

The influence of high CO₂ concentrations on microorganisms in different ecosystems

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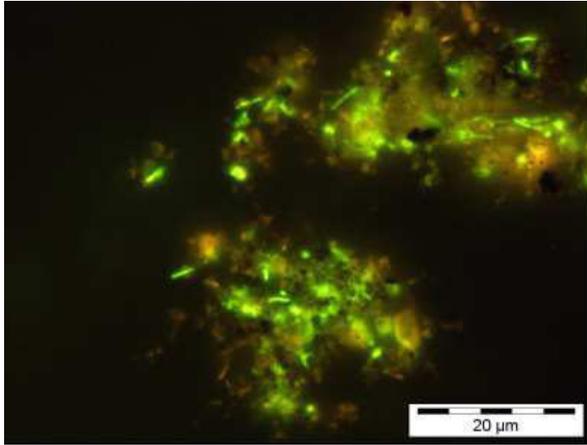
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Für meine Großeltern -
Die immer so stolz auf mich waren!

Zusammenfassung

Die anthropogenen CO₂ Emissionen haben die CO₂ Konzentration der Atmosphäre erhöht und damit Einfluss auf die Funktionen des globalen Kohlenstoffkreislaufes und Klimas. Unsere CO₂-Emissionen beeinflussen so das Ökosystem der Erde und schaden letztendlich auch der menschlichen Gesellschaft. Die Abscheidung von CO₂ und folgende Speicherung im tiefen Untergrund wird zurzeit breit diskutiert, um die CO₂ Emissionen stark und nachhaltig zu reduzieren. Tiefe geologische Strukturen, wie z. B. erschöpfte Erdgas- und Ölfelder, gelten dabei als mögliche Speicherreservoirs mit der nötigen Kapazität. Allerdings können spätere Leckagen des CO₂ aus der Speicherstätte und die daraus resultierenden Umweltfolgen nicht vollkommen ausgeschlossen werden.

In dieser Arbeit wurde der Einfluss von hohen CO₂ Konzentrationen auf die mikrobielle Gemeinschaft beispielhaft in zwei Ökosystemen untersucht: a) die Fluide eines Erdgasfeldes als Modell für ein potentielles Speicherreservoir und b) ein vulkanischer CO₂-Austritt als natürliches Analogon für eine CO₂-Leckage in oberflächennahe Böden. Ziel der Untersuchung war es, die unter Einfluss von CO₂ auftretenden oder sich aus den geochemischen Veränderungen des Ökosystems ergebenden Anpassungen der mikrobiellen Gemeinschaft zu dokumentieren.

Diese Arbeit dokumentiert die dynamische Entwicklung der bakteriellen Gemeinschaft im Erdgasfeld. Die mikrobielle Gemeinschaft war dabei klar von Sulfat-reduzierenden Bakterien dominiert, deren Abundanz und Aktivität im zeitlichen Verlauf deutlich zunahm. Die in einer vorhergehenden Arbeit beschriebenen methanogenen Archaeen wurden in dieser Arbeit als produktionsbedingte Anreicherungen in den Fluiden identifiziert. Die mikrobielle Gemeinschaft veränderte sich stark im zeitlichen Verlauf, was auf produktionsfördernde technische Maßnahmen zurückgeführt werden konnte. Versuche mit superkritischem CO₂ (unter in situ nahen Bedingungen) belegten eine überraschende Widerstandsfähigkeit von thermophilen, Gram-positiven und sporenbildenden *Clostridiales* in den Produktionsfluiden.

Die kombinierten mikrobiellen und geochemischen Analysen am natürlichen CO₂-Austritt dokumentierten Unterschiede in der Verteilung der mikrobiellen Aktivitäten (aerobe/anaerob) zwischen den CO₂-beeinflussten Bodenschichten und den unbeeinflussten Referenz-Böden. Mittels molekularer-biologischer Methoden wurden als potenzielle Indikatorspezies *Thaumarchaeota* in den CO₂-assoziierten Böden identifiziert. Die ökologische Nische der Organismen wurde dabei mit sauren pH-Werten und limitierter Sauerstoff-Verfügbarkeit im CO₂-beeinflussten Boden in Verbindung gebracht.

Zusammenfassend konnten diese mikrobiellen Untersuchungen beider Ökosysteme wichtige Informationen zu der Anwendung und den Auswirkungen der Kohlendioxidspeicherung und einer möglichen Leckage des CO₂ in die oberflächennahen Böden geben.

Summary

The post-industrial anthropogenic CO₂ emissions had a severe effect on the atmospheric CO₂ concentrations and the mechanisms underlying the global carbon cycle and climate functions. Consequently, human-made CO₂ emissions will imbalance the ecosystem of the earth and harm the human society and welfare. Carbon Capture and Storage (CCS) is under political and scientific debate as large-scale solution to reduce the industrial emissions of CO₂. Deep geological structures, like depleted gas or oil reservoirs, are considered as suitable reservoirs providing sufficient storage capacity for CO₂. However, one major concern for CCS is the possible leakage of CO₂ from its storage reservoir and subsequent effects on the environment.

This work evaluates possible effects of high CO₂ concentrations on the microbial population in i) the fluids of a natural gas reservoir as analogue for a potential CO₂ storage site and ii) a terrestrial volcanic CO₂ vent which provides a natural analogue for CCS leakage scenario into the upper soil environment. The overall aim of this work was to investigate the changes and adaptation mechanisms of the microbial community in its structure and function with CO₂ as stress factor affecting the geochemical conditions of the respective habitat.

This work documented dynamic developments of the bacterial population in the gas reservoir. Sulphate-reducing bacteria were predominant in the formation fluids, and their abundance and activities increased in the course of this work. In this work the methanogenic archaea, previously documented for the reservoir, were identified as production-related enrichment and localised within the well head facility. The bacterial community structure revealed temporal variations that were correlated with technical measures to increase the gas productivity. The incubations with supercritical CO₂ (under near in situ conditions) revealed a surprising resistance for thermophilic, spore-forming and Gram-positive *Clostridiales* against the bactericidal effects of high pressurised CO₂.

The geochemical and microbiological parameters at the natural CO₂ vent system revealed differences in the distribution of microbial activities (aerobic/anaerobic) between the CO₂ affected soil surface and the unaffected reference soil environment. The analysis of the microbial community structure identified *Thaumarchaeota* as potential microbial indicator species in the upper soil environment of the vent centre. The predominant *Thaumarchaeota* species were presumably connected to soil acidification and O₂ limitation with hypoxic/anoxic microclimate in ecological niches within the CO₂ affected soil column.

In synthesis, the microbiological investigations in both ecosystems provide important information for the application and consequences of CCS in hydrocarbon reservoirs and in case of a potential CO₂ leakage from the storage reservoir.

Stichwörter: Erdgaslagerstätten, mikrobielle Ökologie, vulkanische CO₂ Austritt

Keywords: natural gas reservoir, microbial ecology, volcanic CO₂ seep

Abbreviations

°C	degree Celsius
<i>16S rRNA</i> (gene)	encoding gene for the small subunit (16S) of ribosomal RNA
<i>amoA</i>	ammonia monooxygenase subunit A
AOA	ammonia oxidising archaea
AOB	ammonia oxidising bacteria
BLAST	basic local alignment search tool
BGR	Bundesanstalt für Geowissenschaften und Rohstoffe
bp	base pair
BP	before present
<i>cbbL</i>	encoding gene for biggest subunit (L) of RUBISCO
CCS	Carbon Capture and Storage
CO2GeoNet	CO ₂ geological storage - European Commission's network of excellence of 6th framework program (2004-2009)
CPR	carbon dioxide production rate
d	day
DR	deep reservoir fluids
<i>dsrA</i> or <i>dsrB</i>	gene of dissimilatory sulphite reductase subunit A or B
EPS	extracellular polymeric substances
EU	European Union
GC	gas chromatography
Gt	giga tones (1 Gt = 1 000 000 t)
GTBP	Isopore™ Millipore product line for black polycarbonate filter discs
h	hour
HPLC	high performance liquid chromatography
ICP-AES	inductively coupled plasma atomic emission spectroscopy
IGÖ	Institut für Grundwasserökologie
IPCA	interacting principal component analysis

IPCC	International Panel on Climate Change
ka	1000 years
LMWOA	low molecular weight organic acids
m-/μM	milli-/micromol L ⁻¹
<i>mcrA</i>	encoding gene of methylcoenzyme M reductase subunit A
mL	millilitre
MPR	methane production rate
NCBI	National Center for Biotechnology Information
PCI	phenol-chloroform-isoamyl solution
PF	production fluids
ppm	parts per million
<i>p</i> -value	probability value (limit of significance 0.05)
qPCR	quantitative PCR
RECOBIO-2	recycling of sequestered CO ₂ by microbial – biogeochemical transformation in the deep subsurface
RUBISCO	ribulose-1,5-biphosphate carboxylase/oxygenase
sc	supercritical (in context with CO ₂)
SD	standard deviation
SEM	standard error mean
SRP	sulphate-reducing prokaryotes
SRR	sulphate reduction rate
TCC	total cell counts
TDS	total dissolved solids (salinity in g L ⁻¹)
TMA	trimethylamine
T _n	Tag _n (n=days of incubation)
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
v/v	volume per volume

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1. Introduction

1.1. The global carbon cycle and involved microbial processes

The global carbon cycle model describes the net exchanges between the main reservoirs of carbon on earth – the lithosphere, hydrosphere, atmosphere, and biosphere (Figure 1). The cycling of carbon is mediated by a network of geological, geochemical and biological processes, like volcanic activity, erosion, weathering, the dissolution/buffering of carbon in the oceans, and biological fixation and mineralisation. By far the biggest carbon reservoir is the lithosphere containing $75 \cdot 10^6$ Gt as sedimentary carbon (carbonate mineral species; $>60 \cdot 10^6$ Gt) and kerogen i.e., deposits of organic matter, which is maturing and thus supplying fossil fuels ($\sim 10\%$ of the global carbon content) [36]. The ocean's hydrosphere comprises the second largest pool of carbon ($38 \cdot 10^3$ Gt), most actively exchanging with the other three compartments. The lithosphere adds carbon mainly as carbon dioxide (CO_2) into the atmosphere via carbonate weathering and volcanic activity [36]. However, the concentration of CO_2 in the atmosphere (735 Gt carbon) is controlled by the dissolution into the ocean's hydrosphere following:

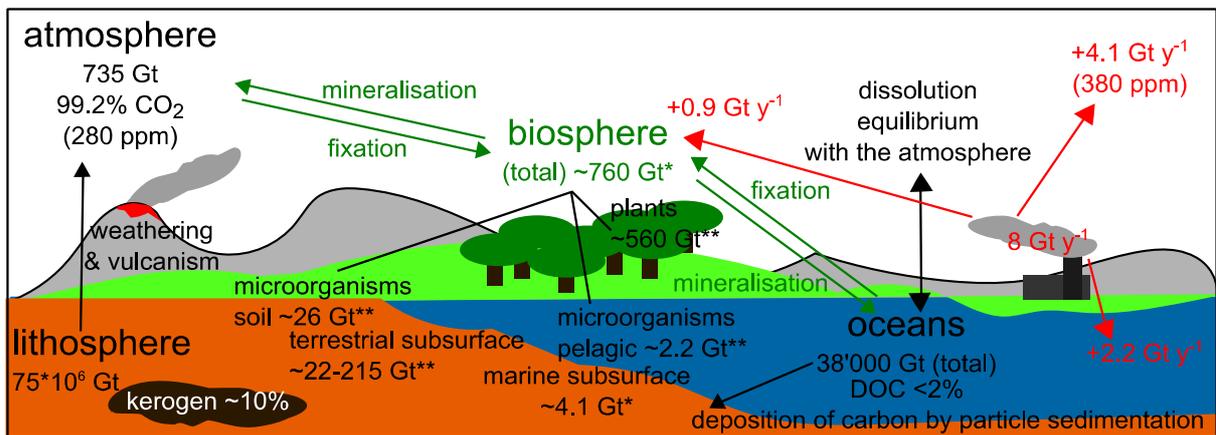
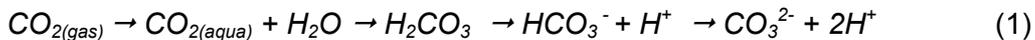


Figure 1: Global carbon cycle with carbon contents of the main reservoirs (following Falkowski *et al.* [36]) and input of anthropogenic CO_2 emissions (red arrows with input rates as Gt year^{-1}). The additional emissions are accumulating mainly in the atmosphere and the oceans (as dissolved CO_2) with a significant proportion remaining in an unknown sink [36, 126]. The carbon content of the biosphere was collected from various references including the recent estimations for the marine subsurface biosphere after *Kallmeyer *et al.* [86] and estimations of the terrestrial biosphere after **Whitmann *et al.* [172]. Biologically mediated processes were indicated with green arrows

The biosphere accumulates organic carbon via the photosynthetic and chemolithotrophic fixation of gaseous/dissolved CO₂ from the atmosphere or hydrosphere. The organic carbon biologically fixed (primary production) is mineralised back to CO₂ and equilibrates with the atmosphere or the dissolved carbon pool of the hydrosphere [36, 150]. The mineralisation of the organic matter is mainly mediated by microorganisms (see below). In the hydrosphere (including freshwater and the ocean) the sedimentation of particulate organic matter comprises a central mechanism for the storage of carbon (carbon sink). In the oceans' hydrosphere, the carbon is transported into deep ocean layers and deposited in marine sediments, effectively storing the carbon for thousands of years [165]. The exchange of carbon from the terrestrial ecosystems with the atmosphere is considered to proceed much faster over decades. The total amount of carbon stored in the (living and dead) biomass of plants comprises the biggest carbon sink within the terrestrial biosphere [36].

1.1.1. Microbial carbon fixation and (re-)mineralisation pathways

The microbial biosphere is actively fixing inorganic carbon (like CO₂) into organic carbon (autotrophy) via several carbon fixation pathways. The most abundant is the Calvin-Benson-Bassham cycle (Calvin cycle) including the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) [105]. This key enzyme is present in *Cyanobacteria*, phototrophic *Proteobacteria*, and is ubiquitously found in the chloroplast-endosymbionts of algae and plants. The enzyme catalyses the first and rate limiting step of the fixation of CO₂ with the carboxylation of ribulose-1,5-biphosphate [7].

Besides the Calvin cycle, five further CO₂ fixation pathways have been documented, some of these restricted to single phyla or metabolic groups: (i) the reductive citric acid cycle (Aron-Buchanan cycle) in green-sulphur bacteria and some anaerobic/microaerobic bacteria [77, 153], (ii) the reductive acetyl CoA (Wood-Ljungdahl) pathway in acetogenic prokaryotes [26], (iii) the 3-hydroxypropionate (Fuchs-Holo) bi-cycle in anoxygenic phototrophic *Chloroflexi* [65], and iv-v) two cycles recently identified for *Crenarchaeota* (*Thaumarchaeota*) together referred to as 4-hydroxybutyrate cycles [11, 70]. The latter

two differ mainly in the activation of the intermediate succinyl-CoA, which is further converted to acetyl-CoA, and in the oxygen sensitivity of the respective enzymes. This oxygen sensitivity restricts their distribution to either (micro-) aerobic *Sulfolobales* [11] or anaerobic *Desulfurococcales* and *Thermoproteales* [70].

The aerobic oxidation of organic carbon, that is, respiration processes using oxygen as final electron acceptor, is conducted by aerobic and facultative aerobic prokaryotes and by eukaryotes, including higher plants and organisms [105]. The anaerobic mineralisation using alternative electron acceptors (like sulphate, metals, nitrate etc.) or fermentation is restricted to prokaryotes and yeast [105]. In the upper aerated soil layers most of the fixed organic carbon is aerobically oxidised (root respiration and microbially mediated mineralisation) but the full extent depends strongly on climate zones, diurnal and seasonal effects [143]. Water saturated habitats with limited oxygen input comprise a terrestrial sink of organic carbon, as the organic matter is mineralised more slowly under anaerobic conditions. In peat lands/bogs, freshwater sediments, rice fields and permafrost soils the organic matter is oxidised by a sequence of anaerobic fermentation, acetogenesis, and methanogenesis [18]. The emitted methane was partially oxidised back to CO₂ at the aerobic/anaerobic interface by aerobic methanotrophic bacteria [94]. In the anoxic compartments of freshwater habitats the methane was oxidised by a syntrophic consortia of anaerobic methanotrophic archaea and sulphate-reducing or nitrifying bacteria [24, 142].

