Azerbaijan and its offshore expanses in the Caspian Sea represent one of the most densely populated regions of mud volcanoes and mud volcanism is one of the major factors controlling oil and gas fields in the region. Unusually high $\delta^{13}C$ values of CO_2 and bicarbonates in the TMVs are thought to result from biodegradation of oil (Feyzullayev

and Movsumova, 2010); however, very little is known about the microbial communities in these volcanoes. Multiple studies have characterized the complex plumbing system of the TMVs that are capable of transporting mud and fluids from origins as deep as 10 km or greater (Planke et al., 2003 and references therein; Mazzini et al., 2009), and records of eruption history as well as geochemical and isotopic compositions of emitted oil, gas, mud and water exist for several prominent TMVs in the region (Guliyev et al., 2001; Planke et al., 2003; Etiope et al., 2004; Mazzini et al., 2009; Feyzullayev and Movsumova, 2010).

Analyses of oil source rocks ejected from mud volcanoes of Azerbaijan revealed low organic sulfur contents (less than 0.03% of the organic matter) with the majority of sulfur occurring as pyrite (Isaksen et al., 2007). Sulfate concentrations appear to vary widely with studies reporting concentrations that are on average ~ 2 mM, but range of values from ~ 10 μM to > 30 mM (Planke et al., 2003; Mazzini et al., 2009). This diverse system of mud volcanoes provides a set of distinct habitats in which to study natural variations in the *in situ* interplay of sulfur-cycling communities and their geochemical environments. Here we conducted a comparative geomicrobiological study of four discrete TMVs in Azerbaijan (D: Dashgil, B: Bakhar, P: Perekyushkul and BJ: Boransyz-Julga) sourced from deep-seated fluids in order to: (i) characterize sulfur-cycling microbial communities in these unique environments, (ii) place these communities in a meaningful context via geochemical and microbial rate measurement analyses, and (iii) examine the potential transport of thermophilic sulfur-cycling microorganisms from the deep subsurface.

4.2. Results

Geochemical characterization

Inorganic and organic geochemistry of pore fluids from four mud volcanoes were analysed with a particular emphasis on sulfur and carbon species (Tables 1 and S4). In general, values were similar to previous reports of geochemistry from TMVs in this area (Planke et al., 2003; Mazzini et al., 2009). Sulfate contents ranged between 0.47 and 1.64 mM in D sediments except for in sample D3, which was below detection (< 0.16 mM). P surface (P1S) and deep (P) samples contained 0.31 and 0.80 mM sulfate respectively. B and BJ had sulfate levels below the detection limit (< 0.16 mM). The sulfate concentrations ranged from less than 0.16 to 1.64 mM and are within the range of

previously reported values from TMVs in Azerbaijan (Planke et al., 2003; Mazzini et al., 2009) as well as those reported from TMVs in Romania (2 mM; Alain et al., 2006), Italy (0.07 mM; Wrede et al., 2012) and China (14 mM; Yang et al., 2011). While values vary widely, none of them approaches that of seawater in general (approximately 28 mM), or the Caspian Sea in particular (approximately 33 mM; Planke et al., 2003 and references therein).

Higher Cl^- values of D salse lakes/pools (312–464 mM) versus gryphons from D, P and BJ (56–68 mM) are in agreement with previously reported trends from this region, and may reflect a deep water source resulting from the dehydration of clays in the gryphons, while the salse lakes and pools may be fed by more shallow meteoric water source with higher solutes resulting from in situ evaporation (Mazzini et al., 2009). Higher Cl^-/Br^- ratios in salse lakes (relative to gryphons) in this region have been previously reported and may be derived from the dissolution of halite crusts which form on the outside flanks of gryphons occurring at higher elevations than salse lakes (Mazzini et al., 2009).

Reduced sulfur species associated with the solid-phase materials were divided into three phases named for the compounds used to liberate them: AVS (acid-volatile sulfur; hydrogen sulfide and monosulfides): CRS (chromium-reducible sulfur, mainly pyrite): DMF (dimethylformamide-soluble fraction, mainly elemental sulfur). Values from all samples analysed were similar, with disulfides comprising > 99% of all reduced sulfur except BJ, which contained relatively lower amounts of disulfides (96% from sample BJ; 72% from BJ2) the difference being made up by monosulfides (Tables 1 and S4).

Microbial microcosm measurements

Biogenic CO_2 production under aerobic conditions (heterotrophic respiration) was at least three orders of magnitude greater than CO_2 production under anaerobic conditions.

In addition, anaerobic sulfide production (in the presence and absence of methane), indicative of active sulfate reduction, was also two orders greater than anaerobic CO_2 production (Table 2). Sulfide and anaerobic CO_2 production rates ranged from 1.4×10^4 to 6.7×10^4 and 2.7×10^2 to 6.3×10^2 (nmol cm^{-3} day^{-1}) respectively. Higher rates of sulfide and anaerobic CO_2 production were measured from D samples suggesting potentially higher in situ SRR in the D salse lakes (D1 and D3).

Table 1: Geochemical and biological characteristics of four terrestrial mud volcanoes of Azerbaijan sampled in October 2008 (for complete data set see Tables S4 and S5).
n/a not available; SRR, sulphate reduction rate.

Mud volcano	D: Dashgil		B: Bakhar	P: Perekyushkul	BJ: Boransyz-Julga
Geographic setting	*Proximal to Caspian Sea* Quaternary sediments		*Proximal to Caspian Sea* Quaternary sediments	*Foothills of Great Caucasus* Oligocene-Lower Miocene deposits	*Foothills of Great Caucasus* Oligocene-Lower Miocene deposits
Coordinates	39°59′47.8″N 49°24′08.8″E		39°59′53.7″N 49°27′20.3″E	0°28′49.9″N 9°26′53.7″E	40°27′59.6″N 49°28′11.4″E
Sample name and feature	Ds: Pool (surface)	Dd: Pool (deep)	B: Gryphon	P: Gryphon (deep)	BJ: Gryphon
Temperature, °C (measurement depth, m)	17.2 (0.2)	20.6 (1.0), 20.8 (1.25)	19.6 (0.2), 20.7 (3.5)	17.6 (2.5)	18.5 (0.2), 18.3 (1.5)
Porewater geochemistry					
Sulfate (mM)	1.65	1.22	< 0.16	0.80	< 0.16
Cl/Br (mass ratio)	220	219	189	> 197.95	206
Acetate (mM)	0.018	0.029	0.027	0.049	n/a
Formate (mM)	0.018	0.024	0.030	0.047	n/a
Reduced sulfur species					
(AVS : CRS : DMF)	n/a	n/a	n/a	0.23: 99.72: 0.05	3.85: 96.13: 0.02
Microbiological analyses					
Desulfotomaculum detection (16S)	n/a	+	+	–	–
Dominant guild (*apr*A clone library)	SRB	SRB	SOB	SRB	SOB
SRR pre-incubation conditions					
24 h: temperature optimum, °C (SRR, nmol cm^{-3} day^{-1})	n/a	32 (9.5)	25 (2.8), 39 (2.0)	n/a	31 (1.0)
48 h: temperature optimum, °C (SRR, nmol cm^{-3} day^{-1})	n/a	32 (8.2), 61 (2.4)	18 (2.0), 39 (2.0)	n/a	29 (3.4)
48 h + VFA: temperature optimum, °C (SRR, nmol cm^{-3} day^{-1})	n/a	35 (2359.9), 63 (424.5)	15 (0.8), 34 (3.7), 54 (1.1)	n/a	30 (2.3)

Potential SRR

Sulfate reduction rates were measured from three of the four mud volcanoes; D (deep pool sample, Dd), B (gryphon sample B) and BJ (gryphon sample BJ). Sediment slurries were incubated for 8 h with sulfate radiotracer at temperatures ranging from approximately 10°C to 82°C, under three different experimental incubation conditions: following 24 h pre-incubation, following 48 h pre-incubation and following 48 h pre-incubation with volatile fatty acids (VFAs). Results were highly variable with SRR spanning four orders of magnitude between sites and depending on incubation temperature, pre-incubation time and VFA addition. Incubations with sediment from BJ,

Table 2: CO_2 and H_2S production (mmol $gdwt^{-1}$ * day) from microcosms inoculated with Dashgil and Bakhar samples collected in 2007.

Sample, depth collected	CO_2 under air	CO_2 under N_2/CO_2	H_2S under N_2/CO_2	H_2S under CH_4
Dashgil (D1) big salse lake, 5.8 m	8.20E+05 ± 1.10E+05	4.30E+02 ± 7.90E+01	2.90E+04 ± 8.00E+03	5.60E+04 ± 4.00E+03
Dashgil (D1) big salse lake, 0.5 m	6.40E+05 ± 8.20E+04	4.80E+02 ± 9.30E+01	1.40E+04 ± 3.00E+03	3.90E+04 ± 1.00E+04
Dashgil (D3) small salse lake, 6.5 m	1.07E+06 ± 2.20E+05	6.30E+02 ± 1.10E+02		
Dashgil (D3) small salse lake, 0.2 m	8.60E+05 ± 1.40E+05	5.80E+02 ± 8.30E+01	3.00E+04 ± 2.00E+03	6.70E+04 ± 1.00E+04
Bakhar small salse lake, 4.2 m	4.90E+05 ± 2.30E+04	3.10E+02 ± 2.00E+01	1.40E+04 ± 3.00E+03	3.80E+04 ± 4.00E+03
Bakhar small salse lake, 0.2 m	4.70E+05 ± 5.60E+04	2.70E+02 ± 4.10E+01	1.60E+04 ± 1.00E+04	3.10E+04 ± 8.00E+03

located inland from D and B (Fig. 1), resulted in a temperature profile for SRR with a narrow temperature optimum around 30°C and no detectable thermophilic activity (Fig. 2C). Results from D and B varied significantly in response to VFA stimulation and revealed different temperature optima for sulfate reduction despite both D and B occurring in the same hydraulic system and rock formation. Sulfate reduction maxima in the thermophilic range were measured for B and D sediments at 54°C and 60°C respectively, with SRR below detection at higher temperatures up to 82°C.