In marine environments the remineralisation of the carbon primary production is mainly proceeding via the microbial loop [5]. Roughly 80% of the fixed carbon is oxidised to CO₂ (released as HCO₃²⁻) by microorganisms within the upper 100 to 200 m of the water column. Depending on the oceans primary production in the photic zone, between 5 to 25% of the primary fixed organic matter can be transported into the deeper ocean layers (biological carbon pump) and 1 to 3% of the carbon finally reaches the sediments [36, 141]. In the coastal shelf regions, where the carbon input is rather high, the organic matter is mainly oxidised by sulphate-reducing prokaryotes in syntrophy with fermenting

microorganisms [83]. In marine sediments, where other alternative electron acceptors are depleted, the organic matter is finally mineralised by methanogenic archaea [66].

1.2. Anthropogenic CO₂ emissions and global climate changes

During the last ~150 years (post-industrial time) the atmospheric CO₂ concentration increased from its pre-industrial concentration (280 ppm) to about 380 ppm by 2006 (Figure 1) [74]. This increase was directly connected to the anthropogenic usage of fossil hydrocarbon resources [36, 61, 74]. Simultaneously, the global average temperature increased by about +0.8°C, which – according to the International Panel on Climate Change (IPCC) – is “very likely” [74] correlated with the increased anthropogenic CO₂ input into the atmosphere.

The additional anthropogenic input of carbon and the warming potential of CO₂ in the atmosphere effectively change the functions of the global carbon cycle. The annual additional input of CO₂ is only to about 50% accumulating within the atmosphere, while about 40% are dissolved in the ocean's hydrosphere. A significant proportion of CO₂ does remain in an unknown sink complicating our estimates of the total mass balance of the carbon cycle [36, 126]. The additional input of CO₂ into the oceans decreases the pH, as result of the dissolution process according to equation (1). Already today the ocean's proton concentration has increased by 30% compared to pre-industrial values. The ocean acidification reduced the ocean's primary production in the photic zones due to decreasing calcification rates of marine plankton e.g. coccolithophore algae [179]. The changed primary production will further affect the transport and storage mechanism of carbon (biologic carbon pump) in the deep ocean layers and sediments. The raising global temperature will further reduce the uptake of CO₂ from the atmosphere and the primary production due to the lesser solubility of CO₂ in warmer surface waters [56]. For terrestrial habitats, the increasing temperature is expected to amplify the carbon mineralisation rate of the organic matter stored within the boreal forests, increasing the net-release of CO₂ into the atmosphere [80]. Thereby, the central storage mechanisms for carbon in the terrestrial ecosystem will be less effective under increasing global temperatures.

Following, the IPCC's best estimate, about 800 ppm in the atmosphere will increase the global average temperature by 3°C [74], which will severely imbalance the global climate system and the carbon fluxes between the earth's carbon reservoirs. To prevent a temperature increase above 2°C, the global emissions of all potential greenhouse gasses (GHGs), e.g. CO₂, CH₄, N₂O (NO_x) and SO₂, must be massively reduced by 2020. Based upon the Kyoto protocol the European Union declared a net reduction by 20% until 2020 compared to the emissions of 1990 (by 30% if other developed countries commit to comparable cuts) [128]. The German government declared the ambitious aim to reduce the GHGs emissions even by 80-95% until 2050 [34].

1.2.1. Options for the reduction of CO₂ emissions for Germany

About 83.5% of Germany's GHG emissions are connected to the production and consumption of energy, including electricity production, transportation, industrial production process, and heat production (Figure 2). In total, about 77% of this energy is produced by fossil fuel combustion, like coal-fired power plants [33].

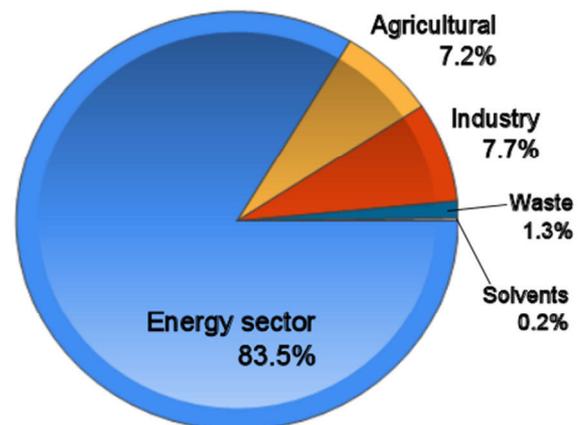


Figure 2: Relative emissions of GHGs subdivided into the producing sectors for Germany in 2010 (in %; obtained from European Environment Agency [35]).

Thereby, the reduction of CO₂ emissions is centrally influenced by two factors: the reduction of our energy demand and the resources or technique used for energy production. The development and establishment of "renewable energy" techniques (e.g. solar or wind energy) as main energy resource will presumably need several decades. Furthermore, the utilisation of fossil fuels as energy resource is expected to buffer variations in the energy demand and production by renewable resources also beyond

2050 [75]. However, the emission of CO₂ from the energy producing sector has to be effectively reduced to achieve Germany's ambitious climate policy aims in the future.

One option currently discussed to reduce the industrial emissions of CO₂ is Carbon Capture and Storage (CCS), which is basically the separation of CO₂ at its emission source and its later storage in appropriate reservoirs [61]. The high CO₂ emissions from the energy sector promote the installation of CCS directly at point emission sources, like coal-fired power plants. The separation of the CO₂ will be integrated into the electricity production chain followed by the transport of CO₂ to the later storage sites [61]. At the moment the storage of CO₂ in deep geological formations, like saline aquifers and depleted gas and oil reservoirs, is considered most suitable for Germany [73, 112]. The qualification of a deep geological formation for CCS is rated according to its overlying geological structure (sealing capacity), its depth (below 800 m depth), and the storage capacity (available pore space) [73]. The documented and evaluated potential CCS reservoirs in Germany will provide a storage volume for about 10 Gt of CO₂. With an estimated emission of 3 Gt in 40 years, this would equal the industrial CO₂ emissions of Germany for several decades [93].

The highest storage potential is seen in saline aquifers which fulfil the site selection criteria in depth and sealing capacity. However, the real extent of these potential reservoirs in Germany is unknown as so far only a small proportion of the saline aquifers have been catalogued. The estimates of the storage potential therefore are ranging between 6.3 to 12 Gt CO₂ [93]. Depleted hydrocarbon reservoirs provide a storage capacity of about 2.7 Gt CO₂ [112]. Despite the lower storage capacity, hydrocarbon reservoirs have some advantages like:

- i) An existing technical infrastructure.
- ii) A good knowledge of the provided pore space (storage capacity) deduced from the volume of produced oil/gas.
- iii) The existence of considerable sealing capacity, since the hydrocarbons accumulated over geological time scales (stratigraphic or structural hydrocarbon traps).

- iv) Enhanced oil/gas recovery (EO/GR) comprises an already well-established application [13], for the (re-)injection of co-produced CO₂ into oil and gas bearing reservoir formations to enhance its productivity (e.g. Weyburn Project [32]).

These factors will considerably promote the development of CCS for the storage in depleted hydrocarbon reservoirs [112]. Thereby, the application of CCS on industrial scales will presumably start earlier in depleted oil and natural gas reservoirs than in saline aquifers (IPCC roadmap [73]).

1.3. Geochemical effects of CO₂ injection into a storage reservoir

The injection of CO₂ into a depleted hydrocarbon reservoir will lead to considerable geochemical and physical changes of the reservoir system, which will affect the storage capacity and long-term safety. A storage depth of more than 800 m below the surface will secure pressure and temperature conditions needed to inject CO₂ in its supercritical (sc) aggregate state (Figure 3a). Supercritical CO₂ is volatile and diffusive like its gaseous state, but relatively incompressible like fluidal CO₂ and is an excellent solvent for non- to moderately polar organic molecules [118]. The injected CO₂ will have a density of >600 g m⁻³ under the storage conditions below 800 m depth. Thereby, scCO₂ will require a smaller pore volume of the storage horizon as gaseous or liquid CO₂. In consequence, the final storage capacity will increase by two orders of magnitude using scCO₂ (Figure 3b).

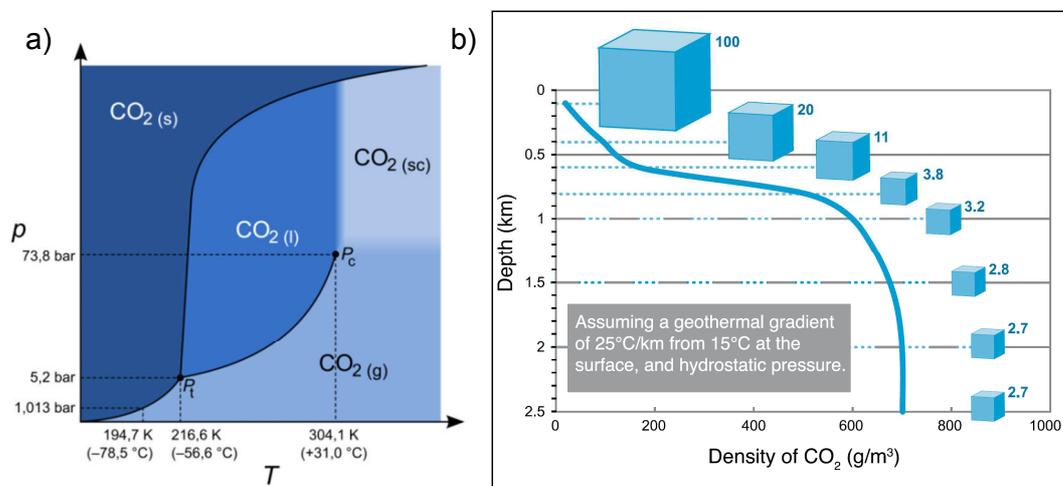


Figure 3: a) Phase diagram of CO₂ (indicating the temperature and pressure of the supercritical point; P_c) and b) depth dependent density and volumetric effect of CO₂ in feasible storage reservoirs [73].

The injected scCO₂ will accumulate at the top boundaries of the storage horizon due to its lesser density compared to the reservoir fluids. Therefore, the overlying geological structure (cap rock) has to be highly impermeable to secure the stratigraphic or structural trapping of CO₂ [73, 110]. Formation fluids and hydrocarbons, present in the pore space prior to the CO₂ injection, will be displaced downwards increasing the pressure in the formation. In case of an overestimation of the maximum injection rate the pressure increase could induce fissures and fractures in the storage horizon or even in the overlying cap rock [154]. Due to capillary forces CO₂ will also be retained as small bubbles (residual trapping) in the pore space or as a thin film of supercritical fluid on the mineral surface. This residually trapped scCO₂ will increase the reactive surface area of the scCO₂ in contact with the formation fluids [110].

Simultaneously with the injection and the migration of the scCO₂ in the reservoir, the CO₂ will dissolve into the fluid (solubility trapping; according to equation [1] shown in 1.1; CO₂ is present as single supercritical phase at the beginning of the injection). The total amount of CO₂ soluble in the formation fluid will depend on the pressure, temperature, and the ionic strength of the formation fluid. Generally, the higher the salinity of the formation fluid is, the lower the saturation point for CO₂ will be [29].

Due to the dissolution of CO₂ into the formation fluids the pH will decrease following the increasing proton concentration. The acidification will influence the saturation equilibrium of dissolved ions leading to the dissolution of the mineral matrix [89, 110]. These reactions will consume protons and thereby vice versa increase the alkalinity. The final buffering capacity of the formation fluids, that is, the proton consuming mineral dissolution processes, depends mainly on the mineral composition of the storage horizon [110]. For example, the injection and dissolution of scCO₂ was shown to increase the concentrations of Ca²⁺, Mn²⁺ and Fe²⁺ in formation fluid systems (e.g. Fri-I formation CCS pilot project) following the dissolution of minerals, like calcite and iron/manganese-oxyhydroxides [89]. Supercritical CO₂ was shown to mobilise also acetate and other organic compounds from a sandstone matrix [89, 149]. It is expected, that the dissolution of the mineral matrix will

further enhance the mobilisation of organic material and heavy metals into the formation fluid [178].

In well-buffered fluid systems with a suitable mineral matrix to consume the protons, the alkalinity will increase up to the oversaturation with CO_3^{2-} . In the presence of divalent ions the CO_3^{2-} will precipitate as carbonate (e.g. CaCO_3 calcium carbonate (calcite)) [110]. Abiotic mineralisation processes are seen as the major long-term trapping factor that will secure a safe detention of the injected CO_2 in the storage reservoir [110, 111]. Both processes, solubility and mineral trapping, will presumably proceed for thousands of years, but at least as long as the trapped scCO_2 will recharge the pool of dissolved CO_2 species [110] according to equation (1).

1.4. Microbial populations in potential storage reservoirs

Hydrocarbon reservoirs represent hotspots within the terrestrial and marine deep biosphere with sometimes elevated population sizes and mineralisation activities compared with soil or sediment subsurface systems [19, 63, 82, 107]. At the moment hydrocarbon reservoirs are representing the best situated option for CO_2 storage, and the following chapter will summarise some microbiological aspects of these geological systems.

1.4.1. The environmental conditions in reservoir ecosystems

The microbial populations in hydrocarbon reservoirs are controlled by the reservoir temperature, salinity (osmotic strength), abundance of essential inorganic nutrients, and appropriate energy resources [102]. The temperature is generally seen as the limiting factor for the presence of living microorganisms, while the other factors are controlling the size and activity of the population [63, 107]. Despite the documented growth at 113°C of the cultured *Pyrolobus fumarii* [12], microbial hydrocarbon degradation is restricted to reservoir systems with *in situ* temperatures below $80\text{-}90^\circ\text{C}$ [173].

The hydrocarbons stored in the reservoir provide ample organic substrates and electron donors for the microbial population. Therefore, the depletion of inorganic

nutrients, mainly phosphorus and nitrogen, and the abundance of electron acceptors are considered as mineralisation rate limiting factors [63, 102, 169]. Suitable electron acceptors besides carbon dioxide are Fe^{3+} , Mn^{4+} and SO_4^{2-} , while oxygen is typically absent [107]. The alternative electron acceptors are provided from embedded or overlying marine evaporites, the mineral matrix (e.g. Fe^{3+} containing siderite), coal, and shale layers. Nitrate and nitrite are generally only present in low amounts [112].

The electron donors in natural gas fields are basically limited to saturated alkanes (C1 to C5), while organic acids (mainly acetate) can be supplied by associated coal and shale-layers [103]. In contrast, oil bearing hydrocarbon formations contain a broad spectrum of hydrocarbons for example, cyclic aromatics, saturated/non-saturated alkanes, naphthalenes and asphaltenes (with >100 carbon atoms per molecule). A considerable proportion of the organic matter in oil reservoirs is not or only badly soluble in the formation fluids. Therefore, the microbial activities are predominantly found at the oil/fluid interfaces [63].

1.4.2. *Microbial community composition and activities in reservoirs*

The limited availability of potential electron acceptors restricts the microbially mediated degradation processes in reservoirs to fermentation, methanogenesis, acetogenesis, reduction of metals, and sulphate [107]. The microbial community is often differentiated into distinct microbial populations for high- and low-temperature environments [20, 72, 99, 134]. The biological degradation of hydrocarbons within the reservoir is well documented [63], e.g. the microbial souring (HS^- production) in oil reservoirs leading to heavier and less valuable oil [47]. Sulphate-reducing bacteria and methanogenic archaea have been cultivated from a variety of oil fields [121, 131, 132, 134, 160] degrading also complex hydrocarbons [1, 22]. Furthermore, thermophilic spore-forming sulphate reducers degrading hydrocarbons were stimulated in arctic marine sediments indicating the input of these organisms from hydrocarbon reservoirs [71, 92]. However, the microbial populations in hydrocarbon reservoirs are presumably acting in complex syntrophic networks specialised on the degradation of hydrocarbons [107, 120] as visualised in Figure 4.