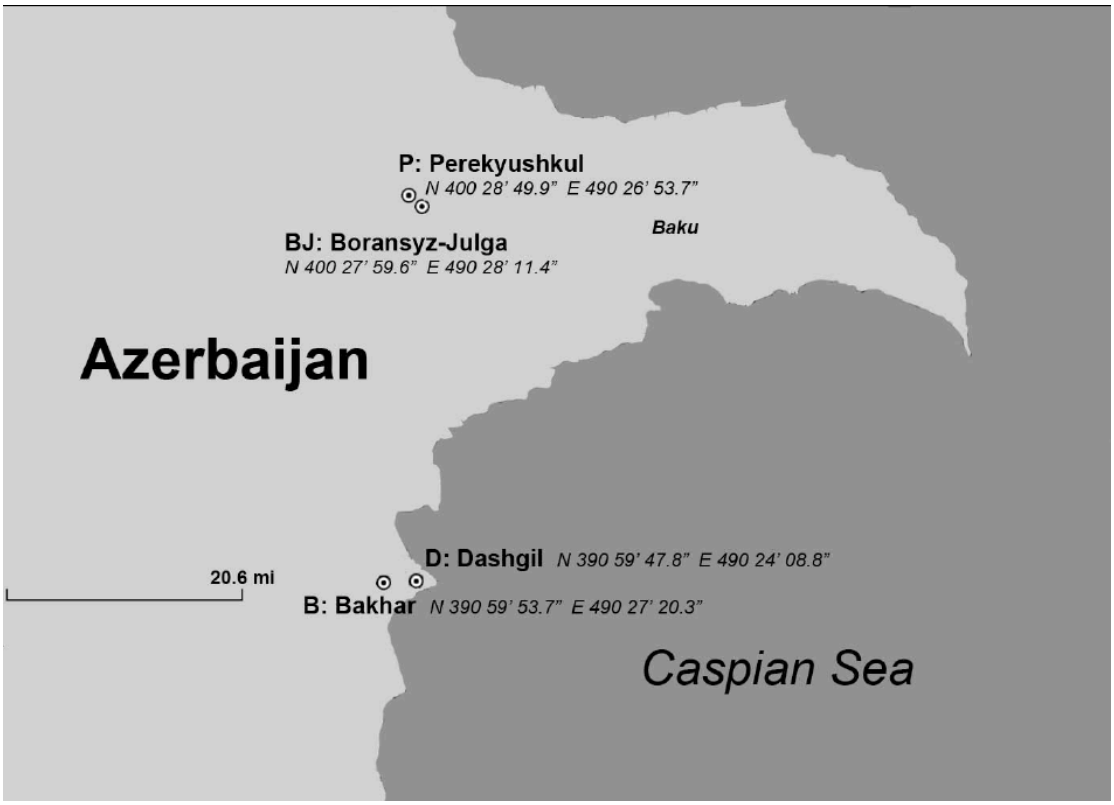

Figure 1: Location of terrestrial mud volcanoes in Azerbaijan sampled in 2007 and 2008.

D samples pre-incubated for 24 and 48 h resulted in similar temperature-activity profiles, with the highest SRR measured at 32.5°C. While sulfate reduction was not detected above 40°C after 32 h (i.e. 24 h pre-incubation followed by 8 h with radiotracer), SRR obtained for 48–56 h revealed a second peak in SRR between 50°C and 70°C. This response was dramatically amplified by VFA amendment, which resulted in SRR up to 250-fold higher at these high temperatures than in the unamended samples (Fig. 2A), similar to previous reports of VFA supporting high SRR in cold marine sediments incubated at these temperatures (Hubert et al., 2010). In B sediments, SRR (ranging from 0.78 to 3.70 nmol cm^{-3} day^{-1}) were much lower than those measured in D sediments (2.38– 2359.88 nmol cm^{-3} day^{-1}) and, as observed for D, the temperature

optima depended on both the pre-incubation time and the addition of VFAs. For the two unamended incubations, the temperature range for sulfate reduction was broad and showed several rate maxima between 15°C and 40°C. Notably, SRR determined after the 24 and 48 h pre-incubations followed slightly different temperature-activity profiles, with rates measured after 24 h being 1.5-fold higher between 25°C and 32°C than after the 48 h treatment. VFA amendment to B samples stimulated SRR that were slightly higher than in unamended samples, with two distinct peaks at 30°C and 54°C and a third minor peak around 15°C (Fig. 2B). The potential SRR were also low in samples from BJ, with one narrow peak at 30°C determined for all three incubation treatments. SRR in samples pre-incubated for 48 h (3.37 nmol cm^{-3} day^{-1}) were approximately threefold greater than in the 24 h sample (1.00 nmol cm^{-3} day^{-1}). Unlike the samples from D and B, VFA addition to BJ samples did not result in higher SRR (2.34 nmol cm^{-3} day^{-1}; Fig. 2C).

Determination of microbial abundance

Quantitative 16S rRNA gene analyses from D and B samples collected in 2007 suggest bacteria are an order of magnitude more abundant than archaea in D salse lakes (D1 and D3), while the two Domains appear equally abundant in the small salse lake sample from B (Table S3). The depth from which the sample was taken did not appear to influence these trends. Across all samples, bacterial 16S rRNA gene copies range from approximately 1 x 10^7 to 1 x 10^9 copies g^{-1}, and archaeal 16S rRNA gene copies range from 1 x 10^7 to 1 x 10^8 copies g^{-1}.

Molecular characterization of sulfur-cycling bacteria

Samples for phylogenetic analysis were selected based on SRR data and *in situ* sulfate concentrations. Here we targeted the aprA gene [adenosine-5-phosphosulfate (APS) reductase] encoding the enzyme required for dissimilatory sulfate-reduction and used in many sulfur-oxidation pathways (Meyer and Kuever, 2007a). Two aprA clone libraries were constructed from samples of the same Dashgil pool; one from the surface (Ds) and one collected approximately 4 m below the surface (Dd). Three clone libraries were constructed from gryphon samples. The B (B) and BJ (BJ) samples were collected at the surface of the gryphon, and the P (Pd) sample was recovered from approximately 2 m below the surface.

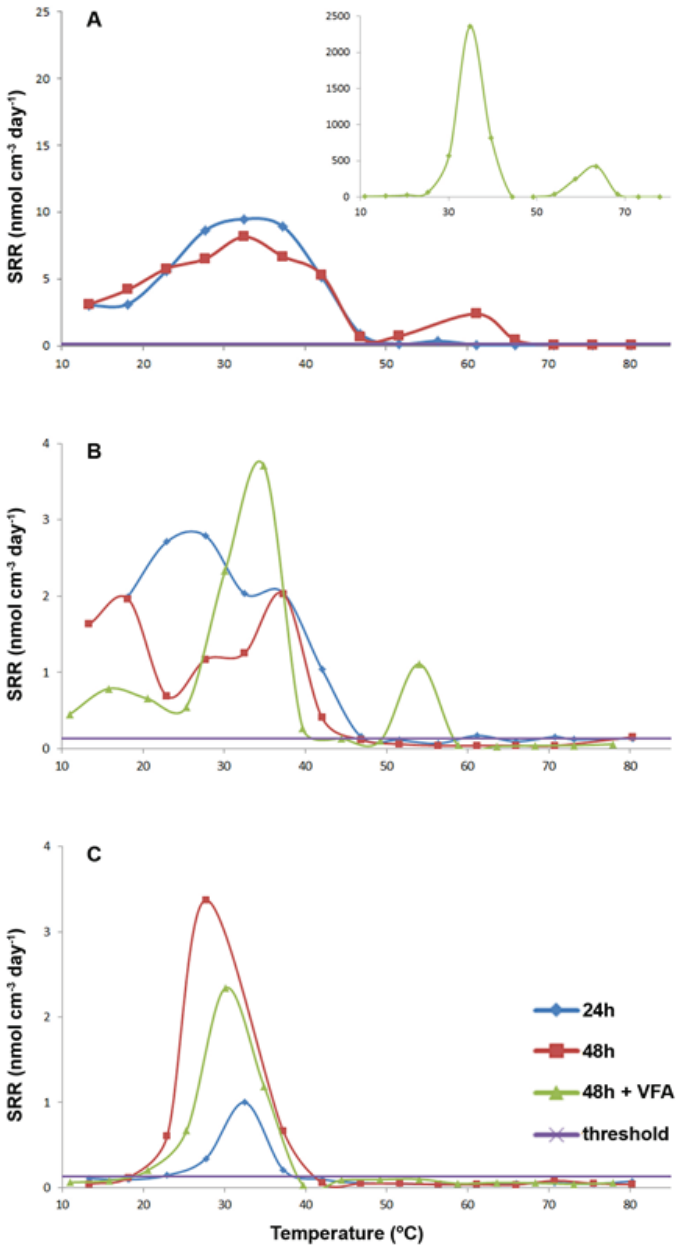

Figure 2: Potential sulfate reduction rates for (A) Dashgil (Dd), (B) Bakhar (Gryphon sample B) and (C) Boransyz-Julga (Gryphon sample BJ). Rate measurement were taken on a thermal gradient block ranging from approximately 10°C to 82°C, with three incubation treatments: 24 h pre-incubation, 28 h pre-incubation and 48 h pre-incubation with volatile fatty acids (VFAs). The dashed line indicates the minimum detection limit was 0.12 nmol cm^{-3} day^{-1}, which is the average of all blank measutrements plus three times the standard deviation.

The aprA gene sequences recovered from four of the five mud volcano samples contained representatives from several groups within the *Gamma*-, *Beta*-and *Deltaproteobacteria r*epresenting multiple SRB and SOB clades. The exception to this was BJ, where 96% of the recovered aprA sequences were affiliated with sulfide-oxidizing *Betaproteobacterial* members related to *Thiobacillus* (Fig. 3A). *Gammaproteobacterial* clones from the aprA lineages I and II (as defined in Meyer and Kuever, 2007b) were present in all but the BJ sample, with lineage I representing the

majority of these clones. Gammaproteobacterial aprA lineage I sequences from B, P and both D samples formed two distinct clusters each of which grouped with distinct aprA sequences from two *Thioalkalivibrio* strains (Fig. 3A). Sequences within lineage I also included representatives grouping within the *Chromatiaceae* (Ds) and environmental sequences reported from the groundwaters of an evaporative, calcareous, salt lake. Putative sulfide-oxidizing gammaproteobacterial aprA lineage II clones from B, P and both D samples also formed two distinct clusters, one affiliated with environmental clones from sulfate-reducing bioreactors treating mine drainage (Hiibel et al., 2008); the other grouping with members of *Thiodicton* sp., within the *Chromatiaceae*. Only one sequence, retrieved from BJ, appears loosely affiliated with an uncultured *Alphaproteobacteria* (Fig. 3A).

All SRB sequences from aprA libraries grouped with mesophilic *Deltaproteobacteria*, with the exception of a clone recovered from Dd, which grouped within the Gram-positive *Desulfotomaculum* subcluster 1b, which includes thermophilic sulfate reducers (Meyer and Kuever, 2007c). The majority of sequences within the *Desulfobacteraceae* were from D surface and deep samples (Dd and Ds), several of which formed a distinct cluster distantly associated with *Desulfosarcina variabilis*. Clones from P, B and D surface samples made up the majority of *Desulfobulbaceae* sequences, mainly grouping into three clusters with either no described relative or a distant association with *Desulfurivibrio alkaliphilus* (Fig. 3B).