The central mechanism for the degradation of complex organic material like hydrocarbon mixtures is the stepwise hydrolysis into smaller organic compounds resulting in a pool of reduced carbon species (short chain alkanes, fatty acids etc.). Acetate comprises a central intermediate (Figure 4) for the final mineralisation of the organic matter. This final step is generally processed either by sulphate-reducing prokaryotes or methanogenic archaea [107, 120]. The acetate can be the product of direct hydrolysis of the complex organic matter, or secondary generated from the reduced carbon pool. Besides, also acetogenic bacteria produce acetate from accumulated CO_2 and hydrogen to generate acetate using reductive acetyl CoA (Wood-Ljungdahl) pathway (Figure 4).

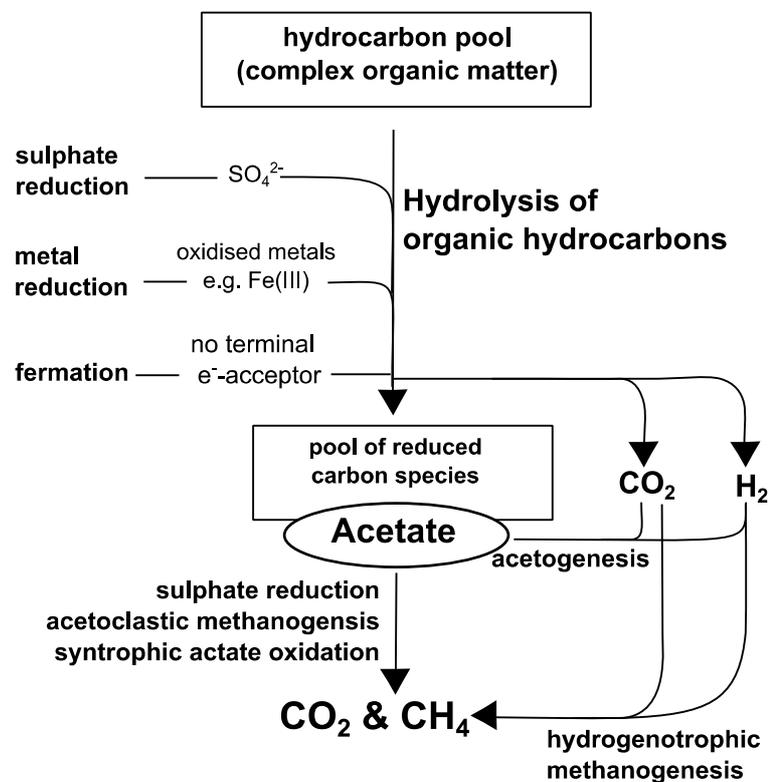


Figure 4: Scheme of hydrocarbon or complex organic matter degradation in reservoirs [105, 107, 120]. The stepwise hydrolysis of the organic matter is leading to a pool of reduced carbon species, CO_2 and hydrogen which are further and finally mineralised to the end products CO_2 and CH_4 . Intermediately produced H_2 and CO_2 can be used to reductively produce acetate via the Wood-Ljungdahl pathway (acetogenesis).

Depending on the abundance of oxidised metals and sulphate, complex hydrocarbons are likely degraded by heterotrophic metal-reducing, sulphate-reducing or fermentative organisms. Bacterial sulphate reducers in hydrocarbon reservoirs were identified as

Deltaproteobacteria, e.g. *Desulfovibrionales* and *Synthrophobacterales* [22, 159], and of the *Firmicutes* from the *Clostridiales* order (e.g. thermophilic *Desulfotomaculum* spp.) [121, 160]. The deeply branching bacterial phylum *Thermodesulfobacteria* is the third sulphate-reducing bacterial taxon detected in hydrocarbon environments [17]. Putative sulphate-reducing prokaryotes seem to be ubiquitously distributed in hydrocarbon reservoirs. Several heterotrophic sulphate-reducing bacteria were documented to reduce besides sulphate also various metals or to grow by fermentation [120]. Furthermore, metal-reducing bacteria that do not reduce sulphate, were identified from the deltaproteobacterial *Desulfuromonadales* (*Geobacteraceae*), and from the thermophilic phylum *Deferrribacteres* [54, 55]. Facultatively anaerobic bacteria, like *Shewanella* and *Pseudomonas* spp., were also repeatedly described for reservoirs [134, 176].

While the complete dissimilatory reduction from sulphate (6+) to sulphide (2-) is mainly restricted to the above mentioned taxa, reduction of more reduced sulphur compounds, like sulphur, thiosulphate or sulphide, is far more distributed. Several heterotrophic fermenters are also capable of the respiration of sulphur compounds. For example, *Geotoga* and *Petrotoga* spp., which are so far exclusively detected in hydrocarbon reservoirs [176], were documented to ferment alcohols and sugar compounds, but also to respire sulphur and thiosulphate [21, 114].

Methanogens are metabolically restricted to the final mineralisation steps due to their low substrate versatility. The major methanogenic phyla comprised *Methanococcales*, *Methanomicrobiales* and *Methanobacteriales* [20, 82, 134]. In low-temperature hydrocarbon reservoirs the generally mesophilic *Methanosarcinales* (*Methanohalobium* and *Methanosarcina*) were repeatedly detected [e.g. 50, 116].

Under thermophilic conditions syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis is thermodynamically more favourable than acetoclastic methanogenesis [82]. For high temperature hydrocarbon reservoirs an enrichment of a hydrogenotrophic methanogen (*Methanoculculus*) and a putative acetate oxidising organism (*Smithella*) was documented [52]. In low temperature environments, both hydrogenotrophic and acetoclastic methanogens were documented [28, 50], leaving a gap

in the knowledge about the limiting factor for the distribution of these organisms. Besides the methanogenic community, several hyperthermophilic sulphate reducers were identified in the genus *Archaeoglobus*. So far several organisms from the family of *Archaeoglobaceae* have been cultivated from high-temperature oil fields that grow with hydrogen/CO₂, acetate and lactate [49, 134]. *Crenarchaeota* were detected by non-cultivation approaches (16S *rRNA* based techniques), but identified as possible mesophilic contaminants in hydrocarbon reservoirs with seawater injection [99].

1.4.3. Inhibitory effects of supercritical CO₂

The bactericidal effect of scCO₂ on bacteria is mainly documented for food borne pathogens. Supercritical CO₂ was applied as disinfectant for food processing machines and medical instruments/material [27, 87]. Food with heat sensitive flavour, nutrients, texture, or colouring can be treated under relatively low temperatures due to the low supercritical point of CO₂ (see also Figure 3). The survival during treatments with highly pressurised CO₂ was studied on pure cultures from typical food-borne pathogens, including *Escherichia coli* (*Gammaproteobacteria*), *Bacillus subtilis* (*Bacilli*, spores), *Salmonella typhimurium* (*Gammaproteobacteria*), and *Listeria monocytogenes* (*Bacilli*, no spores) [44 \reference therein]. Garcia-Gonzales *et al.* [44] reviewed the industrial applications and studies on the biochemical effects of CO₂ on pathogens or spoilage bacteria summarising in seven steps (a-g in Figure 5) the mechanisms for the inactivation of the microorganisms.

The physiological inactivation (Figure 5) starts with a shift of the external pH_{ex}. This will affect the pH homeostasis and increase the maintenance energy costs (a in Figure 5). Interestingly, the negative effect of the external acidification was higher under CO₂-acidified conditions than for a decreased pH without CO₂ [60]. This amplification due to the CO₂ was correlated to the diffusion of aqueous (undissolved) CO₂ and HCO₃⁻ ions into the membrane double layer disturbing its integrity (b in Figure 5). Following the diffusion into the cytoplasm, the internal pH_{in} will decrease (c in Figure 5) which will affect several intracellular mechanisms (d-f), like enzyme activity and structure [3], the physiologically

important electrolyte balance, and the kinetics of carboxylation /decarboxylation reactions. The intracellular CO_2 increase will be enhanced due to the changes in the membrane integrity which will furthermore affect the loss of vital compounds (g in Figure 5), or even lead to the deformation of cells [69].

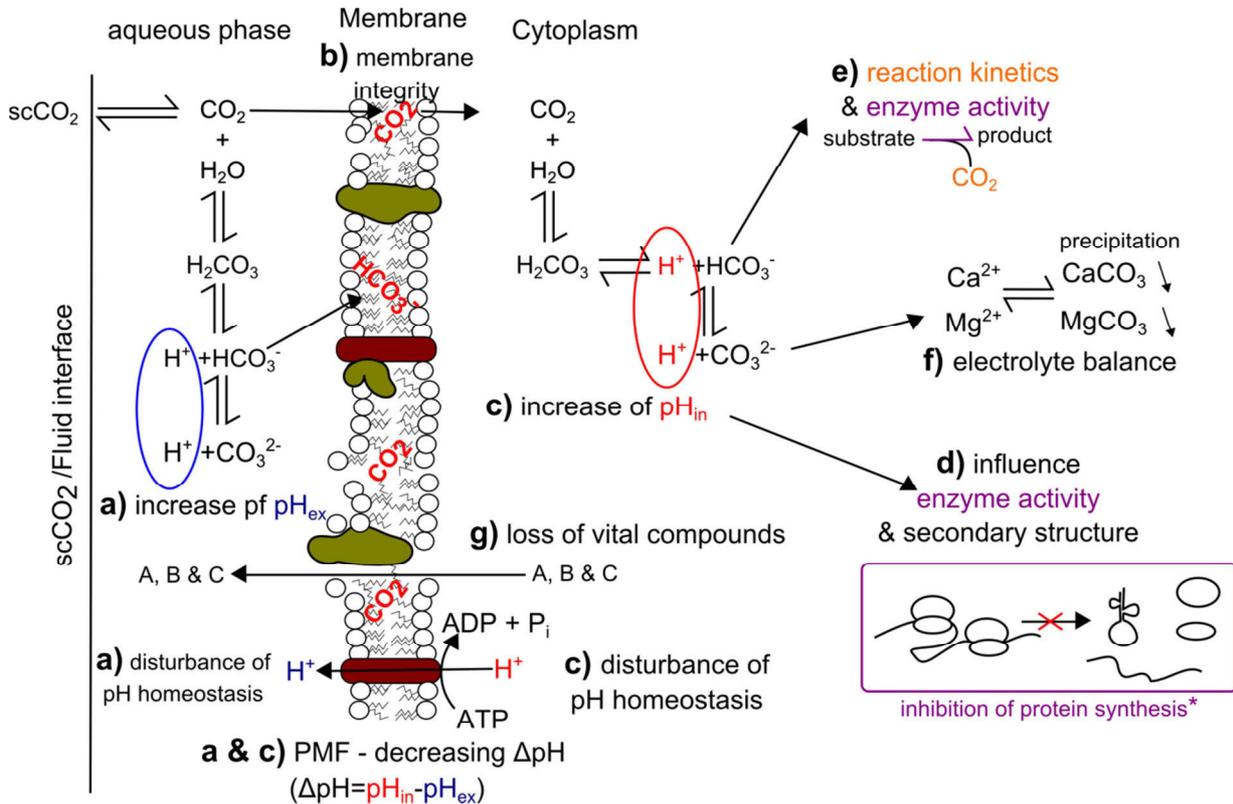


Figure 5: The proposed physiological negative effects of scCO_2 acting in seven steps (a-g) on microorganisms according to Garcia *et al.* [44], including the acidification of external and internal pH, the disturbance of pH homeostasis and proton-motive force (PMF), disturbance of membrane integrity, enzyme inhibition (indicating the proposed effect on the ribosome assembly of Andras *et al.* [3]) and reaction kinetics, action on electrolyte balance and the loss of vital compounds.

Already, lower partial pressures of CO_2 (in gaseous to liquid state) lead to a considerable decrease of the bacterial contamination in the sterilisation goods. However, only very high CO_2 concentrations in aqueous media, above the supercritical point of CO_2 , lead to an effective sterilisation [79]. Furthermore, the inactivation of spores from food spoilage bacteria (e.g. *Clostridium botulinum*) was effective in a combined treatment of scCO_2 and heat for about 30 min and more effective in aqueous solutions than on dry spores [135]. The direct contact with supercritical CO_2 fluid will supposedly lead to the

extraction of non-polar to moderately polar compounds (lipids) from the cells or from extracellular polymeric substances (EPS) of biofilms as described in chapter 1.3.

1.4.4. Biologically controlled mineral precipitation under CCS conditions

Besides the evidence for negative physiological effects of scCO₂, our knowledge about the microbial potential to survive under the actual *in situ* CCS conditions within a storage reservoir is rather scarce. The geochemical gradient of dissolved CO₂ [46, 90] will lead to a zone with a severely affected microbial population close to the injection point. Within this zone of CO₂ saturated formation fluids the microbial population will probably die due to the effects described above. In greater distance to this point the concentration and likewise the negative influence of the CO₂ will gradually decrease and more cells might survive. For such zones with lower CO₂ concentrations, a biologically controlled storage mechanism increasing the capacity and/or storage safety was postulated [115]. However, the tolerable threshold concentration of CO₂ for the indigenous microbial population, mediating such an additional storage mechanism, has not been evaluated so far.

Important for the survival potential of the reservoir microbiome will be the presence of substrates and nutrients for the microorganisms, since the antimicrobial effects will lead to increased maintenance energy demands. Kirk *et al.* [91] calculated the thermodynamic constraints acting on iron(III)-reduction, sulphate reduction, and methanogenesis under reservoir conditions with CCS application. The free energy available (ΔG_A) was positively affected for iron(III)-reducing reactions with different minerals (e.g. goethite) as Fe³⁺ source, while methanogenesis and sulphate reduction were not influenced.

In theory, all reactions consuming protons, decreasing “acidic” substrates (sulphate, CO₂), and/or increasing the alkalinity [10, 163] will influence the storage capacity. These microbially mediated changes in the geochemistry of formation fluids are envisioned to enhance the dissolution trapping and maybe even the mineral trapping of CO₂ [115]. However, so far it remains unclear whether microorganisms will either actively affect the dissolution equilibrium of CO₂ (i.e., bio-mineralisation of CO₃²⁺ into carbonate

precipitates), or simply act as nucleation sites enhancing the abiotic mineralisation mechanism of the CO₂ [109].

Very important for the survival potential of microorganisms will be their vital state. A significant proportion of deep subsurface microbiome is presumably present as spores or inactive starving cells [101]. The abundance of *Firmicutes* in hydrocarbon reservoirs [52, 121, 134] and the documented CO₂ resilience of spores will presumably represent important factors for the survival of the microbial population under reservoir conditions following the injection of scCO₂.

1.5. Potential environmental risks of CCS for soil surface ecosystems

For the injection of CO₂ in deep geological formations also the risks and likeliness of possible leakages have to be assessed. At CCS/EOR pilot sites like Weyburn, Frio-I or Sleipner, the subsurface movements of scCO₂ are intensely monitored. The seismic measurements are used to evaluate the migration pathways of CO₂ within the storage horizon and to detect possible leakages [4, 23]. CCS was estimated to be economically and ecologically insufficient with a leakage rate of more than 0.5% of the stored CO₂ per year from the storage reservoir [180]. So far, the mentioned pilot projects have not documented considerable leakages (rates presumably below 0.0001%). Nevertheless, in concurrency with documented leakages of hydrocarbon reservoirs, the possibilities and environmental consequences of a leakage from CCS-storage sites have to be investigated [73].

The risk scenarios expect different pathways for a leakage of CO₂ (Figure 6), including migration via abandoned wells, diffusion through insufficiently sealing cap rock and along existing (or by the pressure increase induced) fractures. Leakages via abandoned wells will likely occur in a relatively short outburst that can proceed as long as the pressure is elevated in the storage reservoir. Such leakages will presumably be easily detectable and sealed [113]. The rather slow diffusion of CO₂ through insufficient cap rock or fractures will not or only after a certain time period be detectable. Therefore, such leakages

compromise a long-term problem depending on the total injection volume and the leakage rate [73, 178].