The ratio of aprA clones recovered from sulfur-oxidizing versus sulfate-reducing bacteria (SOB : SRB) varied between samples, with SOB clones dominant in BJ (98% SOB) and B (68% SOB). The SOB populations in these samples were distinct, B contained aprA clones within the *Gammaproteobacteria* (43% aprA lineage I, 25% lineage II) while BJ contained 96% betaprotebacterial (aprA lineage II) clones and a single clone putatively from the *Alphaproteobacteria*. B also contained clones (30% of library) from the deltaproteobacterial family *Desulfobulbaceae* while BJ did not contain SRB-affiliated aprA sequences.

P and D surface and deep samples were dominated by aprA clones affiliated with SRB lineages. The D deep sample (Dd) was almost entirely dominated by *Desulfobacteraceae* (83%), with only a single clone each from *Desulfobulbaceae* and *Desulfotomaculum* (Fig. 3B). Similar to Dd, the P sample was collected several metres below the gryphon surface, but had an SRB profile more similar to the Ds surface sample. The majority (56%) of P clones grouped within the *Desulfobulbaceae* with 4% affiliated with *Desulfobacteraceae*. The D surface sample also contained a number of aprA sequences from the *Desulfobulbaceae* and *Desulfobacteraceae* family (63% and 18% respectively).

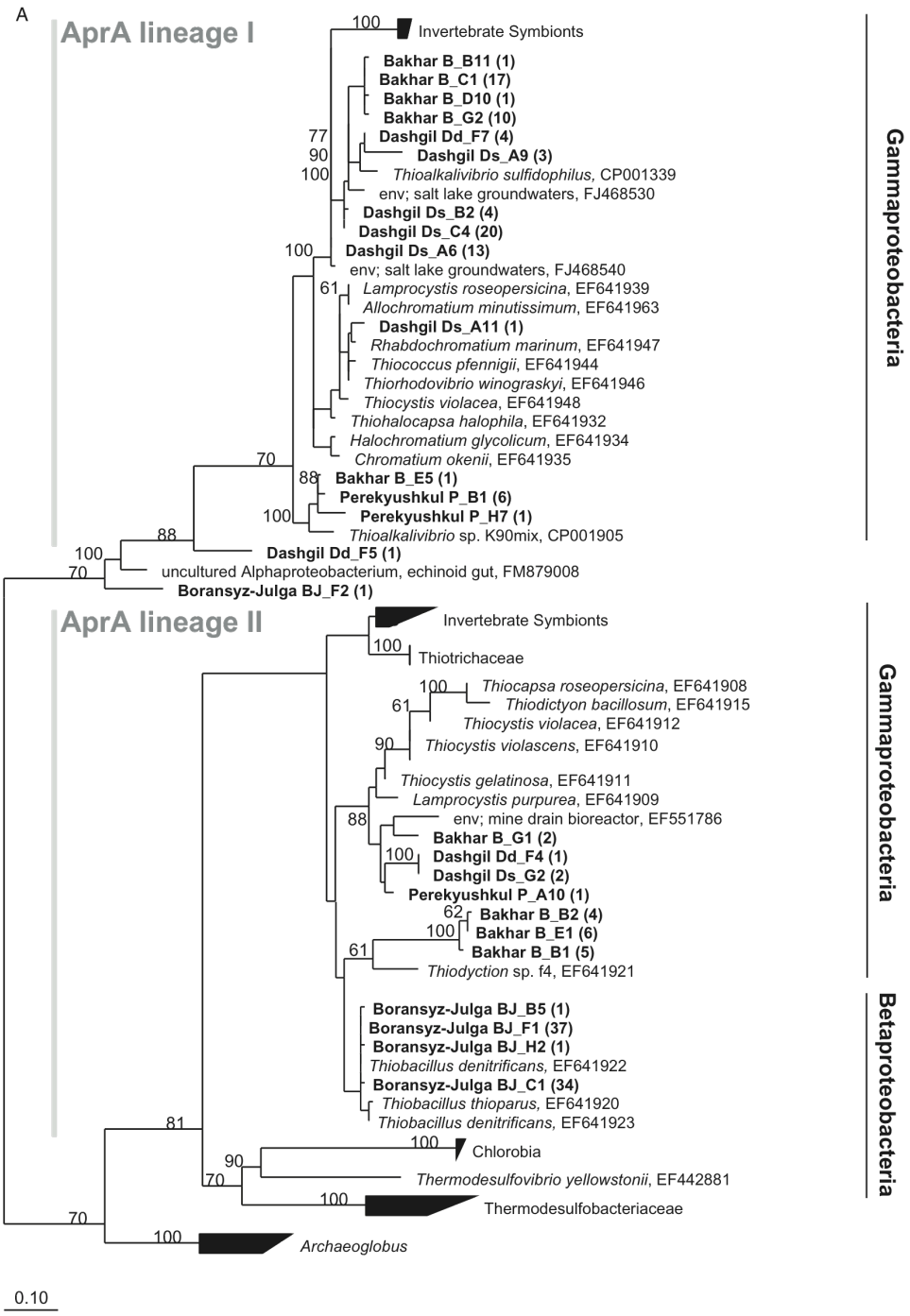
A
AprA lineage I
AprA lineage II
Gammaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Invertebrate Symbionts
Bakhar B_B11 (1)
Bakhar B_C1 (17)
Bakhar B_D10 (1)
Bakhar B_G2 (10)
Dashgil Dd_F7 (4)
Dashgil Ds_A9 (3)
Thioalkalivibrio sulfidophilus, CP001339
env; salt lake groundwaters, FJ468530
Dashgil Ds_B2 (4)
Dashgil Ds_C4 (20)
Dashgil Ds_A6 (13)
env; salt lake groundwaters, FJ468540
Lamprocystis roseopersicina, EF641939
Allochromatium minutissimum, EF641963
Dashgil Ds_A11 (1)
Rhabdochromatium marinum, EF641947
Thiococcus pfennigii, EF641944
Thiorhodovibrio winograskyi, EF641946
Thiocystis violacea, EF641948
Thiohalocapsa halophila, EF641932
Halochromatium glycolicum, EF641934
Chromatium okenii, EF641935
Bakhar B_E5 (1)
Perekyushkul P_B1 (6)
Perekyushkul P_H7 (1)
Thioalkalivibrio sp. K90mix, CP001905
Dashgil Dd_F5 (1)
uncultured Alphaproteobacterium, echinoid gut, FM879008
Boransyz-Julga BJ_F2 (1)
Invertebrate Symbionts
Thiotrichaceae
Thiocapsa roseopersicina, EF641908
Thiodictyon bacillosum, EF641915
Thiocystis violacea, EF641912
Thiocystis violascens, EF641910
Thiocystis gelatinosa, EF641911
Lamprocystis purpurea, EF641909
env; mine drain bioreactor, EF551786
Bakhar B_G1 (2)
Dashgil Dd_F4 (1)
Dashgil Ds_G2 (2)
Perekyushkul P_A10 (1)
Bakhar B_B2 (4)
Bakhar B_E1 (6)
Bakhar B_B1 (5)
Thiodyction sp. f4, EF641921
Boransyz-Julga BJ_B5 (1)
Boransyz-Julga BJ_F1 (37)
Boransyz-Julga BJ_H2 (1)
Thiobacillus denitrificans, EF641922
Boransyz-Julga BJ_C1 (34)
Thiobacillus thioparus, EF641920
Thiobacillus denitrificans, EF641923
Chlorobia
Thermodesulfovibrio yellowstonii, EF442881
Thermodesulfobacteriaceae
Archaeoglobus
0.10

Figure 3: Phylogenetic relationships of (A) putative sulfur-oxidizing bacterial and (B) putative sulfate-reducing bacterial *apr*A sequences retrieved from Dashgil (Dd, Ds; 89 and 47 clones respectively), Bakhar (B; 69 clones), Boransyz-Julga (BJ; 76 clones) and (Perekyushkul (P; 93 clones) mud volcanoes sampled in 2008 and inferred via maximum parsimony using the ARB software package. The number of clones represented by each OTU sequence in the tree is listed in parenthesis after the name. The scale bar corresponds to 10% estimated sequence divergence.

Additionally, there appeared to be a trend between libraries dominated by SRB sequences and the occurrence of gammaproteobacterial lineage I clones comprising the dominant SOB group.

As an independent check of the aprA results, a single dsrA library was constructed from Dd. Similar to the aprA library from the same sample, the recovered dsrA sequences were dominated (84%) by *Desulfobacteraceae*, with one *Desulfobulbaceae* clone and nine clones with no closely described relatives (Table S2). A phylogenetic analysis using known reference sequences placed several of these unidentified clones within a cluster of *Desulfotomaculum* sequences (data not shown).

Molecular characterization of 16S rDNA bacterial diversity

A bacterial 16S rDNA survey was completed from the Dd pool sample. Of the recovered diversity, 59% of the clones were *Epsilonproteobacteria*, followed by *Chloroflexi* (19%), *Deltaproteobacteria* (9%), and a low number of sequences associated with the *Firmicutes* (5%) and *Bacteroidetes* (3%). Epsilonproteobacterial sequences were highly similar (96–99% max identity) to environmental clones from the facultatively anaerobic SOB *Sulfurovum* sp. and all *Chloroflexi* clones were highly similar (96%) to environmental clones from landfill leachate pond sediments (Liu et al., 2011). Among the *Deltaproteobacteria*, clones were similar to environmental sequences from wetlands (three clones, 94% max identity) and a river (two clones, 99%), as well as strains isolated from oil reservoirs (two clones, 95%; Table S2). *Firmicutes* and *Bacteroidetes* clones were similar to environmental sequences from a soda lake (four clones, 93%) and river (two clones, 98%) respectively. To further assess the occurrence and distribution of putative thermophilic SRB contributing to the observed 60°C SRR, we used 16S rRNA primers targeting the genus *Desulfotomaculum* (Stubner and Meuser, 2000), since this genus was detected in both dsrA and aprA gene libraries. This 16S rRNA gene-based approach independently confirmed the presence of *Desulfotomaculum* sequences in Dd and B samples.

4.3. Discussion

Here we characterize the geochemistry and sulfur-cycling communities of four TMVs in Azerbaijan: D (Dashgil) and B (Bakhar), located near the Caspian Sea, and two inland TMVs, P (Perekyushkul) and BJ (Boransyz-Julga), located on the foothills of the Great Caucasses (Fig. 1). Molecular and geochemical data support the presence of active sulfur-oxidizing and sulfate-reducing guilds across a range of physiochemical conditions. While both guilds were present in all four TMVs, the diversity within each guild was unique for each mud volcano suggesting a complex interplay of ecological and environmental factors influence the structure of sulfur-metabolizing communities.