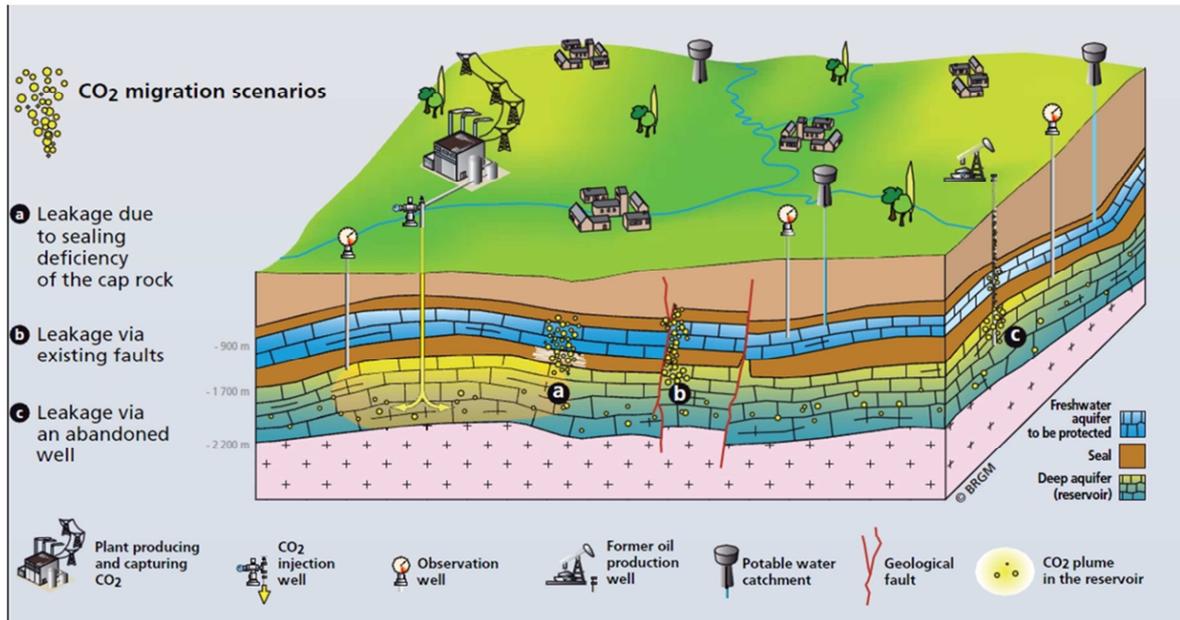


Figure 6: Overview of possible leakage scenarios according the information brochure of the CO₂GeoNet – European Network of Excellence [171].

Insufficient cap rock systems could lead to broad-scale leakage of CO₂ into surface aquifers, thus potentially affecting drinking water reservoirs. The mobilisation of heavy metals (e.g. arsenic) from the mineral matrix of drinking water aquifers could follow the dissolution of leaking CO₂ into the aquifer system [178]. Furthermore, heavy metals, hydrocarbons and saline formation fluids could be co-transported with the leaking CO₂ from the storage reservoir into drinking water resources [113, 178]. Such leakages will reduce the drinking water quality and comprise a severe health risk. The leakage scenario along fractures and faults provides another passage into groundwater resources and also upper soil layers. This scenario is considered to affect the usage and value of surface soils considerably, albeit within a relatively defined area along the geological fracture and fault lineages which provide defined CO₂ leakage pathways [136].

1.6. Natural CO₂ seeps as analogues for CCS surface leakage scenarios

Natural CO₂ seeps are characterised by increased CO₂ concentrations and fluxes in the soil atmosphere or the discharge and following dissolution into groundwater aquifers or

marine bottom waters [130, 166]. The introduction of a similar leakage at a CCS pilot site is neither legally nor ethically considered feasible, as the final consequences might harm humans. Alternatively, the settings of natural CO₂ seeps provide suitable analogues to establish detection and monitoring systems on the effects of a possible leakage of a CCS storage reservoir. The seeps are often evolved in areas with volcanic or seismic activity [136, 166] where the CO₂ migrates from deep geological sources through impermeable layers or along fractures and faults.

1.6.1. *The environmental conditions of surface soils at natural CO₂ seeps*

At terrestrial CO₂ vent systems, the CO₂ reaches the surface soils often in spatially limited areas that are by often visible due to patchy or reduced vegetation [9, 104, 133, 139]. The geochemical conditions in the venting area correlate with the concentration and fluxes of upwards migrating CO₂. The minerals of the affected soils were weathered and reduced in their content of mineral oxides [155]. The buffering capacity of these soils is often reduced as the main buffering minerals (carbonates) are also weathered by the increased influx of CO₂. Therefore, the soil was often shown to be gradually acidified depending on the CO₂ flux rate and concentration compared to the surrounding non-affected pasture or woodland [9, 40, 139]. The aeration of the soil column in the vent area is suppressed due to the flux of upwards migrating CO₂ [9]. Some studies reported an increased water content limiting even more the diffusive exchange of the soil environment with the atmosphere [9, 133].

Plant communities at such sites seem to compensate the geochemical effects up to elevated CO₂ concentrations of 20-30% before plants got dismissed leaving only bare soils [81, 104]. At the Mommouth Mountain volcano (California, USA) the elevated increased CO₂ soil gas concentrations due to volcanic activity had a severe effect on the woods surrounding the volcano [40]. Within two years from the onset of an increased volcanic activity, almost 100% of the conifers died at CO₂ concentrations >30% (between 30-90% measured in 60 cm depth) clearing a total area of 30 ha woodland.

Furthermore, plants growing in or around discharge areas were reported to have reduced photosynthetic pigmentation (showing yellowish to brown leaves) and root respiration rates [138]. But the CO₂ threshold concentrations leading to significant geochemical or physiological changes that eliminate plant growth are unknown. Moreover, these effects are most likely depending on the initial plant coverage, the agricultural utilisation, climate zone [143] and quite importantly on the length of the time-period the CO₂ is present in the soil.

1.6.2. *The influence of CO₂ seeps on the microbial soil community*

The effects of high CO₂ concentrations on the microbial populations are even less understood than for the plant coverage. There are a number of studies concerning elevated atmospheric CO₂ levels [e.g. 84] and their effect on plant and microbial biomass in the rhizosphere. The elevated atmospheric CO₂ concentrations varied between 550 and 800 ppm [25, 84, 174]. Such atmospheric concentrations are expected within the next decades if the CO₂ emissions will not be reduced. These studies showed an increase in photosynthetic activity and net carbon availability for the microbial biosphere that increased the microbial mineralisation activities in the upper soil environment [25, 84, 174]. However, the geochemical conditions in a CO₂ vent, i.e. a possible leakage site, will represent a completely different environment. For a start, the CO₂ concentration within the soil atmosphere of volcanic vent centres reaches nearly 100% CO₂ in the soil atmosphere limiting the oxygen availability [9, 166]. Such high concentrations will result in a loss of plant biomass and reduce the rhizosphere associated microbial populations. Thereby, also the net carbon input from the plants into the soil will be reduced which corresponds to low carbon concentrations previously reported for CO₂ vents [133]. In conclusion, the microbial population structure could be changed by the CO₂ induced soil acidification, oxygen limitation, reduced rhizosphere, and the reduced organic carbon availability.

The decreased aeration can affect the microbial carbon mineralisation activity either by changing microbial metabolisms (aerobic/anaerobic) or simply decreasing microbial activity rates. Such a reduced mineralisation activity was indicated in CO₂ affected soils by

reduced ATP content and bacterial cell counts [170]. In case of the Latera volcanic vent system, a natural CO₂ vent in Italy, the ATP content and bacterial cell counts were also reduced [9, 133]. Another indication for effects on the microbial activity was the distribution of CO₂ fixation genes in CO₂ emitting moffetes (terrestrial volcanic CO₂ seeps). The encoding gene (*cbbL*) and protein variance of RuBisCO allows distinguishing between two major types: green-like *cbbL* type and red-like *cbbL* type [168]. The latter was dominantly present in the CO₂-affected soil samples, indicating a CO₂-fixing community of *Alpha*- and *Betaproteobacteria* which are mainly carrying this gene variance [164]. However, the total abundance of the gene was significantly reduced within the CO₂-affected soil column. The authors concluded that the high CO₂ concentrations inhibited the CO₂ fixing community and noted that especially sites with elevated CO₂ concentrations of about 20% showed a considerable reduction in the gene copy abundance of *cbbL* [164].

Besides CO₂, volcanic seeps often emit further gases, like CH₄ and H₂S. For example, the Latera volcanic system emitted H₂S with the CO₂, and showed a considerable increase of sulphate reduction activity for the vent centre. The soil column of the vent centre was almost anoxic up to shallowest layers due to the high flux rates of CO₂ [9]. Anaerobic processes, like sulphate reduction and methanogenesis are of special importance in the risk scenario of a potential leakage. For example, methane is a very potent greenhouse gas with about 23 times the global warming potential of CO₂ [74], while the toxicity of H₂S provides a severe health risks for humans and animals [146]. However, neither process is regarded as a typical mineralisation activity in the upper soil environment. Anaerobic mineralisation activities in terrestrial soils other than nitrate reduction [45] are generally more documented for water-saturated habitats, like freshwater sediments [24, 147], rice fields [94], and permafrost soils [145]. Nevertheless, an increase in anaerobic mineralisation activities, as postulated for Latera [9, 133], could represent severe risk factors that need to be included into the safety estimations of CCS operations.

1.7. Ecosystems description

1.7.1. The Schneeren natural gas field

The gas field Schneeren-Husum is located close to the Steinhuder Meer about 40 km northwest of Hannover in the North German Basin (Figure 7). The reservoir is built of a large Zechstein salt diapir which is structurally trapping the natural gas within a sandstone formation of the Upper Carboniferous (Figure 8) [67]. The exploration of the hydrocarbons started 1986. Today the field is operated by Gaz de France Suez E&P Deutschland GmbH, with actually about 10 wells in production. The whole reservoir can be separated into two massifs, Husum and Schneeren, with the latter enclosing the investigated well heads Z2 and Z3. The site is used as CCS analogue in a series of BMBF-funded projects since it is meeting the selection criteria for CCS [73]. Furthermore, it produces sufficient amounts of formation fluids together with the gas to allow a broad geochemical and microbial study approach.

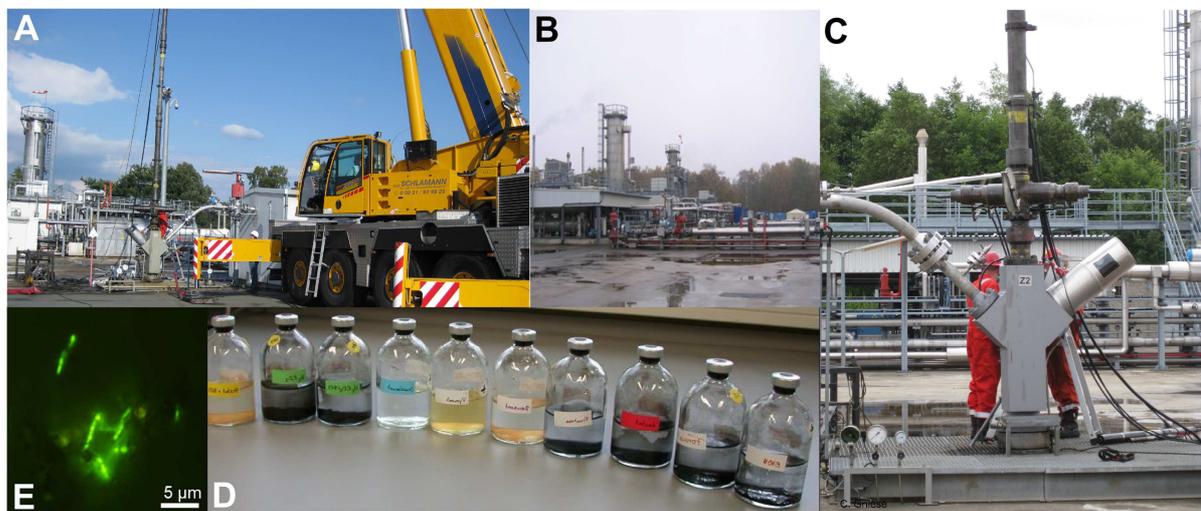


Figure 7: Schneeren-reservoir - A) tower crane for deep reservoir fluid sampling; B) industrial site of the gas facility; C) well head with surface head-valves; D) microcosms setup for activity measurement in production fluids; E) cell assembly in production fluids (may 2010) – scale 5 μm .

The gas and the formation fluids were extracted from a depth of 2000-2800 m with an approximated *in situ* temperature 60-80°C (geothermal gradient of 3°C per 100 meter [73]). The produced gas consisted of about (v/v) 89% methane, 7% nitrogen, 3% CO₂, 0.38% ethane and further gaseous hydrocarbons between 0.01 to 0.005% (personal

communication). The hydrocarbons were formed in the Upper Carboniferous and late Mesozoikum in the source rock by thermal stress of coal layers and are not associated with oil deposits [67]. Coal- and shale-bearing layers are also embedded within the reservoir rock system. These layers presumably also provide the source for ample organic material and ammonium detected in the formation fluids [31, 103]. The formation fluids' salt concentrations reached up to 230 mg L^{-1} [103], but were also found to be moderately saline. The salinity depended on the massif and its sub-compartment in which the production horizon was located. For example the included well heads Z2 and Z3, located within the central part of the Schneeren massif, contain only $30\text{-}60 \text{ mg L}^{-1}$ [31, 103]. Isotopic analysis to evaluate the fluids origin revealed the evaporation of meteoric Paleozoic waters (low salinity) and/or seawater (high salinity) as main source for the formation fluids. The mixing of formation fluids with recent meteoric waters was excluded for the reservoir from the isotopic analysis [54].

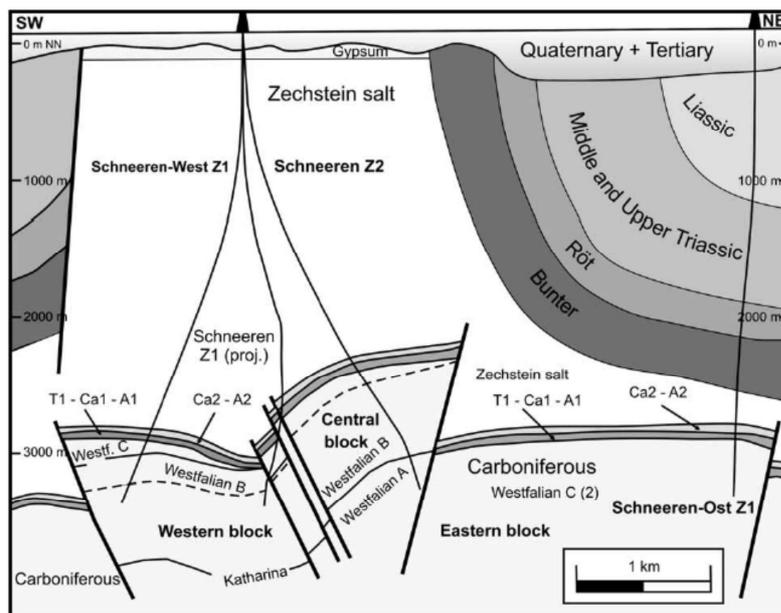


Figure 8: Cross-section through the Schneeren-Husum natural gas field (according to Hollmann *et al.* [67]) indicating the location of Schneeren Z2 within the central massif of the Schneeren reservoir.

In a previous study, the archaeal microbial community of Z2 and Z3 was already closer examined. Ehinger *et al.* [31] described differences within the archaeal community for both well heads. At the beginning of their study, *Methanlobus* sp. was dominant in both formation fluids. While in the course of the study, *Methanoculleus* sp. became predominant in the fluids of Z3. Ehinger *et al.* [31] also briefly described the abundance of several bacterial taxa, like *Marinobacter*, *Desulfotomaculum*, *Petrotoga* spp., albeit the

temporal and spatial (differences between the wells Z2 and Z3) dynamics in the bacterial community were not reported. In the course of this work, the geochemical conditions, microbial activities and community structure of the Schneeren gas reservoir were documented in detail from April 2008 to May 2011 (April 2012 for Z3).