Molecular data revealed SOB were present in mud volcanoes with low and high sulfate levels and co-occurred with an active SRB population. AprA phylotypes affiliated with SOB revealed putative chemosynthetic metabolisms including sequences clustering with obligate aerobes and facultative anaerobes collectively capable of coupling the oxidation of a variety of sulfur species to the respiration of both oxygen and nitrate. SRB community analyses together with SRR measurements support a predominance of *Desulfobulbaceae* members and active sulfate reduction across a range of temperatures with potential rates reflecting ambient sulfate levels. Thermophilic sulfate reduction was also detected in c. 20°C fluids of these mud volcanoes and together with molecular detection of putatively thermophilic *Desulfotomaculum* spp., supports the hypothesis that mud volcanoes transport themophilic microorganisms from deep warm habitats up to the cooler surface where they have been previously reported as endospores (Hubert et al., 2009).

Microbial ecology of the Azerbaijan mud volcanoes

The investigated mud volcanoes support microbial assemblages similar in abundance to other TMVs with bacterial abundance being greater than or equal to that of archaea (Table S3), depending on site and depth examined (Alain et al., 2006; Schulze-Makuch et al., 2011; Chang et al., 2012). Molecular analyses and rate measurements presented here indicate that bacterial diversity is moderate and dominated by meso-and thermophilic sulfur-cycling microorganisms.

Geochemical data suggest the large salse lakes and smaller pools, both of which continuously emit gas and water but very little sediment, have a shallower fluid source than gryphons (conical-shaped mounds emitting viscous mud, gas and water). These data are similar to prior studies from this region (Etiope et al., 2004; Mazzini et al., 2009), and may also explain differences observed in SRB phylotypes retrieved from these distinct habitats. In situ sulfate concentrations were highly variable and did not appear to be correlated with specific habitats (salse versus gryphon) or proximity to the Caspian Sea, and may therefore result from deep sources. Measurements of abundance and composition of reduced sulfur-species (Tables 1 and S4) are consistent with published studies from this region, revealing sulfur to be primarily in the form of disulfides such as pyrite (Isaksen et al., 2007). Monosulfides and elemental sulfur may therefore have a short residence time in these systems due to rapid sulfur cycling. Rock-Eval pyrolysis revealed higher concentrations of free hydrocarbons in sulfate-depleted

volcanoes (B and BJ) suggesting more labile carbon may be available to microbes in these sites, perhaps resulting from a scarcity of heterotrophic SRB.

Relevant microbial processes affecting carbon cycling that were not directly measured here include methanogenesis and methanotrophy. Studies of TMVs have reported evidence of anaerobic methane-oxidizing ANME archaea by CARD-FISH, molecular and rate analyses (Alain et al., 2006; Schulze-Makuch et al., 2011; Yang et al., 2011; Chang et al., 2012; Wrede et al., 2012). In the present study, CARD-FISH analyses also revealed the occurrence of ANME/SRB aggregates (Fig. S2), and sulfide production was observed in anaerobic microcosms incubated with methane (Table 2).

Sulfur cycling

Microbial rate measurements and molecular analyses were used to further examine sulfur cycling in these dynamic environments. Unlike marine MVs, very little is known about chemosynthetic communities such as SOB populations in TMVs (Niemann and Boetius, 2010), although these communities can be a significant source of primary production (Levin and Michener, 2002). aprA phylotypes associated with putative SOB were recovered from all samples and were similar in three out of the four TMVs. Phylogenetic analyses reveal that phylotypes from B, D and P cluster together to the exclusion of reference sequences from cultured species (Fig. 3A). The majority of these sequences have as their closest relative *Thioalkalivibrio* spp., an aerobic gammaproteobacterial genus isolated from soda lakes (Sorokin et al., 2011). The occurrence of these potential alkaliphilic SOB phylotypes in P is also consistent with the recovery of SRB phylotypes from the same clone library (48/93 total clones), which fell within a cluster of sequences most closely related to the alkaliphile *D. alkaliphilus* (Fig. 3B). The pH values of all investigated TMVs were approximately 8.0; with waters of P (and BJ) with a total mineralization in the range of 1.6–2.6 g l^{-1}, defined as highly alkaline, hydrocarbonate-sodium type water compared with D (and B) which show total mineralization in the range of 2.5–8.2 g l^{-1} (data not shown). BJ, while sharing some physical and geochemical features with other TMVs, had distinct aprA phylotypes, most of which (96%) grouped closely together in one cluster of *Betaproteobacteria* including sequences from *Thiobacillus denitrificans*, a facultative anaerobe capable of respiring nitrate and oxygen. 16S rDNA phylotypes from D also provided evidence for the presence of SOB with *Epsilonproteobacteria* comprising a significant proportion of recovered sequences. As both putative aerobic and nitrate-respiring SOB phylotypes were recovered, these data suggest the sulfur-oxidizing guild may be common to TMVs

in the region with the environment dictating the dominant SOB lineage (Table S2). Total nitrogen and $\delta^{15}N$ were measured with similar values across all TMVs. Although the composition of nitrogen species was not measured here, nitrate versus oxygen availability could be a driver of dominant SOB lineages. Future studies focusing on nitrogen cycling in TMVs may reveal SOB as an important microbial component.

Sulfate-reducing bacterial communities can be broadly categorized into two groups based on their potential carbon oxidation pathways; complete oxidizers are capable of complete mineralization of organic substrates to CO_2, while incomplete oxidizers excrete acetate as a final by-product (Canfield et al., 2005). There exists a taxonomic relationship between carbon oxidation pathway and many genera or even families of SRB; most members of the *Desulfobacteraceae* family are complete oxidizers while most members of the *Desulfobulbaceae* are not (Kuever et al., 2005). CARD-FISH analyses revealed the occurrence of *Desulfobacteraceae* (Fig. S2) and *Desulfobulbaceae* cells (data not shown). Of the SRB phylotypes retrieved from the investigated mud volcanoes, putative incomplete carbon-oxidizing genera within the *Desulfobulbaceae* family were dominant at all sites except the deep Dd sample, which is dominated by *Desulfobacteraceae* (Fig. 3B). All aprA clone libraries originated from gryphon mud volcanoes, except for the D libraries, which were associated with a mud volcano pool.

While gryphons contain viscous mud that may be more susceptible to mixing by rising gas bubbles throughout the depth column, pools contain water with comparatively minor amounts of fine sediment overlying a more discrete benthic layer. Interestingly, the diversity recovered from D, sampled at the surface (Ds) and at the bottom sediment (Dd) showed a dominance of complete carbon oxidizer SRB in the underlying sediment layer but not in the surface sample. DsrA gene surveys from deep Dd also confirmed a significant fraction of recovered sequences were associated with complete carbon-oxidizing *Desulfobacteraceae* (Table S2). The P gryphon was also sampled at depth (2 m) however here showed a dominance of putative incomplete carbon-oxidizing *Desulfobulbaceae*. Cultured representative of incomplete oxidizing SRB are known to have comparatively faster growth rates, and outcompete complete oxidizing SRB in enrichments with substrates like lactate and thus may have a selective advantage in habitats where organic matter input is variable (Canfield et al., 2005). It is possible that gryphons and the shallow surfaces of salse lake waters are more dynamic environments giving incomplete carbon oxidizers an advantage.

Microbial rate measurements were performed in order to assay carbon utilization and temperature optimum among active SRB communities. Previous studies have confirmed an approximate 2:1 stoichiometry of CO_2 produced per sulfate consumed in habitats

where sulfate reduction represents the primary means of carbon mineralization (Thamdrup and Canfield, 1996; Vandieken et al., 2006). In the present study CO_2 and sulfide production rates based on microcosm experiments revealed anaerobic CO_2 production was several orders of magnitude less than sulfate consumption (Table 2), consistent with the interpretation of incomplete carbon oxidation during sulfate reduction. In most TMVs sampled, the addition of VFAs did not substantially stimulate SRR suggesting these samples were not limited by bioavailable carbon (Fig. 2). These observations are also consistent with Rock Eval analyses. The exception was the deep Dd sample (dominated by complete carbon-oxidizing *Desulfobacteraceae*) in which VFA addition caused a several orders of magnitude increase in SRR. Notably, aprA phylotypes recovered from this sample were uniquely related to putative autotrophic genera suggesting a potential adaptation to carbon limitation.

Evidence of deep biosphere activity

Terrestrial mud volcanoes have been proposed to serve as conduits that connect earth's surface environments with the underlying deep biosphere whereby the same processes that trigger mud volcanism also lead to a transport of materials from great depths (Hubert et al., 2009; Niemann and Boetius, 2010). While ambient surface mud temperatures of approximately 20°C were measured in the current study (Table 1), conduits of > 10 km have been modelled from Azeri mud volcanoes (Planke et al., 2003) suggesting a potential for the transport of organisms from deeper warmer underlying strata. Maximum SRR in the thermophilic range were observed from 20°C mud collected at B and D, exhibiting temperature optima (55°C and 60°C respectively) within the activity range of thermophilic SRB belonging to the genus *Desulfotomaculum*. SRR at these high temperatures were stimulated by the addition of VFAs, which members of this genus are known to use as electron donors (Rabus et al., 2006). Targeted 16S rRNA primers specific for the genus *Desulfotomaculum*, initially detected in both dsrA and aprA gene libraries, independently supported the presence of *Desulfotomaculum* sequences in those samples exhibiting sulfate reduction at high temperatures.

Desulfotomaculum have been detected in several deep subsurface habitats such as 5 km deep faults and 3.2 km gold mine boreholes (Baker et al., 2003; Moser et al., 2005) and mud volcanoes were hypothesized as a mechanism of transport of themophilic SRB of the genus *Desulfotomaculum* from deep warm habitats to the arctic seabed (Hubert et al., 2009). This idea is strongly supported by our observation of high SRR in mud volcano samples originally at 20°C in situ that are heated to 50–70°C, and by the

detection of putatively thermophilic *Desulfotomaculum* spp. in samples from the same mud volcanoes. Based on our *in situ* temperature measurements (Tables 1 and S4), and the reported geothermal gradient in the South Caspian Basin (Planke et al., 2003; and references therein) temperatures of 50–65°C (optimal SRR as shown in Fig. 2 and consistent with the thermal range of several *Desulfotomaculum;* Rabus et al., 2006) exist at approximately 2–3 km below the surface. Consistent with a lack of SR detection above 60°C, we saw no molecular evidence of hyperthermophilic sulfate reducers, either archaea or bacteria.

4.4. Conclusions

Mud volcanism in Azerbaijan is one of the controlling factors for the vast oil and gas fields in this area; thus elucidating microbial processes in this region is important as these processes can play an important role in the degradation of hydrocarbon inside the reservoirs. The ability to analyse the geochemistry and microbial diversity and processes of distinct TMVs afforded by their high density in this region gives new insights into the interplay between S-cycling microorganisms and their environment.

Sulfur cycling in TMVs has been largely unexplored and here we provide a comparative view of the microbial communities and processes involved in sulfur cycling in multiple TMVs of Azerbaijan. SRB and SOB guilds were present in all TMVs but differed at the genus level between individual mud volcanoes. Detection of thermophilic SRB in 20°C habitats suggest that these TMVs, with conduits extending 10 km or more into deep thermic sediment, may actively transport microorganisms adapted to the deep biosphere.

4.5. Experimental Procedure

Site descriptions

Four mud volcanoes in the South Caspian Basin of eastern Azerbaijan were investigated: Dashgil (D), Bakhar (B), Perekyushkul (P) and Boransyz-Julga (BJ). Samples were taken from the following TMV features (described in Mazzini et al., 2009): gryphons (conical shaped features less than 3 m in height which continuously emit gas, water, oil and viscous mud from their craters), pools (small round features with a diameter up to 2 m, which continuously release water and gas with a minor amount of fine sediment) and salse lakes (lake-like features up to 30 m in diameter and 10 m deep,

which vigorously vent large quantities of gas and water with only a limited amount of mud).

Despite regional and geological differences there is evidence that all four investigated mud volcanoes are sourced from the Maikop Series (Berner et al., 2009), which is Oligocene-Low Miocene in age and rich in organic carbon. Sediments of the Maikop Series are unconsolidated due to rapid burial and overpressure. Overpressuring is enhanced by biogenic and thermogenic gas generation, and results in upward migration at tectonically weak zones or due to earthquakes. Although all four mud volcanoes likely share this common source, D and B are located near the Caspian seaside lying on young Quaternary sediments while BJ and P are situated on the SE foothill of Great Caucasus and lie on comparatively older Oligocene-Miocene deposits (Fig. 1).

D was photographed in 1997 by Hovland and colleagues, and together with B and P can be found in published maps of the South Caspian Basin along with background geochemical data (Guliyev et al., 2001; Planke et al., 2003; Etiope et al., 2004; Mazzini et al., 2009; Feyzullayev and Movsumova, 2010). D has more than 60 gryphons and salses, and has erupted at least six times since 1882; B has around 30 gryphons and salses, has erupted 11 times since 1853, the last recorded eruption occurred in 1992 (Etiope et al., 2004 and references therein). B is considered to have higher eruptive potential (Etiope et al., 2004), but both D and B have high seep activity (Planke et al., 2003). To our knowledge, there are no prior studies of BJ. Both P and BJ are visible from public satellite imaging (see Tables 1 and S4 for GPS coordinates) and appeared to be dominated by uplifted clusters of gryphons during the October 2008 trip (Fig. S1). Several of the gryphons in P and BJ had white crust on the dry outer flanks. In BJ, dead arthropods (mainly beetles) were observed floating on the surface along the sides of several gryphons, along with a visually identified microbial mat. While mud volcanoes are considered dormant in the interval between eruptions (Mazzini et al., 2009), all four TMVs investigated exhibited active seepage of gas and mud.

Sampling

Samples were collected in 2007 from two D salse lakes (D1 and D3 in 2008 data) and one small salse lake within a satellite vent of B (see Planke et al., 2003 and Mazzini et al., 2009 for location of satellite relative to main vent). During a second collection trip in October 2008, samples were collected from D, the same B satellite vent, BJ and P. Unless stated otherwise, all samples were collected directly into sterile 50 ml falcon tubes. Due to the morphological diversity of D and abundance of published background data,

multiple features were sampled within this volcano. Sample D1 was taken from a large, actively bubbling salse lake (referred to as 'Salse A' in Planke et al., 2003 and Mazzini et al., 2009). These samples were taken from the surface of the lake approximately 1 m from the shore using an extendable pole. Ds and Dd were taken from the surface and depths, respectively, of a small pool approximately 3 m from D1 (see Fig. S1 for photograph). Deeper sample Dd was collected from approximately 4 m below the surface of the salse using a 65 cm drop core. Mud for microbial analyses was sampled from the centre portion of the core. D3 samples were taken from a smaller salse lake ('Salse B' in Planke et al., 2003 and Mazzini et al., 2009) with visibly clearer water than that of D1. Samples were scooped from the bottom of this salse using a large ladle attached to the end of an extendable pole. D4 samples were collected directly into a falcon tube from the thick bubbling surface of a gryphon. B samples were also taken directly from the surface of an actively bubbling gryphon at a site known as the Bakhar satellite vent. At the BJ site, surface material from a viscous gryphon was sampled (BJ) along with sediments from the bottom of a less viscous gryphon (BJ2). Ps and P were taken from the surface and depths, respectively, of a gryphon in P. Deeper sample P was taken from the bottom of a 65 cm drop core extended approximately 2 m below the surface.

Field conditions required that all samples be kept at ambient temperature until the end of each sampling day (approximately 5 h) when they were processed and stored at the Geology Institute of ANAS (The Azerbaijan National Academy of Sciences) research lab in Baku prior to their shipment to either GFZ, Potsdam, Germany or CIT, USA.

Geochemical analyses

Air-dried mud material was investigated by all methods further mentioned except for organic-petrographical analyses, for which the mud was freeze-dried. For determination of organic acids the freshly collected mud samples were immediately amended with 5% (v/v) of 10 N NaOH to stop microbial activity. Water samples were collected by removal of the supernatant after centrifugation, followed by filtration. The samples for turnover measurements were immediately transferred into glass flasks, filled without headspace and once back in Baku lab, stored at 4°C. Organic geochemical parameters were determined on samples that were collected in pre-cleaned Teflon cups and stored in liquid nitrogen within a few hours after sampling.

Determination of anions

All water extracts were analysed in replicate by ion chromatography with conductivity detection (ICS 3000, Dionex Corp.). For chromatographic separation of the anions the analytical column AS 11 HC (Dionex Corp.) was used at a temperature of 35°C. The sample was eluted by KOH solution of varying concentration over time. The initial KOH concentration was 0.5 mM, maintained for 8 min. After 10 min, 15 mM KOH solution was reached and kept constant for 10 min. After 30 min analysis time, 60 mM KOH concentration was reached, followed by a rapid increase to 100 mM after 32 min. At 32 min analysis time, KOH concentration was again at the initial level of 0.5 mM and kept there for an additional 15 min to equilibrate the system. For quantification of organic acids (formate, acetate) and inorganic anions (F^-, Cl^-, Br^-, SO_4^{2-}) standards containing all of the investigated compounds were measured in different concentrations every day. Standard deviation of sample and standard quantification is below 10%.

Determination of reduced sulfur species/fractionated distillation

Solid reduced sulfur species were quantified separately based on the extraction scheme of Zhabina and Volkov (1978) with some modifications for the separation of elemental sulfur. The sample is placed into a cold distillation apparatus (Kallmeyer and Boetius, 2004). In a first step 8 ml of 6 M hydrochloric acid is used to liberate the AVS fraction, comprising hydrogen sulfide and monosulfides. In the second step 16 ml of a 1 M chromous chloride ($CrCl_2$) solution is added to the sample to liberate the CRS fraction, comprising mainly pyrite and other disulfides. In the third step 20 ml of N,N-dimethylformamide is added to the sample to obtain the elemental sulfur fraction (ES).

A constant flow of nitrogen is used to strip the liberated hydrogen sulfide gas from the sample and quantitatively collect it in a trap filled with 7 ml of 5% (w/v) zinc acetate solution. For each fraction a fresh trap is used; the reagents are simply added to the existing slurry. Each distillation step takes 2 h to ensure sufficient time for the reaction.

The zinc acetate from the traps interferes with the spectrophotometric sulfide quantification (Cline, 1969), therefore the precipitated zinc sulfide is separated by centrifugation, the clear supernatant carefully decanted off and the pellet resuspended in demineralized water for analysis.

Potential SRR

The samples used for quantification of potential SRR were collected during October 2008 from three different mud volcanoes: at Dd from a depth of approximately 4 m, at B

and BJ from the surface of an actively mud-emitting pool. For SRR quantification the mud samples were diluted with anaerobic saline solution (for composition see Table S1) in a 1:1 ratio (w/v) in a 500 ml Duran flask. The flask was pre-flushed with Gas (N_2/CO_2 80/20) for 5 min before quickly scooping in the mud, followed by flushing for another 5 min before screwing on the cap. After determination of the exact volume of mud by weighing, anaerobic saline solution was added to the flask and the slurry stirred for 1 h. Aliquots of 3 ml were dispensed anaerobically into 30 autoclaved 16 ml screw cap culture tubes using a syringe and a needle. A mixture of six VFAs (acetate, butyrate, lactate, propionate, pyruvate, succinate) and ethanol, each with a final concentration of 1 mM were added to the remaining slurry. From this VFA slurry again 3 ml each were dispensed into each of 15 anoxic autoclaved screw cap culture tubes. Because of the storage in the cold room the samples were pre-incubated at their respective incubation temperature for 24 or 48 h and labelled '24' and '48' accordingly. The VFA-amended samples were pre-incubated for 48 h and labelled '48-VFA'.

In order to avoid any sulfate limitation and thereby causing potential biases when comparing the microbial activity between the different sites, all samples were incubated with the same final sulfate concentration of 20 mM. A thermal gradient block (TGB) was used for experiments requiring different incubation temperatures (e.g. Elsgaard et al., 1994; Sagemann et al., 1998). One end of the block was heated to 95°C while the other was cooled to 5°C resulting in a temperature gradient from 82.6°C to 10.8°C. The linearity of the thermal gradient was checked after 1 day of equilibration with a digital thermometer. The positions of the culture tubes in the TGB were chosen to achieve a resolution of 5°C. All experiments were performed in duplicates.

After 24 or 48 h of pre-incubation, approximately 1 MBq (15 ml) of radioactive $^{35}SO_4^{2-}$ tracer was added to each sample with a syringe. A carrier-free tracer radiotracer stock solution was diluted with a saline solution containing the same major salts as the anoxic saline solution that was used to prepare the slurry. Incubation with the tracer lasted 8 h and was terminated by adding 3 ml of 20% (w/v) zinc acetate (ZnAc) solution and immediate vortexing (Sagemann et al., 1998). The ZnAc-fixed slurry was poured into a 50 ml centrifuge tube. Remaining sediment was washed out of the tube with twice 3 ml of 20% ZnAc. The sediment-ZnAc slurry was then centrifuged (5 min, 4500 g) and the supernatant carefully decanted off. A small amount of supernatant was kept for quantification of the total radioactivity. The pellet was used for SRR measurements. The samples were processed according to the cold chromium distillation protocol of Kallmeyer and Boetius (2004). In short, the pellet was washed out of the tube with 20 ml of DMF into a three-neck round-bottom flask, then 8 ml of HCl and 16 ml of $CrCl_2$

solution was added. The liberated H_2S was flushed with N_2 into a 5% ZnAc trap. The radioactive sulfide was quantified using a Packard 2900 TR Tri-Carb scintillation counter. The SRR was determined according to Jørgensen (1978). Control samples that were first fixed in ZnAc prior to radiotracer addition were processed together with the regular samples to quantify the background and to calculate the minimum detection limit.