Two different formation fluids were examined from both wells (Figure 7; A and C): the production fluids, referring to formation fluids that are co-transported with the gas and separated at the surface (gas/water-separation system), and unproduced deep reservoir fluids. These deep reservoir fluids were sampled directly at the production horizon in ~2000-2500 m depth, where the formation fluids are effusing from the gas-bearing sandstone layers into the well tubing. For the first time such unproduced deep reservoir samples from this reservoir were microbiologically analysed. The comparison of unproduced deep reservoir and production fluids included analyses of the water chemistry, potential microbial activities, and molecular-biological analyses using quantitative PCR (qPCR), fingerprinting techniques (terminal restriction fragment length polymorphism; T-RFLP) and state of the art bidirectional 454-amplicon sequencing.

1.7.2. The Laacher See natural CO₂ vent system

The Laacher See volcanic centre is situated right in the middle of the East Eifel volcanic field. This field is part of the Rhenish Massif extending further south building the Ardennes in France. The eruption of the Laacher See volcano is the only known large explosive eruption (phreatomagmatic) during late Quaternary times (13.1 ka BP) [127]. Its ashes were transported as far as Gotland (Sweden) and over the Alps and are used as isochrones for geological dating purposes [14].

The morphological centre of the volcano is built by an eruption crater (caldera) that is today filled by a lake – the Laacher See. The lake is with 3.3 km² and a maximum depth of 52 m the biggest caldera lake (morphologically not a maar) in the Eifel region [95, 175]. The lake is surrounded by a 90 to 240 m (above lake level) high ring wall that contained no natural outlet (Figure 9 A). Already in the 12th century the lake's water level was regulated by a tunnel through the southern crater wall lowering the water table by

approximately 10 m [57]. After this structure collapsed, a new tunnel was constructed in the mid-19th century which lowered the lake level by another 5 m. The pastures surrounding the lake at its southern to north-western shore line were obtained due to the man-made drainage of the lake. Nowadays, these pastures are mainly used for extensive cattle farming and sociocultural purposes.

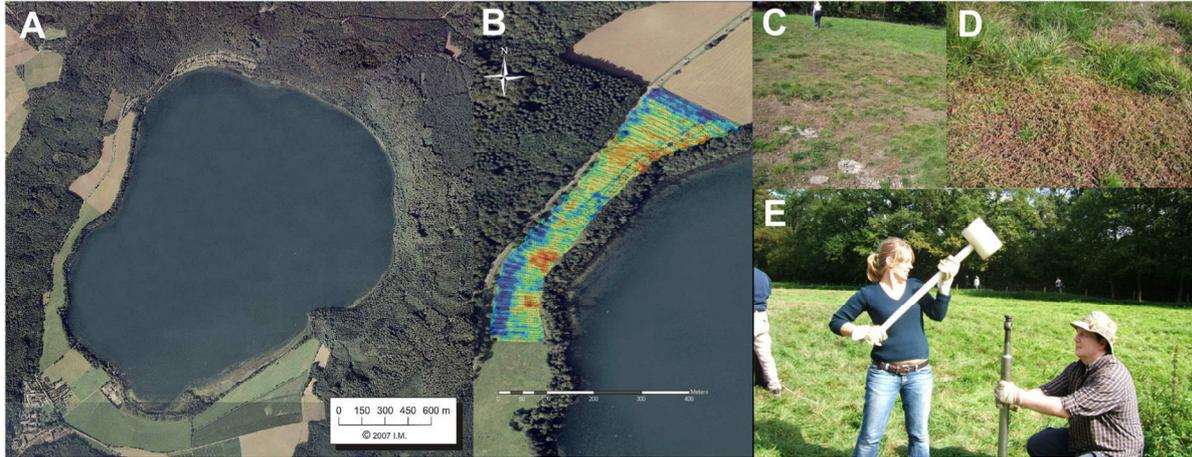


Figure 9: Laacher See ecosystem – A) air photo with B) enlarged section of western shore line indication the increasing soil gas concentration (red high concentration; blue background concentration) according to Krüger et al. [95]; C) reduced vegetation in the vent centre with D) enlarged picture of the dominant plants surrounding the vent centre (plant community analysis included in chapter 3.3.); E) Dr. rer. nat. Birte Oppermann and Dr. rer. nat. Martin Krüger at the sampling session (with friendly permission of both).

Discharge of gaseous CO₂ along the (north-) eastern shore of Laacher See is well documented [2, 6, 161]. The isotopic analysis of CO₂ dissolved in the groundwater and lake body indicated a magmatic origin from the upper mantle hemisphere [48, 58]. The gas consists predominantly of CO₂ (~ 99%) and the fluxes into the Laacher See have been estimated with about 5'000 tons of CO₂ per year [2]. Besides the emission of CO₂ from the water body, several vents were identified along the shore line (Figure 9 B). The distribution of these mofettes indicated a fault depended distribution of the CO₂ seeps [95].

In the frame of an international EU network of excellence the pasture at the western shore line of the Laacher See was investigated [95]. Along the shore line several soil gas anomalies were detected with two new vent centres showing CO₂ concentrations above 80% in the soil atmosphere. One of those vents was subject for the microbiological and geochemical survey including the weather conditions, gas concentrations/fluxes and

geochemical soil profile. In two sampling campaigns, in September 2007 and July 2008, the surface soils at three positions along a transect of increasing CO₂ concentrations was sampled. Samples were taken at the maximum zone of CO₂ fluxes in the centre of the vegetation free zone (vent centre), as well as in a zone with elevated CO₂ concentrations at the outer rim of the venting area (medium site). The reference sample was taken in proximity of the CO₂ vent, in an area where no elevated gas concentration was observed (reference site) [81]. In September 2007 additional soil depth profiles were sampled for the vent centre and the reference site.

1.8. Motivation and main working objectives

The study in both ecosystems was motivated by one major hypothesis: The effects of CO₂ in reservoir formation fluids and soil surface environments will be reflected in changes in the microbial community (activity and/or population structure). The environmental changes will promote organisms which either have a higher resilience potential against the negative effects of CO₂ or an environmental advantage due to the induced changes in their habitat.

The working objectives of this work are defined as:

- i) This work examines the dynamics of the microbial activity rates, metabolic versatility and the microbial community structure (gene copy abundance and diversity) with focus on the bacterial population of the Schneeren gas field in the period from 2008 to 2012 (chapter 3.1. and 3.2.).
- ii) It gives new insights on the origin of the microorganisms living in the formation fluids and examines the influence of the production processes and technical measure on the distribution pattern of microorganisms in the reservoir (chapter 3.1.).
- iii) It evaluates the effect of very high CO₂ concentrations (up to saturation) on the microbial population in high-pressure incubations using original formation fluids and enrichment cultures obtained from the Schneeren formation fluids (Chapter 3.2.).
- iv) It identifies environmental parameters that differentiate a CO₂ affected from an unaffected soil ecosystem to estimate the critical soil gas concentration of CO₂ (thresholds) for the microbial biosphere (Chapter 4.3. and 4.4.).
- v) It examines and identifies the occurrence of putative indicator species for CO₂-induced changes of the soil environment and evaluates the significance of such community changes for possible leakage scenarios. (Chapter 4.4.)

2. General Discussion

This work investigated the effects of high CO₂ concentrations on the microbial population in hydrocarbon reservoirs, representing a potential CCS storage reservoir, and the soil surface layers of natural volcanic CO₂ seeps in relation to the environmental consequences of CCS-leakages. The overall aim of this work was to investigate how CO₂ and its anticipated geochemical effects will influence the structure, activity, and abundance of microorganisms. Both ecosystems represent distinct environments that were investigated for spatial and temporal variations. The following chapters will summarise the results and amend the discussion given in the publications (chapter 3.1 to 3.4).

2.1. Dynamics of the deep biosphere of the Schneeren gas field and consequences of gas-production related technical measures

The bacterial community structure of the Schneeren reservoir showed a generally diverse community including *Gammaproteobacteria*, *Deltaproteobacteria*, *Firmicutes*, *Synergistes* and *Thermotogales*. According to other reports this community structure seems representative for the hydrocarbon-associated deep biosphere in high-temperature reservoirs (temperature >50°C) [20, 49, 51, 72, 134]. In a previous study of the Schneeren reservoir, the archaeal microbial community of Z2 and Z3 was already documented showing a limited diversity of only one potentially methylotrophic *Methanolobus* sp. and another hydrogenotrophic *Methanoculleus* sp. [31]. The detailed microbiological description in this work examines the origin of the bacterial and archaeal organisms in the formation fluid system to evaluate which organisms and microbial processes are actually important under *in situ* conditions in the reservoir. The detailed analysis of the microbial community structure revealed some surprising temporal and spatial dynamics of the Schneeren reservoir biosphere that were correlated with technical measures conducted during the study.

2.1.1. Evidence for a sulphate-reducing community in the Schneeren reservoir and the abundance of methanogenic archaea in the fluids

In the course of this work, the capability of the microbial population to reduce sulphate and to produce methane was tested with various substrate additions. These microcosms were compared to non-stimulated microcosms to evaluate the substrate specific potential to stimulate the microbial activity. Accompanying the microbial activity measurements, the abundance of specific gene markers for sulphate-reducing prokaryotes (subunit A of the dissimilatory sulphide reductase; *dsrA*) and methanogenic archaea (subunit A of the methyl Co-enzyme M reductase; *mcrA*) were monitored in the formation fluids. Both processes are important mineralisation activities for hydrocarbon reservoirs [30, 47, 82] and were previously briefly described in the Schneeren formation fluids [31].

The production fluids of both wells showed strongly induced sulphate reduction activity with various substrates, including hydrogen, low molecular weight organic acids, monosaccharides, and alcohols. For example, the sulphate reduction activity was hundred fold higher with hydrogen amendment in comparison to non-stimulated microcosms. The potential to metabolise different carbon species indicated the presence of a diverse community of sulphate utilising species. Furthermore, sulphate was reduced under high temperature conditions close to the *in situ* values (up to 80°C) in production and deep reservoir fluids.

The quantitative molecular-biological community analyses (qPCR) supported the presence of an active sulphate-reducing community in the Schneeren reservoir. The abundance of specific gene copy numbers of sulphate-reducing prokaryotes correlated positively with the sulphate reduction rates, as both increased simultaneously in the course of this work. Likewise, also the abundance of the gene marker specific for methanogenic archaea was correlated with the microbial methane production activity. At the beginning of this work (2008), the methane production rates and *mcrA* abundance were rather high compared to other gas field formation fluids [116]. The methanogenic activity was strongly stimulated by the addition of methanol indicating the activity of the

methylotrophic *Methanlobus* sp. However, after two years the methanogenic activity was almost completely dismissed and the *mcrA* gene abundance strongly decreased. Interestingly, the methanogenic activity was significantly reduced at temperatures above 40°C in incubations with production and deep reservoir fluids.

Magot *et al.* [107] summarised two central paradigms to distinguish between organisms that were enriched during the production process (in the well head facilities) and those indigenous in the reservoir formation. Indigenous organisms detected in the production fluids should either be capable to withstand (or be active) under the *in situ* temperatures, and/or should be globally distributed in hydrocarbon reservoirs under similar conditions (high- or low-temperature reservoirs)[107]. As stated initially, the global distribution of the predominantly detected bacterial taxa showed their presence within high temperature reservoirs. For the previously documented archaeal community, Ehinger *et al.* [31] correlated the presence of these taxa also with other community descriptions from high-temperature environments [20, 116].

However, in contrast to Ehinger *et al.* [31] this work gave evidence for an enrichment of the methanogenic archaeal lineages during the production as no methanogenesis was detected above 40°C. The gradually decreasing temperature from about 60-80°C in the reservoir to 36-42°C in the separation system at the well head (at surface level) likely affected the distribution of *Archaea*. The gradual enrichment of the methanogenic archaea in the tubing and gas/water-separation system is in good agreement with reports of the methanogenic community of low-temperature reservoirs around 40°C [50, 162] and the documented temperature limits at 30-40°C of their cultivated next relatives [85, 117, 177].

In conclusion, this work provides evidence for an active sulphate-reducing bacterial population under reservoir and well head conditions, while an ecological *in situ* importance of the predominantly detected *Methanlobus* sp. and *Methanoculleus* sp [31] is questionable. Respectively, the detailed community analyses and the later following experiments with high CO₂ concentrations were concentrated on the bacterial community structure, which seemed of predominant importance for the Schneeren reservoir.

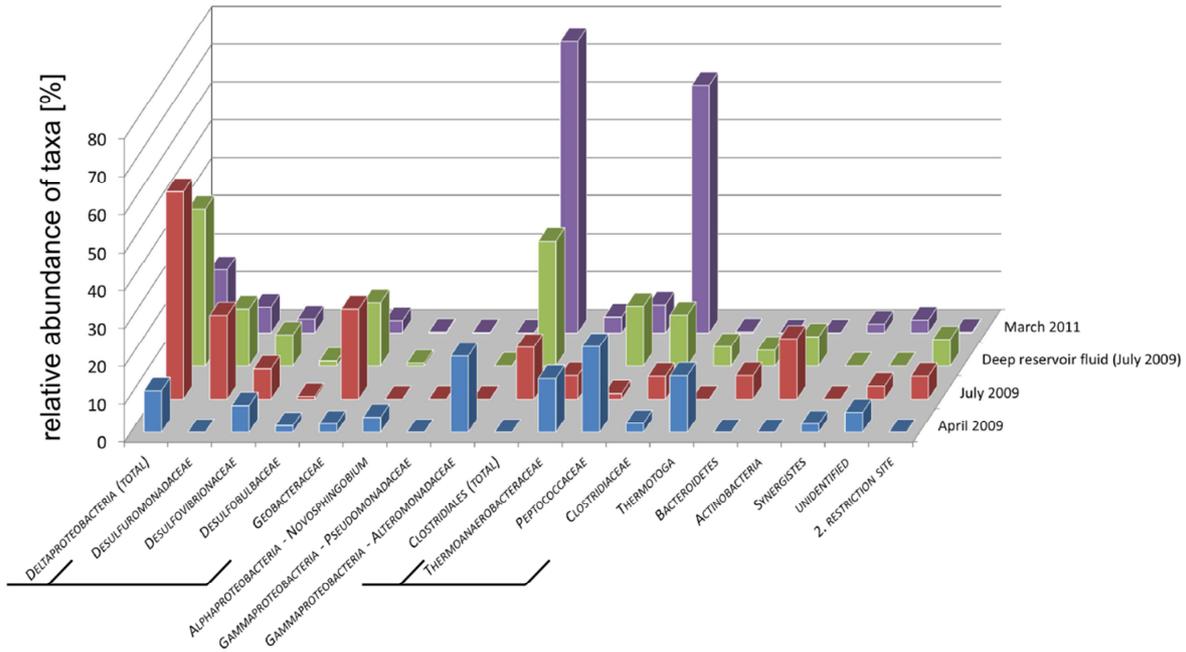
2.1.2. Temporal dynamics of the deep biosphere

The bacterial community structure was monitored from 2009-2011 (included in chapter 3.1) using a combination of fingerprinting techniques (T-RFLP) and bidirectional 454-amplicon sequencing for the identification of abundant terminal restriction fragments (T-RFs). The community description is further amended with respective analyses conducted in the high pressure incubations experiments (only for Z3; November 2011 and April 2012). The majority of the identified bacterial taxa in both deep reservoir and produced fluids were related to organisms metabolising various sulphur redox states. For example, *Desulfovibrionaceae*, *Desulfobulbaceae* and *Peptococcaceae* are capable to reduce several sulphur redox states with a variety of electron donors including alcohols, fatty acids and carboxylic acids, and were repeatedly reported for different hydrocarbon associated environments [8, 51, 106, 121, 125].