Microcosms to test sulfide and CO_2 production

Mud and water samples were collected in 2007 from the D and B mud volcanoes. Experiments were carried out in glass tubes (20 ml) sealed with butyl-rubber stoppers and screw caps. Sediment samples (from 2007 trip) were mixed 1:1 with artificial mineral medium (after Widdel and Bak, 1992; similar in composition to medium used for potential SRR measurements) to obtain homogenous slurries. Subsequently, 9 ml of medium were added to 3 ml of sediment slurry. All manipulations were performed under an atmosphere of nitrogen in an anoxic glove box. The headspace of the incubation tubes consisted either of methane (100%), air (100%) or of N_2/CO_2 [90/10 (v/v)]; with CO_2 levels similarly unlimited as with gas composition used for potential SRR measurements]. CO_2 was determined in all incubations using a GC 14B gas chromatograph (Shimadzu) as described in Nauhaus and colleagues (2002). Sulfide was determined in anaerobic incubations using the formation of copper sulfide (Cord-Ruwisch, 1985).

Gene quantification by qPCR

Mud and water samples were collected in 2007 from D (salse lakes D1 and D3 in 2008 data) and B (small salse within same B satellite visited in 2008) mud volcanoes. DNA extraction was carried out using a Fast DNA for Soil Kit (Fast DNA Spin Kit for soil, BIO 101, MP Biomedicals, Germany). To block sedimentary nucleic acid binding capacities, 10 ml of a 1% polyadenylic acid solution were added in the initial step (Webster et al., 2003). Directly before PCR, 125 ml of 0.3% bovine serum albumine (BSA) in ultra pure water was added as blocking agent to the Taqman master mix (Applied Biosystems, Germany) or the SYBR green® master mix (Eurogenetec, Germany). A real-time PCR instrument (ABI Prism 7000, Applied Biosystems) was employed to determine the 16S rRNA gene copy numbers of Archaea (Takai and Horikoshi, 2000) and Bacteria (Nadkarni et al., 2002).

Molecular biological determination of sulfur-cycling bacteria

DNA extraction. Mud samples collected from D, B, BJ and P during October 2008 for molecular analyses were stored at approximately -20°C before and after room temperature shipment. DNA extractions were conducted using the MoBio Ultraclean soil kit following a previously published protocol (Orphan et al., 2001). Due to inconsistencies in mud viscosity, the following starting material was used from each sample: Ds, 500 ml; Dd 0.5 g weight wet; B, 250 ml; BJ, 0.5 g weight wet; P, 50 ml.

PCR amplification and cloning

Unless otherwise noted, amplification reactions followed published PCR mixtures and conditions (Harrison et al., 2009) with 0.5 ml of Hotmaster Taq polymerase (Eppendorf AG, Hamburg, Germany). Bacterial 16S rRNA genes were amplified from Dashgil (Dd) using bacterium-specific forward primer BAC-27F and universal reverse primer U-1492R (Lane, 1991). Thermocycling conditions included an initial 94°C denaturating step for 2 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, and then a final 72°C elongation step for 6 min. The products of two reactions were pooled and cleaned using a Multiscreen HTS plate (Millipore). The resulting purified 16S rRNA gene amplicons were ligated into pGEM-T Easy vector and used to transform JM109 chemically competent cells (Promega, Stoughton, WI). Genusspecific 16S rRNA primers DEM116F and DEM1164R were used to check for presence of *Desulfotomaculum* (Stubner and Meuser, 2000). Thermocycling conditions included an initial 94°C denaturation step for 1 min followed by 40 cycles of 94°C for 45 s, 57.5°C for 45 s and 72°C for 1 min, and then a final 72°C elongation step for 6 min.

An equimolar mix of forward primers DSR1F, DSR1Fa, DSR1Fb, DSR1Fc and DSR1Fd and reverse primers DSR4R, DSR4Ra, DSR4Rb, DSR4Rc, DSR4Rd and DSR4Re (Zverlov et al., 2005) was used to amplify the 1.9 kb dsrAB fragment from Dashgil (Dd). The 1.9 kb PCR product was excised from a 1% agarose gel and purified using a Quiaquick Gel Extraction kit (Qiagen Corp., Valencia, CA). Resulting DsrAB amplicons were ligated into pGEM-T Easy vector and used to transform JM109 chemically competent cells (Promega, Stoughton, WI).

The primer set AprA-1-FW/AprA-5-RV was used to amplify the approximately 0.4 kb fragment of the aprA gene (Meyer and Kuever, 2007a) from 2 ml of Dd and BJ and 1ul of Ds, B and P DNA extractions. Thermocycling conditions included an initial 94°C denaturation step for 3 min followed by 40 cycles of 94°C for 30 s, 54°C for 55 s and 72°C for 1 min, and then a final 72°C elongation step for 6 min. The products (one

reaction each from Dd, Ds, B and P templates, and two pooled reactions from the BJ template) were cleaned using a Multiscreen HTS plate (Millipore). The resulting purified aprA gene amplicons were ligated into pGEM-T Easy (Dd and BJ; Promega) or pCR 4.0 TOPO TA (Ds, B and P; Invitrogen Corp., Carlsbad, CA) vectors and used to transform JM109 (Dd and BJ; Promega, Stoughton, WI) or One-Shot TOP10 (Ds, B and P; Invitrogen Corp., Carlsbad, CA) chemically competent cells according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

HaeIII restriction fragment length polymorphism (RFLP) analysis was performed on products amplified with the standard primer set M13F/M13R (Pernthaler et al., 2008; Harrison et al., 2009; New England Biolabs, Ipswich, MA) from all 16S rDNA, dsrA and aprA clones. One RFLP pattern from Ds had representative clones from two distinct phylogenetic groups; all clones from with this pattern were further digested with PstI (New England Biolabs, Ipswich, MA) and the pattern divided accordingly. From the Dashgil_DD samples, 62 dsrA clones were screened (11 unique RFLP patterns), and 74 bacterial 16S rDNA clones were screened (11 unique RFLP patterns). The following number of aprA clones were retrieved (with number of unique RFLP patterns in parenthesis) from each TMV: Ds: 89 (16), Dd: 47 (9), B: 69 (14), BJ: 76 (6), P: 93 (21). Representative clones with unique restriction patterns were cleaned using Multiscreen HTS plates (Millipore) and sequenced unidirectionally either in house with a CEQ 8800 capillary sequencer according to the DTCS protocol (Beckman Coulter, Fullerton, CA), or at the ASGPB DNA Sequencing Facility of the University of Hawai'i at Manoa. All sequences were manually edited using Sequencher 4.5 software (Gene Codes, Ann Arbor, MI) and closest relatives in the GenBank database were identified using BLASTN (Altschul et al., 1997). All aprA sequences (131 translated amino acid characters each) were manually aligned using the ARB software package (version 7.12.07org, ARB_EDIT4; Ludwig et al., 2004) into an alignment of full-length aprASRP and SOB reference strains and closest relatives recovered from the public databases. The aligned sequences were then added to the existing full-length aprA tree using the quick add maximum parsimony method with a filter to mask regions outside of the 131 amino acid characters. GenBank accession numbers for aprA, dsrA and 16S rRNA sequences are JX908299-JX908360 and JX889577-JX889588.

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4.7. References

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Supporting information

Additional Supporting Information may be found in the online version of this article:

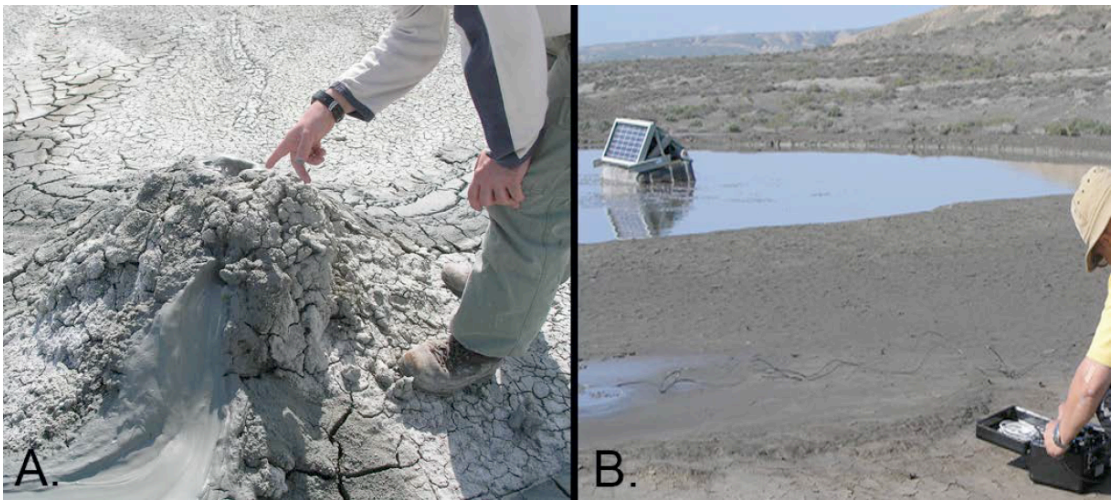

Figure S1: Photographs taken in October 2008 of (A) Boransyz-Julga gryphon and (B) Dashgil salse lake.