The overall community structure supported the model of a Schneeren reservoir formation dominated by sulphate reduction (i.e. the reduction of more reduced sulphur compounds). However, the community monitoring revealed some surprising temporal dynamics in the bacterial distribution pattern (chapter 3.1 & 3.2). Such temporal dynamics of the deep biosphere have never been documented before for hydrocarbon reservoirs and might give some important information about the mechanisms acting on the microbial community structure in this type of environment (see 2.1.3.).

Two phases can be distinguished in the development of the microbial community structure (Figure 10). At the beginning (2009), the microbial community was dominated by several proteobacterial families of the *Deltaproteobacteria* (Z2) and *Alpha-/Gammaproteobacteria* (Z3) subclasses. Especially the high abundance of putative metal-reducing organisms (*Deltaproteobacteria*: *Desulfuromonadaceae* and *Geobacteraceae*) was prominent in Z2 production and deep reservoir fluids in July 2009. Interestingly, the abundance of *Geobacteraceae* specific-16S *rRNA* genes was also strongly increased in July 2009 compared to a lower *Geobacteraceae* abundance in the production fluids sampled before and after July 2009. Thereby, the quantitative community profile supported the community structure pattern of the T-RFLP analysis.

Z2 community composition April 2009 - March 2011



Z3 community composition April 2009 - April 2012

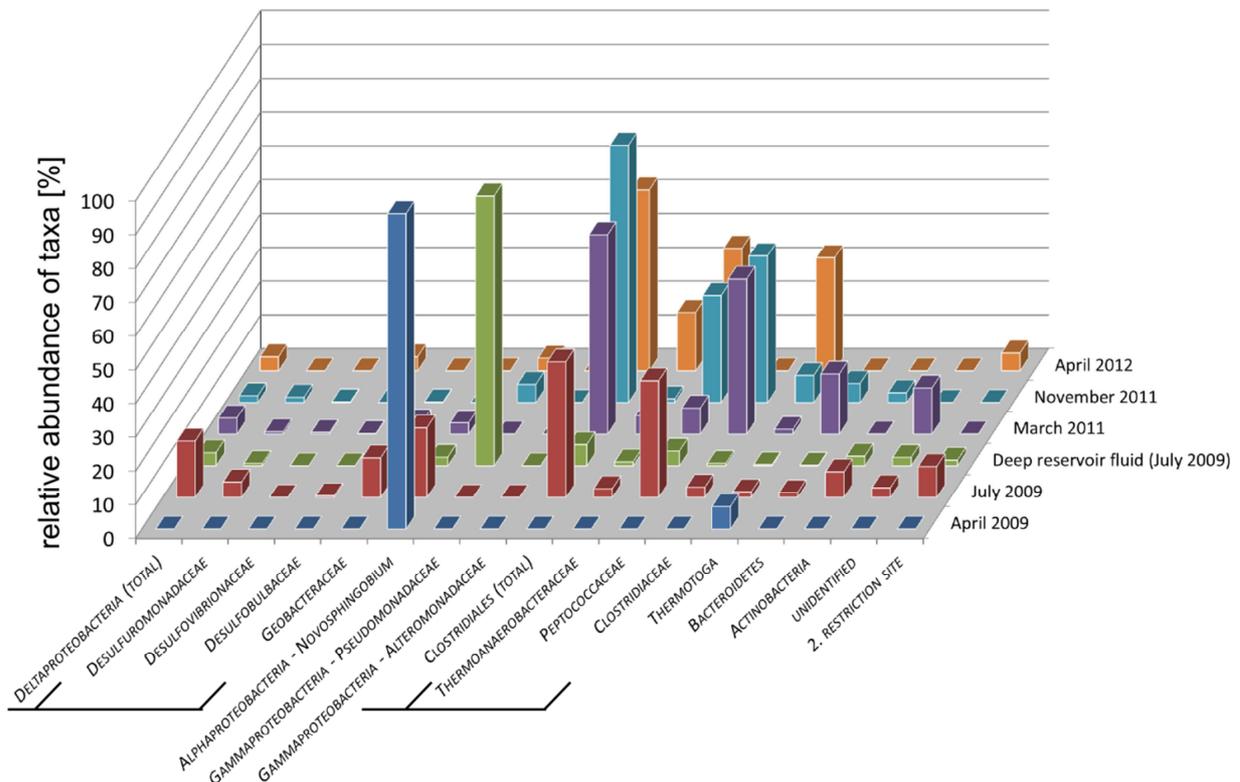


Figure 10: Dynamic developments of the bacterial community structure in Z2 and Z3 production fluids (2009-2011/2012) and the deep reservoir community (July 2009). Community composition was analysed by T-RFLP with the identification of abundant T-RFs using 454-amplicon pyrosequencing libraries. Unidentified resembles T-RFs without identification 2. Restriction site: fragments cut at the second restriction site resulting in longer terminal fragments.

At the end of the monitoring period, *Clostridiales* became dominant starting with the samples analysed for March 2011. Further analyses (only Z3 Figure 10) showed dynamic developments during the period of only six months from March till November 2011. During this time several proteobacterial taxa “reappeared” (relative increase of abundance) in the production fluids of Z3. For both wells a general increase of putatively fermentative organisms related to *Thermotogales*, *Thermoanaerobacteraceae*, and *Clostridiaceae* was observed after July 2009. In case of the *Petrotoga* spp. and *Thermoanaerobacter* spp., the next relatives were described to reduce sulphur and thiosulphate in addition to the fermentation of saccharides and amino acids [16, 37, 114]. The increase of thermophilic spore-forming *Clostridiales* (including *Clostridiaceae* and *Peptococcaceae*) in the formation fluids could represent an ecological advantage or resistance of the organisms, which could be connected to the production of spores.

In general, the microbial diversity seemed to increase in the course of eight months (until Nov 2011) showing the appearance of several groups that had been absent in March 2011. Since the samples of May 2010 were not analysed using T-RFLP, we cannot exclude similar seasonal variations in 2010 as in 2011. However, the documented changes proved a surprisingly strong dynamic within the Schneeren system and showed considerable variations of the community structure especially in the last year from March 2011 to April 2012 (Figure 10).

2.1.3. Indications for the effects of technical measures on the reservoir microbiome

Since the whole Schneeren-Husum massif is economically exploited, technical measures were implemented to increase or stabilise the reservoir’s gas productivity. A sequence of technical measures was conducted starting in spring 2009 until late 2010. The technical measures conducted in this time frame included a repeated acidification to solve mineral precipitates (scales) in the tubing system. The scale prevention was followed by a perforation approach (in July 2009) that included small-scale explosions in the tubing to open new gas bearing sandstone layers in the reservoir (about 1500 m depth). Only

because this approach was accompanied by the demounting of several valves in the tubing system, the deep reservoir fluids were obtained. Thereby, the formation fluids sampled in July 2009 were not directly influenced by the perforation but likely by the acidification approach conducted about eight weeks in advance. In addition to these technical measures, the gas production rate from the reservoir is controlled and adjusted to cope with the seasonal variations in the gas demand. Although the wells are not closed during summer, the production rates are decreased. Thus, the flux rate of the formation fluids from the reservoir and residence times in the tubing and/or separation tank are changed.

The long-term evaluation of the microbial community structure using different molecular-biological methods (qPCR, T-RFLP and 454-pyrosequencing) showed considerable temporal dynamics, which could be the consequences of the described measures. For example, it is likely that the rather high gene copy numbers of various gene markers (incl. universal bacterial, archaeal, and taxon-specific 16S *rRNA* genes and functional genes) during July 2009 were connected with the scaling prevention approach which mobilised mineral precipitates in the production horizon.

The scales dissolving during the acidification might have mobilised biofilms which were likely present (following the description of biofilms in the deep subsurface [167]) in the production horizons or in the fractures around these zones. The mobilisation of such biofilms could in consequences lead to the increased copy numbers of the monitored gene markers. Besides the mobilisation of precipitates and biofilms, the acidification likely increased the temporal availability of oxidised metals for putative metal-reducing organisms [68]. Thereby, the elevated abundance of specific 16S *rRNA* genes for metal-reducing *Geobacteraceae* (July 2009) could represent a stimulation by increased oxidised metals.

The appearance of *Clostridiales* corresponded with the increased water production for both wells in succession of the perforation approach (personal communication). Therefore, the increased abundance of *Clostridiales* after July 2009 could resemble the influx of so far unproduced formation fluids changing the community fingerprint. Likewise,

the later reappearing *Proteobacteria* could resemble a time-dependent recovery of these organisms related to the production of formation fluids.

In combination with the previous chapters, this study revealed a pronounced temporal dynamic in relation to the technical measures. In the months (years) following July 2009, the microbial community structure seemed to recover from the massive human intervention. For a more precise correlation, however, the frequency of the sampling approach has to be directly aligned with the technical measures. The results of the Schneeren reservoir community structure propose a more throughout monitoring of the deep biosphere to understand the underlying feedbacks of the reservoir ecosystem to technical measures and gas production rates. For closer inspections and interpretations one would need further down-hole sampled deep reservoir fluids, which were unfortunately not available for this study.

2.2. Survival of reservoir organisms despite the bactericidal effects of supercritical CO₂

The microbial community in November 2011 and April 2012 of the Z3 formation fluids mainly consist of several *Clostridiales* taxa, *Thermotoga*, *Gammaproteobacteria* and *Bacteroidetes* (see Figure 10 in chapter 2.1.2.). The community structure after the *in vitro* experiment with scCO₂ showed that almost exclusively the spore-forming, thermophilic, and Gram-positive *Clostridiales* survived the incubation under high pressure. This result is in direct conflict to other previously reported studies about the bactericidal effects of scCO₂ (e.g. for spores and spore-forming *Firmicutes* [27, 79]) and suggests further examinations for the resistance of these reservoir associated organisms.

2.2.1. Effects of supercritical CO₂ on the microbial community structure

Two high pressure incubations with scCO₂ were designed to mimic different conditions within a potential storage reservoir during the injection of CO₂ (zones according to Gaus *et al.* [46]): (i) close to the injection point resulting in a completely CO₂ saturated formation

fluid (ii) within the diffusion plume in distance to the injection point where the fluids are undersaturated with CO₂. Both experiments were conducted with formation fluids from the Z3 well head (described above). The latter experiment (ii) was conducted with a stimulated microbial population directly obtained from Z3 production fluid. In the following discussion, the two experiments are described as (i) “saturation experiment”, referring to the CO₂ saturated conditions in the reactor system, and the (ii) “stimulation experiment”, according to the stimulation phase in advance of the actual incubation with CO₂. For a detailed description of the experimental setup please refer to section 3.2 Figure 22; p. 118.

During both experiments no sulphate reduction activity was detected over the course of the high pressure incubations with scCO₂. The monitoring included the evaluation of the total cell density (cell counts using SybrGreen staining Figure 11) and the abundance of sulphate reducers, via qPCR of *dsrA* gene copy numbers. During the stimulation experiment no significant changes of cell density and *dsrA* gene abundance were detected, while both parameters declined during the saturation experiment.

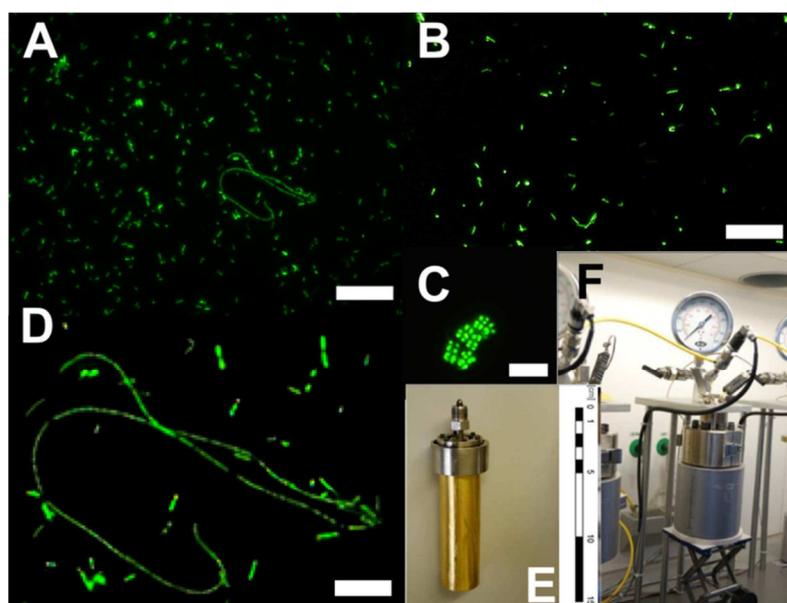


Figure 11: High pressure incubation with scCO₂ A) stimulated batch culture (stimulation experiment), scale 20 μm; B) cell density at the end of the high pressure incubation with scCO₂ (after 40 days), scale 20 μm; D) enlarged image section of A), scale 10 μm; C) sarcina-like cells detected in the formation fluids, scale 5 μm; microbial cells were stained with SybrGreen® fluorescence dye; Reactor setup with E) the 100 mL bag made of gold foil and F) the reactor incubation system (BGR; ©Dr. C. Ostertag-Henning)

The accompanying microbial community analysis (T-RFLP) revealed only minor changes during the saturation experiment. The stimulation experiment showed structural community changes similar to the control setup indicating that a not CO₂-related effect was changing the community. After pressure and CO₂ were released from the incubations

systems at the end of each experiment, fluid material from the reactor systems and the accompanying controls was transferred (reactivation approach). All transfers were further incubated without CO₂ (under ambient pressure) to evaluate whether the detected microorganisms were able to cope the prolonged CO₂ stress. The reactivation approach of both experiments revealed surprisingly minor differences in the microbial sulphate reduction activity comparing the previously scCO₂-affected systems with the control setups that were not incubated with CO₂ (Figure 27, p. 142).

In conclusion, the microbial reactivation approach revealed an actively growing microbial population after the incubation with scCO₂ for several weeks. For both experiments thermophilic, Gram-positive and putative spore-forming *Clostridiales* were predominant in the reactivation approach after the scCO₂ incubations were finished. For example, in the reactivation approach of the saturation experiment even ~95% (*Peptococcaceae*) of the population were affiliated to the taxon.

This result was rather surprising considering the ample documentations for the bactericidal or bacteriostatic effects of scCO₂ already on the basis of several minutes or hours of incubation time (reviewed by Garcia *et al.* [44]). The potential to survive under elevated CO₂ concentrations was indicated for sulphate-reducing prokaryotes in a report on the German CCS pilot project “Ketzin” [119]. In case of the drilling and CO₂ injection test site “Ketzin” (Berlin, Germany), microorganisms plugged the sandstone pores with produced biomass and FeS precipitates in advance of the CO₂ injection. These microorganisms were presumably fed by the drilling mud (containing organic solvents, emulsifiers etc.) and likely also originated from the drilling fluids. The plugged pore space was cleaned with the injection and release of nitrogen removing the microbial biomass and FeS precipitates from the injection well. In the following injection of CO₂ the cell numbers were strongly reduced, but still a significant proportion of the microorganisms survived the injection [119].

Following the CO₂ dispersion in the “Ketzin” reservoir, the abundance of active cells increased and sulphate-reducing bacteria reappeared in the formation fluid. However, the authors missed to document the microbial community induced by the drilling mud and/or

the initially present population before the drilling approach was started. Additionally, the documented community structure remained ambiguous about the phylogenetic relation for the detected sulphate-reducing bacteria (*Desulfovibrionales* or *Desulfotomaculum*) [119]. In contrast, the here documented phylogenetic affiliation of the organisms in Schneeren fluids gives important indications for the possible survival mechanisms.

2.2.2. Mechanisms increasing the survival potential of microorganisms

The high pressure incubations with scCO₂ implied a rather high survival potential of spore-forming *Clostridales* (see above). The most abundant families were *Peptococcaceae* and *Thermoanaerobacteraceae* in the community of the reactivation approach. The first include a *Desulfotomaculum* sp. that was affiliated to sulphate reducers from various high temperature oil fields [96, 121, 125, 160]. The *Thermoanaerobacter* sp. was affiliated with fermentative organisms from geothermal sources and hydrocarbon reservoirs [16, 38, 39, 122]. However, the DNA based analysis directly after the saturation experiment showed the presence of several additional taxa in the fluid, e.g. a rather high abundance of *Pseudomonas* spp. Nevertheless, this has to be interpreted with caution since the presence of cells and amplifiable DNA does not necessarily indicate the survival of these organisms, and likely included also dying cells with damages beyond repair [3, 78].

The Gram-positive cell wall structure and/or the formation of spores seemed to be of importance for the survival of the organisms. The higher CO₂ resistance of vegetative *Firmicutes* cells was connected to their Gram-positive cell wall structure in previous studies [27, 79]. For example, the spore-forming species *Bacillus subtilis* (aerobic growth) was included in a study investigating the simulated leakage of CO₂ into an aquifer system [152]. This study showed that all tested pure cultures reacted negatively to high CO₂ concentrations in growth and cell yield with the highest resilience for the tested Gram-positive *Bacillus* species. Although the pH in the microcosms was buffered to minimise pH shifts, all cultures reacted quite pronounced on the high CO₂ concentrations [152]. Independent from the acidification by CO₂, the diffusion of CO₂ and HCO₃⁻ into the cell membrane and cytoplasm (see Figure 5; p. 24) was shown to severely reduce the

potential of microorganisms to survive [60, 76]. In case of Gram-positive cells, the dense peptidoglycan structure was interpreted to represent an effective diffusion barrier [27]. Accordingly, also the dense structure of spore coat and cortex, besides the low water content of the spore's cytoplasm, could likewise reduce the negative effects. Although the artificial groundwater systems does not resemble an adequate analogue for a deep CCS storage horizon in CO₂ concentrations and physical conditions [152], the survival of facultative aerobic *Bacilli* further emphasises the ecological advantage of Gram-positive and spore-forming organisms.

Despite this environmental advantage for *Firmicutes*, also several non-sporulating and Gram-negative organisms “reappeared” in the reactivation microcosms after the scCO₂ was released. For example, *Geotoga* sp. and *Petrotoga* sp. were not detected directly after the stimulation experiment, but detected later in the reactivation assessment. The lower abundance of these taxa in comparison to the control setups indicated a certain sensitivity or inhibition of these organisms during the scCO₂ incubations. Their later detection in the reactivation phase might represent a grown population that survived below the T-RFLP-detection limit. The *Thermotogae* might survive due to their typically present enlarged outer membrane. This “toga”-like outer membrane contains up to 20 cells per sheath, structuring *Thermotoga*-biofilms and possibly also helping the cells to adhere to mineral and particle surfaces [21, 100, 114, 144].

The formation of extracellular polymeric substances (EPS) could also enhance the resilience of organisms during the conducted experiments and especially in the real reservoir system. Microorganisms embedded in biofilms are usually surrounded by a complex matrix of polysaccharides, glycoproteins, glycolipids and even extracellular DNA [41, 158], which builds a diffusion barrier and/or zone with buffering capacity for the CO₂ [156]. For example, embedded in a biofilm the survival rate of *Bacillus mojavensis* was increased in incubations with scCO₂, albeit the exposure did not exceed 20 minutes [115]. The experiments compared the resilience of biofilm assembled cells with planktonic cells, showing after the scCO₂ contact a decrease in the cell numbers by several orders of

magnitude for the planktonic culture, and a better regrowth potential of the biofilm cells [115].

2.2.3. Consequences for potential CCS storage reservoirs

The potential of microorganisms to survive the presence of scCO₂ (i.e. under *in situ* conditions CO₂-saturated fluids) is of importance for the geochemical storage mechanisms (see chapter 1.3.). The storage mechanisms can be described by the injectivity of CO₂, as a function of the permeability of the storage site, and the final storage capacity, which include the available pore space volume and the CO₂ fraction dissolved in the formation fluids [110]. However, the question is whether microbial activity will more likely enhance mineral trapping mechanisms [110, 111, 115] or decrease the permeability and storage potential for CO₂ in the reservoir [119].

The here documented experiments with scCO₂ showed the survival potential of putative fermentative (e.g. *Thermoanaerobacteraceae*) and sulphate-reducing bacteria (*Desulfotomaculum*). *Desulfotomaculum* represents the genus with the largest number of thermophilic sulphate-reducing cultures from hydrocarbon reservoirs and geothermal fluids that are able to metabolise a broad spectrum of organic substances [see e.g. 37, 121]. The substrate versatility of *Desulfotomaculum* spp. makes these organisms to generalists globally distributed in high temperature environments [20, 51, 122]. Fermentative organisms play an important role in the degradation of organic matter, the production of hydrogen, and are also globally distributed in hydrocarbon reservoirs [51, 53].

In conclusion, the surviving organisms are most likely present in the selected storage reservoirs in future. Their resilience for high CO₂ concentrations under realistic *in situ* conditions (in pressure and temperature) has to be taken into account for CCS site selection and applications. Corresponding to the effects at the “Ketzin” pilot project [119], microbial sulphate reducers might actually plug the pore space with produced biofilm/biomass and FeS particles, thus reducing the available pore space for CO₂. However, the here documented experiments showed that in the direct injection zone

(saturated CO₂ concentrations) likely no cell growth or respiration activity will occur. Thereby, the effective plugging of the pore space around the injection zone by biomass seems unlikely.

Since a real storage reservoir does not resemble a closed reactor system, the diffusion of CO₂ in the reservoir will lead to considerable CO₂ concentration gradients [46, 90], with zones of completely unaffected formation fluids. Geochemical *in situ* processes might further limit the bacteriostatic effects, as the mineral matrix provides a certain buffering capacity decreasing the concentrations of free HCO₃⁻ and CO₃²⁻ ions [89, 110]. Furthermore, the demonstrated mobilisation of dissolved organic matter might provide suitable microbial substrates [149]. In conclusion, organisms with the highest resistivity, which are according to this work *Clostridiales*, will presumably find suitable conditions within a relative broad area of the storage horizon. Within these areas a putative syntrophy of surviving fermentative and sulphate or metal-reducing prokaryotes can enhance mineral precipitation increasing the storage capacity, via mineral trapping mechanisms, as postulated previously ([110, 115, 163] see also Introduction 1.3. and 1.4.4.).

Further studies will have to evaluate the influence of EPS and biofilm formation, spore formation and the mineral buffering capacity on the long-term (100's-1000's of years) survival of microorganisms during and after CO₂ injection. With this knowledge it will be possible to better estimate the CO₂ threshold of the microbial population, and to determine which effects the microbial activity will have on the storage potential of the CCS storage reservoir formation.

2.3. CO₂ induced differentiation of affected and unaffected soil environments

The flux of CO₂ into the upper soil environment induces changes in the soil geochemistry as reported previously [9, 40, 81, 136], and will likely also affect the microbial community structure. For a better differentiation between CO₂-affected and unaffected soil environments, we have to evaluate which microbial parameters (activity, cell abundance, genetic community fingerprint etc.) are actually influenced by CO₂. Only based upon a significant differentiation it will be possible to determine the ecological consequences of high CO₂ concentrations in soil ecosystems, and accordingly the risks of leakages from possible CCS storage sites.

The here studied soil ecosystem on the pastures along the shoreline of the Laacher See showed variations in microbial parameters with increasing CO₂ concentrations in the soil (Figure 12). The evaluated microbial activities were rather heterogeneously distributed for the uppermost 20 cm of soil surface. While in the upper ten centimetres no methanogenesis activity was detected at any site (chapter 3.4), it increased with depth (e.g. 10-20 cm) in the vent centre compared to the reference site (chapter 3.3). However, no significant CO₂-induced changes in the geochemical profile (e.g. pH and organic carbon content) were revealed for the uppermost 20 cm, despite of the microbial activities.

Besides their heterogeneous distribution in the soil column, seasonal effects lead to additional variation in the distribution of microbial activities in the soil surface. In July 2008 both aerobic and anaerobic mineralisation activities were relatively high corresponding to enhanced microbial respiration activities during summer [143]. The distribution of the activities during summer 2008 showed a significant correlation of anaerobic microbial processes (sulphate reduction) with the increased CO₂ concentrations at the medium site and the vent centre, respectively. However, the microbial activities measured in September 2007 showed no likewise correlation with CO₂, and were much lower than during summer 2008. To differentiate seasonal from CO₂-induced variations in the

microbial activities, a high spatial and temporal resolution of sampling is needed which was unfortunately not obtained during this work.

In conclusion, the results of activity measurements indicated a reduced aeration status in the vent centre soil environment during summer, corresponding to the anoxic conditions described for other volcanic CO₂ vents [9, 40, 104, 133] (Figure 12).

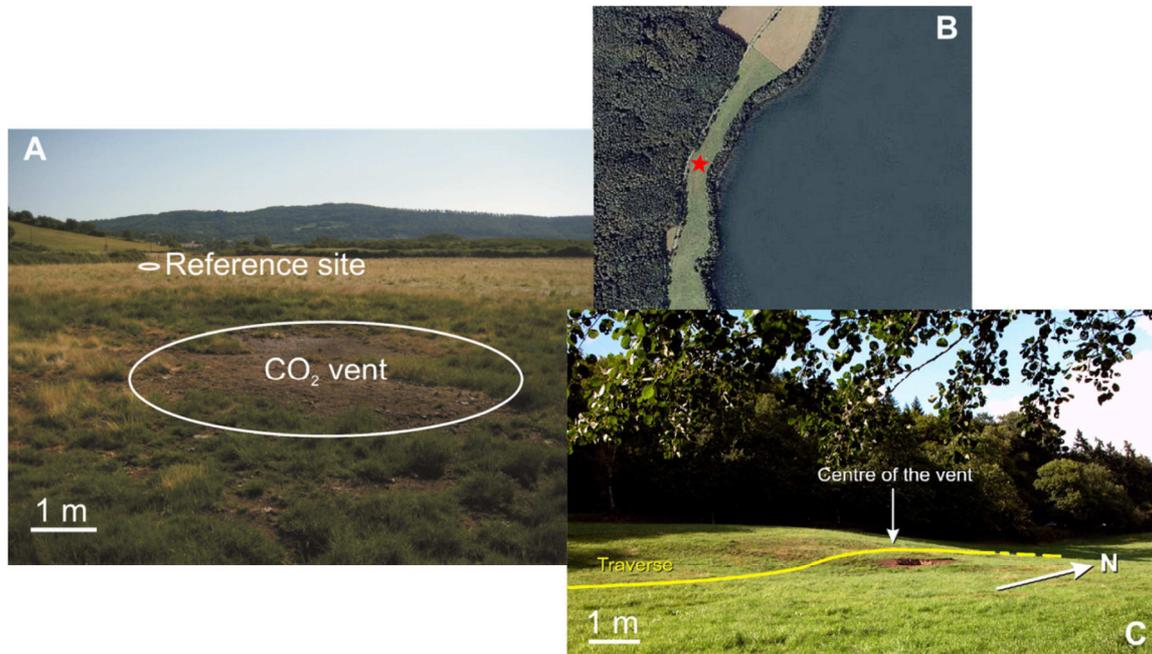


Figure 12: Ecosystem setting of the natural CO₂ vents at Latera (A) and the Laacher See (B air photograph & C at the pasture). For both vents the typical reduction of plant coverage is visible with a circle of bare soil surrounding the CO₂ emitting seep. The pictures originated from A) Oppermann *et al.* [133]; B) final report on joint geoecological research of natural CO₂ sources in the East Eifel, Germany (Krüger *et al.* [95]) – modification: detail of the western shore line with the location of the vent centre indicated with the red mark; C) picture originally included in the publication chapter 3.3.

2.3.1. Identification of putative indicator species in the soil surface

The microbial community composition was investigated in more detail to identify possible indicator species promoted by CO₂ and the changes of the soil environment using denaturing gradient gel electrophoresis (DGGE). For the bacterial community no other environmental factor besides the sampling depth was identified influencing the community composition. In contrast, the archaeal community composition showed a pronounced differentiation with increasing CO₂ concentration; with a predominance of *Thaumarchaeota* associated sequences in the vent centre (nine out of ten sequences from the vent surface soils).

The *Thaumarchaeota* (incl. group 1.1a and 1.1b) have been reclassified from the *Crenarchaeota* phylum and contain the only so far cultivated ammonia-oxidising archaea (AOA) [15]. In the soil surface samples of the vent centre also the abundance of 16S *rRNA* gene copies (qPCR assay [129, 137]) of the phylum was increased, as was the abundance of ammonia-oxidising archaea (using an AOA specific qPCR assay [43, 98]). Thus, the gene copy abundances and the phylogenetic fingerprint showed a good correlation and revealed also a pronounced predominance of the *Thaumarchaeota* and AOA in the archaeal community of the vent centre. The differentiation of the archaeal community allowed a significant correlation with the CO₂ induced effects.

Several soil ecosystem studies revealed the importance of the *Thaumarchaeota* (mainly group 1.1b) as possible ammonia oxidisers for acidic habitats [59, 88, 97, 123, 124]. However, at the Laacher See vent centre the measured pH of the bulk soil showed no CO₂ induced acidification in the uppermost soil surface. Only in the deeper soil column the pH showed a sharp decrease in the vent centre (chapter 3.3). Nevertheless, the microclimate in soil aggregates can be quite different from the actually measured bulk pH [42]. Respectively, the abundance of *Thaumarchaeota* in the surface soils of the vent centre could reflect a small scale acidification promoting these organisms. Despite the problems to verify bacterial indicator species, the predominant position of *Thaumarchaeotes* proposed them as possible indicator species for high CO₂ concentrations in combination with the accompanied soil acidification.

2.3.2. Ecological niches for *Thaumarchaeota*

Despite the qualitative and quantitative predominant position of *Thaumarchaeota* in the surface soil environment, the organisms were also very abundant in the depth profiles of both sites, reference site and vent centre, respectively. However, the cluster analysis showed a significant separation of the depth-associated sequences from those identified within the surface layers (included in the supporting information of the Chapter 3.4.). Especially the reference site showed a high phylogenetic differentiation towards the next cultivated relative (between 90-97% sequence similarity) with depth.

In addition to the distribution of AOA in acidic habitats, these organisms were abundant and active in water saturated habitats with limited oxygen input, like peat bogs [157] and freshwater sediments [64], and even in the suboxic water column of the Black Sea [43]. Marine AOA, phylogenetically related to group 1.1a *Thaumarchaeota*, have a higher substrate affinity for ammonia than ammonia-oxidising bacteria (AOB) and heterotrophs using ammonia as N-source [108]. Furthermore, studies documented the uptake and oxidation of ammonia at low oxygen concentrations which might enhance the environmental advantage of these organisms in oxygen limited environments like the Black Sea suboxic zone [43, 62, 140].

These findings need to be verified for the terrestrial AOA community (dominantly group 1.1b *Thaumarchaeota*) in the surface and deeper soil layers of terrestrial ecosystems or the CO₂ vent environments. Their environmental advantage in microaerobic and acidic habitats has to be defined for soil habitats. Previous findings already proposed a physiologic differentiation for AOA in terrestrial habitats from the cultivated marine representatives [148]. Hence, a stimulation by organic fertiliser indicated a heterotrophic AOA community (group 1.1b in [148]) in contrast to the autotrophic marine community. In this context, their share in the global cycling of organic carbon and nitrogen has to be evaluated to actually link the phylogenetic variability of *Thaumarchaeota* with the abundance of AOA and an ecological function [151]. In conclusion, the here documented results clearly emphasise a more detailed consideration of the nitrogen cycle in the CO₂ vent-associated soil environment. Such volcanic CO₂ vents might represent suitable ecosystems to evaluate the effects of oxygen limitation and acidification affecting the distribution of *Thaumarchaeota* and/or AOA in soil ecosystems.

2.4. What can we learn from the microbial community of the Laacher See soil environment for the risk assessment of CCS leakages?