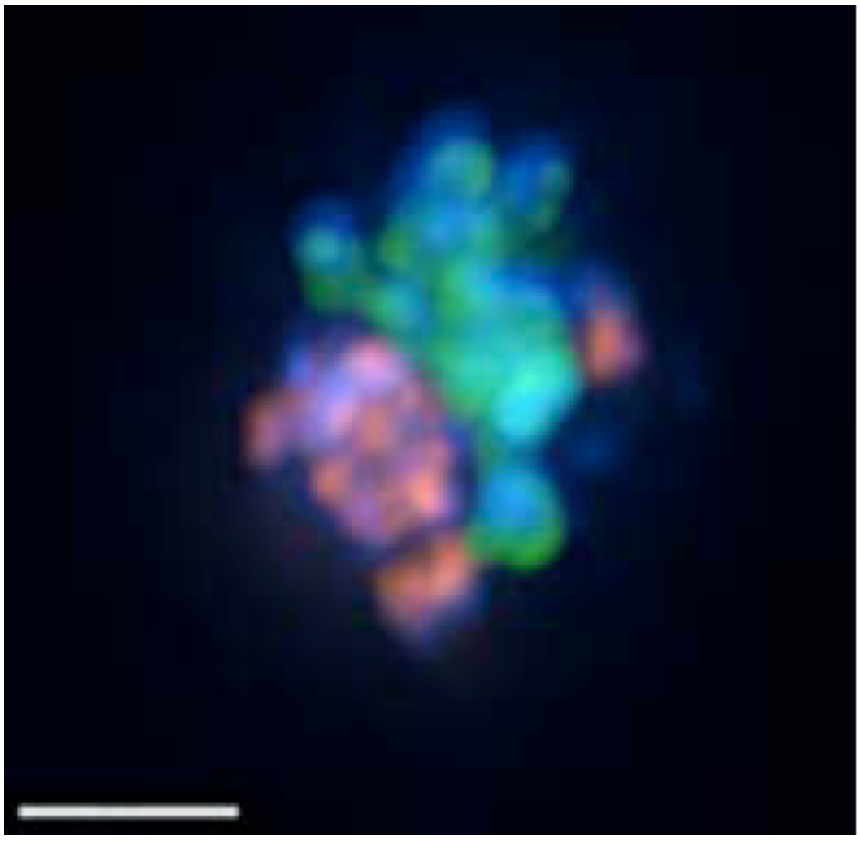

Figure S2: Putative methane-oxidizing aggregates from Dashgil (D1) mud samples identified by CARD-FISH after hybridization with oligonucleotide probes specific to anaerobic methane-oxidizing archaea ANME-2 (EelMsMX_932; red), and Desulfosarcina-Desulfococcus members (DSS_658; green). The general DNA stain DAPI is shown in blue. Scale bar = 2 mm.

Table S1: Composition of the synthetic saline solution; the solutions were added after the anoxic cooling post autoclaving.

	14 g NaCl, 2.1 g $MgCl_2$*$6H_2O$, 0.105 g $CaCl_2$*$2H_2O$, 2.8 g Na_2SO_4, 0.175 g NH_4Cl, 0.14 g KH_2PO_4, 0.2 g KCl per Liter
Add after anoxic cooling past autoclaving:	
1 ml trace elements mixture	50 ml H_2O + 2.1 g $FeSO_4$*$7H_2O$ + 13 ml HCl (25%), 30 mg H_3BO_3, 100 mg $MnCl_2$*$4H_2O$, 190 mg $CoCl_2$*$6H_2O$, 24 mg $NiCl_2$*$6H_2O$, 2 mg $CuCl_2$*$2H_2O$, 144 mg $ZnSO_4$*$7H_2O$, 36 mg Na_2MoO_4*$2H_2O$ per Liter,autoclaved
2 ml SeW-solution	200 mg NaOH, 6 mg Na_2SeO_3*$5H_2O$, 8 mg Na_2WO_4*$2H_2O$ per Liter, autoclaved
60 ml 1 M $NaHCO_3$-solution	84 g $NaHCO_3$ per Liter, anoxic, autoclaved
2 ml 7-vitamin mixture	100 ml H_2O + 4 mg 4-amino benzoic acid, 1 mg D(+)-biotin, 10 mg nicotinic acid, 5 mg Ca-D(+)-pantothenat, 15 mg pyridoxine hydrochloride, 10 mg thiamine chloride hydrochloride, filtered sterile
2 ml B_{12}-solution	5 mg cyan cobalamine in 100 ml H_2O, filtered sterile

Table S2: Closest relatives of phylotypes retrieved from (A) 16S rRNA and (B) dsrA gene clone libraries of Dashgil sample (deep pool Dd).

A.

Classification	clone (frequency)	Accession	Description	Similarity
Bacteroidetes	D12 (2)	JF806919	env., river	98%
Chloroflexi	A10 (6)	HQ183882	env., landfill leachate pond sediment	98%
Chloroflexi	B6 (4)	HQ397021	env., landfill leachate pond sediment	97%
Chloroflexi	C8 (30	HQ689286	env., landfill leachate pond sediment	96%
Chloroflexi	G4 (1)	HQ183892	env., landfill leachate pond sediment	99%
Delta-proteobacteria	B4 (3)	FJ517079	env., wetlands	94%
Delta-proteobacteria	F1 (2)	JF806819	env., river	99%
Delta-proteobacteria	H2 (1)	NR_044429	*Geoalkalibacter subterraneus* strain Red1	99%
Delta-proteobacteria	G5 (1)	AF177428	env., oil reservoir	95%
Epsilon-proteobacteria	A1 (39)	JF806914	Uncultured *Sulfurovum* sp.	96%
Epsilon-proteobacteria	G1 (5)	JF806947	Uncultured *Sulfurovum* sp.	99%
Firmicutes	C12 (4)	GQ848205	env., soda lake	93%

B.

Classification	clone (frequency)	Accession	Description	Similarity
Desulfobacteraceae	B2 (29)	EU496887	env., methane-seep sediment	82%
Desulfobacteraceae	A3 (15)	AF482455	*Desulfobacterium anilini*	79%
Desulfobacteraceae	D5 (4)	AY626030	*Desulfonema ishimotoni*i strain DSM 9680	76%
Desulfobacteraceae	E2 (1)	AY626031	*Desulfonema limicola* strain DSM 2076	75%
Desulfobulbaceae	G4 (1)	CP001940	*Desulfurivibrio alkaliphilus* AHT2	80%
	F7 (3)	AB124917	Uncultured sulfate-reducing bacterium, hydrothermal vent	87%
	H2 (5)	FJ748851	Uncultured sulfate-reducing bacterium, estuarine sediment	82%
	H7 (3)	JF950248	Uncultured bacterium, anoxic fjord sediments	77%
	H4 (1)	FJ748848	Uncultured sulfate-reducing bacterium, estuarine sediment	77%

Table S3: Archaeal and Bacterial 16S rRNA gene abundance from D (Dashgil) and B (Bakhar) samples collected in 2007.

Sample, depth collected	Total Archaea			Total Bacteria		
Dashgil (D1) big salse lake, 5.8m	2.99E+07	+/-	1.81E+07	2.23E+08	+/-	1.83E+07
Dashgil (D1) big salse lake, 0.5m	7.62E+07	+/-	5.12E+07	3.77E+08	+/-	3.14E+07
Dashgil (D3) small salse lake, 6.5m	8.80E+08	+/-	8.74E+07	1.29E+09	+/-	4.78E+08
Dashgil (D3) small salse lake, 0.2m	1.97E+08	+/-	7.65E+07	1.09E+09	+/-	1.53E+08
Bakhar small salse lake, 4.2m	1.30E+07	+/-	6.06E+06	1.02E+07	+/-	1.26E+07
Bakhar small salse lake, 0.2m	2.11E+07	+/-	2.66E+06	3.43E+07	+/-	2.02E+07

Table S4: Geochemical characteristics of terrestrial mud volcanoes of Azerbaijan sampled in October 2008.

Mud Volcano	**D: Dashgil**			**P: Perekyushkul**	**BJ: Boransyz-Julga**
Geographic Setting	*Proximal to Caspian Sea* Quaternary Sediments			*Foothills of Great Caucasus* Oligocene-Lower Miocene Deposits	*Foothills of Great Caucasus* Oligocene-Lower Miocene Deposits
Coordinates	N 39^0 59' 47.8" E 49^0 24' 08.8"			N 40^0 28' 49.9" E 49^0 26' 53.7"	N 40^0 27' 59.6" E 49^0 28' 11.4"
Sample Name and Feature	**D1: Salse Lake**	**D3: Salse Lake**	**D4: Gryphon**	**Ps: Gryphon (surface)**	**BJ2: Gryphon**
Temperature, °C (measurement depth, m)	19 (0.2), 18.7 (5.5)	n/a	n/a	16.3 (0.2)	n/a
POREWATER GEOCHEMISTRY					
Fluoride (mM)	0.18	0.14	n./a	n/a	< 0.05
Sulfate (mM)	0.75	< 0.16	0.47	0.32	< 0.16
Chloride (mM)	378	464	209	68	64
Bromide (mM)	0.82	0.97	0.54	0.16	0.16
Cl/Br (mass ratio)	205	213	173	183	177
Acetate (mM)	< 0.010	0.020	n/a	n/a	0.016
Formate (mM)	< 0.013	0.027	n/a	n/a	0.034
STABLE ISOTOPE COMPOSITION					
$d^{15}N$ ‰	2.2	2.5	2.0	2.7	3.0
N_{total} wt. %	0.09	0.09	0.10	0.11	0.09
C_{total} wt. %	2.55	2.74	2.67	2.41	1.77
TOC wt. %	1.05	0.78	0.95	1.29	0.71
$d^{13}C_{org}$ ‰	-25.1	-26.4	-24.9	-26.6	-26.3
$CaCO_3$ calc %	12.5	16.3	14.4	9.3	8.8
$d^{13}C_{carb}$ ‰	0.42	-0.59	-1.04	2.71	4.83
$d^{18}O_{carb}$ ‰	-3.47	-2.50	-4.03	-2.41	-1.23
ORGANIC ANALYSIS					
S1, S2, S3 (mg/g)	0.42, 2.22, 1.01	0.13, 1.46, 1.21	0.33, 2.62, 0.71	0.48, 4.87, 1.26	0.24, 2.16, 1.51
Tmax (°C)	431	431	433	431	428
Hydrogen Index HI (mg HC/gTOC)	193	150	224	316	214
Oxygen Index OI (mg CO_2/g TOC)	88	124	61	82	150
TOC (%)	1.15	0.97	1.17	1.54	1.01
REDUCED SULFUR SPECIES (AVS:CRS:DMF)	n/a	0.01: 99.91: 0.08	0.00: 99.95: 0.05	n/a	27.68: 72.30: 0.02

Table S5: Geochemical characteristics of four terrestrial mud volcanoes of Azerbaijan sampled in October 2008.

Mud Volcano	**D: Dashgil**		**B: Bakhar**	**P: Perekyushkul**	**BJ: Boransyz-Julga**
Geographic Setting	*Proximal to Caspian Sea* Quaternary Sediments		*Proximal to Caspian Sea* Quaternary Sediments	*Foothills of Great Caucasus* Oligocene-Lower Miocene Deposits	*Foothills of Great Caucasus* Oligocene-Lower Miocene Deposits
Coordinates	N 39^0 59' 47.8" E 49^0 24' 08.8"		N 39^0 59' 53.7" E 49^0 27' 20,3"	N 40^0 28' 49.9" E 49^0 26' 53.7"	N 40^0 27' 59.6" E 49^0 28' 11.4"
Sample Name and Feature	**Ds: Pool (surface)**	**Dd: Pool (deep)**	**B: Gryphon**	**P: Gryphon (deep)**	**BJ: Gryphon**
Temperature, °C (measurement depth, m)	17.2 (0.2)	20.6 (1.0), 20.8 (1.25)	19.6 (0.2), 20.7 (3.5)	17.6 (2.5)	18.5 (0.2), 18.3 (1.5)
POREWATER GEOCHEMISTRY					
Fluoride (mM)	0.03	< 0.05	< 0.05	0.06	n/a
Chloride (mM)	312	321	255	56	60
Bromide (mM)	0.63	0.65	0.60	< 0.13	0.13
STABLE ISOTOPE COMPOSITION					
$d^{15}N$ ‰	2.0	2.4	2.6	2.7	3.0
N_{total} wt. %	0.10	0.07	0.05	0.10	0.09
C_{total} wt. %	2.93	2.33	2.88	2.56	2.12
TOC wt. %	1.50	0.70	0.58	1.26	1.24
$d^{13}C_{org}$ ‰	-25.6	-25.3	-25.1	-26.6	-26.7
$CaCO_3$ calc %	11.9	13.6	19.2	10.8	7.3
$d^{13}C_{carb}$ ‰	0.20	n/a	-0.28	3.02	1.41
$d^{18}O_{carb}$ ‰	-4.02	n/a	-4.33	-2.46	-2.91
ORGANIC ANALYSIS					
Tmax (°C)	423	432	392	432	425
TOC (%)	1.63	0.83	0.72	1.58	1.46

5. Conclusion

The aim of this study was to estimate the abiotic release of low molecular weight organic acids (LMWOAs) from different coals from New Zealand and Germany and to evaluate their potential to provide a feedstock for indigenous subsurface microbes. Furthermore the effect of LMWOAs on microbial communities in surface ecosystems, which are linked with subsurface environments, was evaluated. Mud volcanoes represent such a link between subsurface and surface. The influence of abiogenically produced LMWOAs from buried macromolecular organic matter on sulphate reduction was explored on samples from terrestrial mud volcanoes (TMVs) from Azerbaijan.

LMWOAs are liberated from buried macromolecular organic matter (e.g. coal), the rate of release is controlled by geochemical conditions (pore water composition, temperature, pressure, pH). To simulate the liberation of LMWOAs under *in-situ* conditions, a high-pressure high-temperature incubation system was constructed, which allowed incubations with different gas compositions and concentrations. The system can be used for incubations of geological as well as biological samples. Furthermore, the system allows sub-sampling without major loss of pressure, thereby avoiding cell rupture due to depressurisation.

Using the new incubation system sub-bituminous coal from New Zealand was extracted in order to evaluate the effect of pressure on the extraction yield of LMWOAs in comparison to the commonly used Soxhlet extraction method that works at atmospheric pressure and with demineralised water. As elevated pressure is a key feature in the deep subsurface, incubations with our new high-pressure system are much closer to true *in-situ* conditions of geological settings. Furthermore, in our new system the liberated LMWOAs are not removed from the extraction medium, we could simulate acidification of the pore water, which is a further improvement in comparison to the Soxhlet method.

As CO_2-containing flue gas from power plants is injected into geological reservoirs for enhanced recovery of coal bed methane (ECBM) or in the scope of carbon capture and storage (CCS), porewater becomes acidified due to the formation of acids upon the injection of CO_2. To determine the effect of acidification of the reservoir porewater, experiments with highly CO_2-enriched water were carried out. As most research focuses on the effects of supercritical carbon dioxide, our experiments were carried out at subcritical conditions in order to gain information about this rather overlooked issue.

Experiments with low-ranked lignite from East Germany were carried out to determine the content of extractable LMWOAs as they act as a feedstock for the indigenous microbial ecosystem. Furthermore the effect of flue gas impurities (SO_2, NO_2) on the extraction yield was evaluated, as these impurities form even stronger acids than CO_2, causing a further acidification of the porewater.

To link the deep biosphere and the production of LMWOAs with surface processes, samples from terrestrial mud volcanoes of Azerbaijan were investigated. In these structures LMWOAs are produced at depth and transported to the surface together with the upwards-flowing mud where they are utilized by microbes. Mud volcanoes represent a conduit for deeply buried macromolecular organic matter, liberated LMWOAs and mud of marine origin. As sulphate is abundant in these mud volcanoes, sulphate reduction is a common process, using LMWOAs as an electron donor. Therefore the effects of LMWOAs on the sulphate reduction rate (SRR) in mud volcanoes of Azerbaijan were determined. Furthermore reduced sulphur species in the mud were identified to illustrate the different pathways in the sulphur cycle of mud volcanoes.

Temperature-dependent SRR should provide evidence for a connection between the deep hot subsurface and the much cooler surface biosphere. Additional to biogeochemical experiments, phylogenetic analyses were performed to obtain an overview of the microbial community composition.

The extraction of low molecular weight organic acids from sub-bituminous coal of the DEBITS-1 well of the Waikato coal area of New Zealand (North Island) under elevated pressure resulted in the extraction of the same LMWOAs (formate, acetate, oxalate) as those extracted with a Soxhlet apparatus (Vieth et al., 2008; Glombitza et al., 2009). However, high-pressure extraction yields were up to four times higher than those without pressurisation (see figure 5.1). This shows the importance and necessity of elevated pressure for the simulation of true *in-situ* conditions in underground reservoirs.

The injection of CO_2/flue gas into underground reservoirs was simulated by the addition of CO_2 to the extraction medium. Many experiments are conducted with supercritical carbon dioxide, which kills all microbes. Therefore this PhD project focused on subcritical conditions and thereby remains within the limits of microbial habitability, despite a decrease in pH due to formation of carbonic acid. When adding pure (food grade) carbon dioxide to the extraction medium at subcritical conditions, the overall extraction yields were lower or remained unchanged in comparison to extractions with

pure water. However, the various LMWOAs behaved differently. Extraction yield of formate remained unchanged, whereas there was an increase for acetate and a decrease for oxalate upon acidification of the pore water. Possible reasons for this effect have not yet been identified but several possible scenarios were discussed for oxalate.

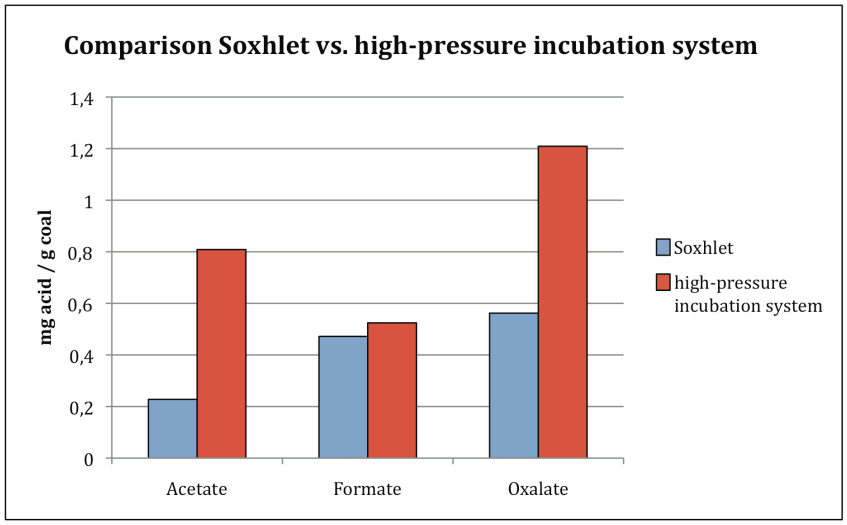

Figure 5.1: Comparison of extractions yields of sub-bituminous coal (from DEBITS-1 well of Waikato Basin, North Island, New Zealand) extracted with Soxhlet (blue, 80°C) and high-pressure incubation system (red, 90°C and 5 MPa pressure). Extractions were performed for 48 hours.

When extracting lignite from the Niederlausitz brown coal district in Eastern Germany the same LMWOAs as in the slightly higher-ranked coals from New Zealand were detected. Therefore it can be speculated that the LMWOA pattern, which was found in both types of coal, represents the common pattern of extractable LMWOAs. Upon the addition of CO_2 and mixtures of CO_2 and SO_2/NO_2, extraction yields for formate and acetate increased, leading to an increase in the nutrient supply for indigenous microbes. As formate and acetate are the main microbial substrates, microbial growth and activity will strongly be promoted, even though the liberation of oxalate decreases, but it is less important as a microbial substrate. Furthermore, as methane is the main product of microbial metabolism in coal seams, the production of natural gas from coal seams may be increasing over time.

LMWOAs from deeply buried sediments also promote microbial ecosystems in mud volcanoes, where they are ascending from great depth together with large amounts of hydrocarbons. As sulphate concentrations are high in terrestrial mud volcanoes of Azerbaijan, sulphate reduction is a major process in this environment. The presence of a wide rage of reduced sulphur species showed the presence of a full sulphur cycle.

Biological sulphate reduction is the most important process in the production of reduced sulphur species, and we could demonstrate that the addition of LMWOAs massively promoted the microbial sulphate reduction, showing an influence of the availability of nutrients on sulphate reduction rates (SRR). The increase in SRR towards higher temperatures and the presence of thermophilic sulphate reducing bacteria (SRB) in a mesophilic environment supports the assumption that mud volcanoes – as well as other hydrocarbon-expelling systems – transport microorganisms from the deep subsurface to the surface.

The findings of this thesis support the assumption that - despite its recalcitrance - buried macromolecular organic matter can liberate significant amounts of microbial substrates, at least much more than previously thought. Furthermore the injection of CO_2 could potentially increase the nutrient supply for subsurface microbes. Together with microbes, which are adapted to deep subsurface conditions, the substrates generated at depth can be transported to the surface by active fluid flow like in mud volcanoes or seeps. Such windows to the deep biosphere allow us to study processes subsurface processes in surface environments.

Outlook

So far rather little research has been dedicated to the liberation of LMWOAs from macromolecular organic matter. Especially the influence of non-supercritical anthropogenic CO_2 has rarely been studied. Unlike in marine mud volcanoes, the microbial community in TMVs has also not yet been studied to a greater detail. It is still quite difficult to obtain uncontaminated samples from deep subsurface environments, and many techniques for the exploration of processes and microorganisms are still not sensitive enough to detect and quantify the small rates of activity. The results from this thesis suggest several possibilities for future work:

- Incubations with highly CO_2-enriched water should be performed with fresh coals instead of freeze-dried ones in order to determine the effect of microbes on the extraction yields of LMWOAs as microbes are present in the lignite and the surrounding sediments.
- Another possible topic would be a characterization of the indigenous microbial community before and after incubation with CO_2-enriched water.

- Furthermore, microbes of the sediments surrounding the coal could be incubated under elevated gas partial pressure to obtain a better overview of the spatial distribution of microbial processes in the entire geological unit.
- In order to identify more compounds that were liberated from lignite and could act as a possible microbial feedstock the detection of the extracted compounds could be improved, using more advanced analytical techniques.

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