The survey at the Laacher See CO₂ vent, a natural analogue for a CCS leakage, was focused on the identification of indicator species in the microbial community and/or shifts in the microbial activity profile. The results suggest the abundance of anaerobic/acidic niches and a predominance of *Thaumarchaeota* in the CO₂ affected soil column. However, not all results were as significantly CO₂-related as previously reported for the Latera vent system [9], non-volcanic CO₂ moffetes [164], and artificial test fields for CCS leakage scenarios [170]. Despite these studies, still important questions for the transformation of the results to CCS-leakage scenarios remain unanswered. The time dependence factor for the development from an unaffected soil column towards a CO₂-affected ecosystem could not be deduced from the Laacher See soil environment study. However, it is expected that at least one or two vegetation periods would be needed to produce significant differences in the plant coverage in a vent centre (i.e., leakage site) [40, 104, 170]. For the effects of CO₂ on the microbial biosphere such time frames may not be accurate, and the changes in the community structure are going to occur faster.

For an accurate estimate of the time-related development within the plant and microbial community, artificial test fields are most likely the best option. Under defined environmental conditions the time scale for induced changes can be evaluated and also possible recovery rates of CO₂-affected ecosystems. Nevertheless, the natural setting at the Laacher See with gradually increased CO₂ concentrations in the soil revealed some significant dynamics in the microbial community. These dynamics can help to evaluate specific concentration thresholds for CO₂ in the soil environment.

The soil gas survey (chapter 3.3) documented spatial variations in the soil atmosphere for the CO₂ concentrations especially for the medium site. The CO₂ concentrations at this site varied from 10-60% (in 60 cm soil depth; 12.5 m in Figure 13). The isotopic signature of the CO₂ indicated a mixture of biotic and magmatic CO₂ at the medium site. The variations in the gas concentration at the medium site corresponded well with the model of

a horizontal diffusion of CO₂ from a point emission source (fault/fracture) localised at the vent centre.

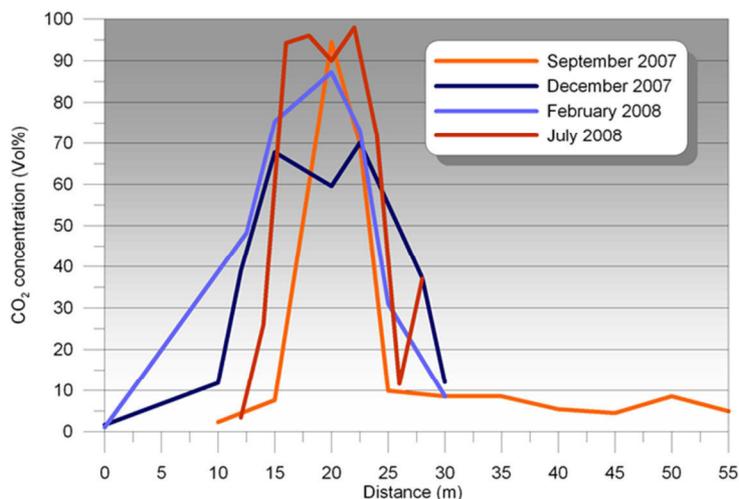


Figure 13: Seasonal variation of CO₂ concentrations at 60 cm depth along the N-S traverse at vent "1". The vent centre is located at 20 m. Source: [95; p. 53].

Horizontally diffusing CO_{2a} is possibly channelled along permeable soil layers [166]. Additionally, the gas concentration at the Laacher See medium site reflects temporal variations, caused by e.g. water content, air pressure, and wind speed (chapter 3.3), which have a pronounced influence on the final flux rate of CO₂. Generally, the medium site showed the highest seasonal variability in the evaluated microbial mineralisation activities of all sites (chapter 3.4). The abundances for specific gene markers for anaerobic processes and AOA were elevated corresponding to the numbers in the vent centre. However, also similarities with the reference site were documented, as the cluster analysis (DGGE fingerprint) showed a significant correlation for the community composition of medium and references surface soil samples (chapter 3.4 and supporting information).

The microbiological analyses revealed an intermediate position of the medium site showing similarities with both the references site and the vent centre. In correspondence to these results, the microbial CO₂ fixing community was especially affected under medium CO₂ concentrations in terrestrial, volcanic mofettes [164]. The lowest *cbbL* (red-like type) genetic variability and strongest variation in the gene abundance was detected in the soil profile with 10-30% CO₂ in the soil atmosphere. Videmšek *et al.* [164] postulated that the CO₂ fixing community might be more severely affected in soil columns

with strong fluctuations in the CO₂ concentrations. Accordingly, other soil ecosystem studies reported a decreasing root respiration, changes in plant species composition, and altered microbial activities in the upper layers of the soil column already at CO₂ concentrations of 5-20 % in the soil atmosphere [9, 40, 104, 138, 139]. Consequently, the CO₂-induced alteration of the microbial community at the Laacher See medium site indicated a tolerance threshold of about 10-20% CO₂ in the soil atmosphere.

In conclusion, the microbiological monitoring of the natural analogue revealed important factors about the development of soil ecosystems under CO₂ influence. The results identified *Thaumarchaeota* as predominant species in the vent centre and thus as potential indicator species at least for the Laacher See vent systems. The ecological niche for these organisms and their function as ammonia oxidisers is not fully understood, but seems to correlate with specific environmental conditions in their habitat, like suboxic conditions and acidic soil environment [e.g. 43, 124]. Furthermore, the microbial monitoring supported the findings of geochemical investigations that the impact of a possible CCS leakage will be locally centred at defined fault/fracture zones [95, 136, 166]. Further this work documented dynamically affected soil layers surrounding the leakage centre (i.e. medium site) with strong variations in the microbial community profiles and activity distribution.

3. Short summary of publications and author's contribution

Chapter 4.1.: Pronounced microbial population dynamics in a Northern German gas reservoir connected to production-related technical measures

With a previous study approach on the reservoir formation fluids considering mainly the methanogenic archaeal community [31] this report comprises to date the longest microbiological monitoring with new investigations regarding the bacterial community. For the first time, unproduced deep reservoir fluids (sampled down-hole at the perforation horizon) were microbiologically analysed from this reservoir. These samples enabled a closer comparison with the produced formation fluids for spatial differentiations in the microbial community structure. Furthermore, the microbial community inhabited in saline formation fluids revealed a surprising temporal variability in its potential mineralisation activity and community structure. In conclusion, this investigation obtained a detailed picture of the bacterial community in the formation revealing significant anthropogenic influences on the deep biosphere by technical measures related to the gas-production.

Authors: Janin Frerichs, Claudia Gniese, Tillman Lueders, Nils Hoth, Michael Schlömann, and Martin Krüger

Author's contribution:

Janin Frerichs obtained the fluid samples, conducted the activity measurements, the majority of the molecular investigations (qPCR, T-RFLP), and evaluated the respective results. Claudia Gniese participated in several sampling approaches and conducted additional T-RFLP analyses that are included in the supplemental material. The 454-amplicon pyrosequencing was prepared and interpreted by Janin Frerichs with the help of the groundwater research group under the supervision of Tillmann Lüdgers (IGÖ Helmholtz Zentrum Munich). Claudia Gniese, Michael Schlöman and Tillmann Lüdgers reviewed and amended this manuscript. Janin Frerichs and Martin Krüger designed the experiments and wrote the manuscript.

Chapter 4.2.: Viability and adaptation potential of indigenous microorganisms from natural gas field fluids in high pressure incubations with supercritical CO₂

The application of CCS will lead to a diffusion gradient within the reservoirs' environment that were simulated in experiments mimicking conditions (i) close to the injection point with interface contact of scCO₂ fluid and formation brine (ii) within the diffusion plume in distance to the injection point. The later was conducted with an actively mineralising community under sulphate-reducing conditions referring to the postulated mobilisation of organic matter from the sandstone matrix. The results of this study proved the survival potential of thermophilic, spore-forming *Clostridiales* which were predominant in the microbial community after the high pressure experiments with scCO₂. The approach with formation fluids from an analogue CCS storage reservoir comprises the first investigation of an indigenous microbial population with a close evaluation of survival and adaptation potential for the microbial community.

Authors: Janin Frerichs, Jana Rakoczy, Christian Ostertag-Henning, and Martin Krüger*

Author's contribution:

Experiments were designed and conducted by Janin Frerichs under the supervision of Martin Krüger. The technical application of the high pressure reactors was supervised by Christian Ostertag-Henning. The activity measurements of reactivation incubations and total cell counts were prepared by Janin Frerichs with the help of Jana Rakozy. Chemical analysis were prepared and interpreted by Janin Frerichs. The dataset of the water chemical analysis was obtained by the BGR in-house facilities for water chemistry. Microbial community analyses (T-RFLP) were conducted and statistically evaluated by Janin Frerichs. Janin Frerichs prepared the full manuscript while Jana Rakozy, Christian Ostertag-Henning, and Martin Krüger reviewed and amended the manuscript.

Chapter 4.3.: Effects of elevated CO₂ concentrations on the vegetation and microbial populations at a terrestrial CO₂ vent at Laacher See, Germany

The description of the Laacher See volcanic vent system at the pastures of the north/western shore line included a broad gas measurement approach verifying the fluxes and extension of the terrestrial vent system and the origin of the CO₂ from the upper mantle hemisphere. The study was amended with geochemical parameters (pH, oxygen concentrations) and a botanical survey evaluating the influence of the CO₂ on the plant community/coverage. For the respective soil horizon (10-20 cm) microbiological results were included regarding the bacterial/archaeal 16S *rRNA* gene copies and activities. The report comprises an introductory presentation of the Laacher See natural CO₂ vent ecosystem for the following microbiological study approach on the distribution/identification of putative microbial CO₂ indicative organisms.

Published as:

Krüger M, Jones D, Frerichs J, Oppermann BI, West J, Coombs P, Green K, Barlow T, Lister R, Shaw R, Strutt M and Möller I (2011).

“Effects of Elevated Co₂ Concentrations on the Vegetation and Microbial Populations at a Terrestrial Co₂ Vent at Laacher See, Germany.” *International Journal of Greenhouse Gas Control* **5**: 1093-1098.

Author's contribution:

Samples were obtained by the whole party of authors (except for Janin Frerichs) integrated into the CO₂GeoNet community. Janin Frerichs provided activity measurements and qPCR results and wrote respective parts of the manuscript. David Jones, Tom Barlow, Bob Lister, Richard Shaw, Michel Strutt, and Ingo Möller conducted the gas measurement on the sampling sites and evaluated the data set. Julia West, Patricia Coombs, and Kay Green conducted the botany survey and wrote respective parts of the manuscript. Birte I. Oppermann conducted the geochemical analysis and provided the data set. Martin Krüger and Ingo Möller designed the experiments and wrote the paper.

Chapter 4.4.: Microbial community changes at a terrestrial volcanic CO₂ vent induced by soil acidification and anaerobic microhabitats within the soil column

The study revealed differences in microbial activity and community structure for the vent centre which were highly affected by annual factors that interacted with the CO₂ and its secondary induced factors of aeration, soil pH, mineral weathering etc. The community analyses revealed a significant predominance of *Thaumarchaeota* for the vent centre that was evident also in the monitoring of marker genes (Ammonia oxidising archaea (AOA) and 16S *rRNA* specific for *Crenarchaeota/Thaumarchaeota*) and the analyses of lipid biomarker distribution in the soil column.

The distribution pattern and the discussed dominance of *Thaumarchaeota* for acidified environments lead to the conclusion that the phyla represent indicator species that were promoted by the environmental conditions induced by the seeping CO₂. In general, the results indicated a spatially highly restricted centre for the CO₂ influence that was partially overprinted by interaction with depth dependent and seasonal variations. Carbon dioxide provides an environmental factor that affects the habitat conditions (pH, aeration etc.) and influences via these secondary factors the microbial community structure.

Published as:

Frerichs J, Oppermann BI, Gwosdz S, Möller I, Herrmann M, Krüger M (2013).

„Microbial Community Changes at a Terrestrial Volcanic CO₂ Vent Induced by Soil Acidification and Anaerobic Microhabitats within the Soil Column.” *FEMS Microbiology Ecology* **84**: 60-74.

Author's contribution:

Samples were obtained by Martin Krüger, Birte Oppermann and Ingo Möller. Janin Frerichs conducted activity measurement, qPCR and DGGE analyses of soil samples with the accompanied statistical analyses. Birte Oppermann conducted the lipid biomarker analyses and geochemical analyses (total organic carbon etc.) and wrote the respective

parts of the manuscript. Ingo Möller conducted the gas measurement at the site and provided the data interpretation in connection to the IJGHG publication. Simone Gwosdz and Martina Hermann conducted qPCR analyses of ammonia oxidising archaea and bacteria. Martina_Hermann, Birte Oppermann, and Ingo Möller reviewed and amended the manuscript. Janin Frerichs and Martin Krüger designed and wrote the manuscript.

3.1.1. Publications not included

Krüger, M., J. West, J. Frerichs, B.I. Oppermann, M.-C. Dictor, C. Jouliand, D.

Jones, P. Coombs, K. Green, J. Pearce, F. May, and I. Möller (2009):

“Ecosystem effects of elevated CO₂ concentrations on microbial populations at a terrestrial CO₂ vent at Laacher See, Germany.” *Energy Procedia* 1:1933-1939.

Oppermann, B.I., W. Michaelis, M. Blumenberg, J. Frerichs, H. M. Schulz, A.

Schippers, S. E. Beaubien, and M. Krüger (2010):

“Soil microbial community changes as a result of long-term exposure to a natural CO₂ vent.” *Geochimica et Cosmochimica Acta* 74:2697-2716.

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4. Manuscripts

4.1. Pronounced microbial population dynamics in a Northern German gas reservoir connected to production-related technical measures

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Running title: Microbiological long-term monitoring of a natural-gas field formation fluids

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1 **Abstract**

2 Natural gas reservoirs represent extreme but nevertheless often densely populated habitats
3 for microorganisms with regional but also local and sometimes temporal variations in their
4 geochemical conditions, which are also influenced by gas production-related measures. So
5 far, in such ecosystems only little is known about the natural and man-made dynamics in the
6 microbial biosphere.

7 To get a better understanding of these dynamics and it's the underlying environmental
8 factors, a detailed micro- and molecular biological study was conducted over three years in
9 the Schneeren-Husum natural gas field in North Germany. Saline formation fluids from two
10 neighbouring wells (Z2 and Z3) differed in various geochemical parameters revealing
11 spatially separated compartments already within one reservoir formation. For the first time *in*
12 *situ* sampled deep reservoir formation fluids were studied for the Schneeren reservoir, thus
13 allowing a detailed perception of the microbial population and its distribution within the
14 reservoir and the gas production system.

15 The molecular biological and microbial investigations indicated a general functional
16 advantage sulfur metabolism for the Schneeren formation. Despite previous findings the
17 methanogenic community likely comprised a gas production-related enrichment within the
18 tubing system. Finally, the long-term monitoring of the bacterial population revealed a
19 remarkably high variability in the microbial community structure, i.e. its size, activity potential
20 and composition. The taxa *Desulfotomaculum*, *Desulfovibrio*, *Geobacteraceae*,
21 *Pseudomonadaceae*, *Acteobacterium* and *Alphaproteobacteria* were detected with varying
22 abundance in the different fluids. However throughout this study, a significant shift in the
23 community towards thermophilic spore-forming *Clostridiales* was observed that was
24 correlated with production-related measures to increase gas-productivity.

4.1.1. Introduction

The knowledge about microbial populations in deep geological formations, the so-called deep biosphere, has become increasingly important in the last two to three decades [48]. Although mostly hidden to human perception, these organisms fulfill important functions in global elemental cycling [38]. Hydrocarbon reservoirs often represent microbial hotspots within the deep biosphere exceeding in both, cell numbers and mineralization activities, other subsurface habitats [38, 39, 48].

The activity of methanogenic and sulphate-reducing prokaryotes is of special importance for hydrocarbon reservoirs as they are considered as final physiologic active units in organic matter degradation [18, 30]. In consideration of the specific reservoir temperature, the microbial community present in reservoirs above 50°C generally consist of *Clostridiales* (*Desulfotomaculum*, *Thermoanaerobacteraceae*), *Proteobacteria* (mainly *Deltaproteobacteria*), *Bacteroidetes*, *Deferribacteres*, *Thermotogea* and methanogenic Archaea, e.g. *Methanococcoales*, *Methanobacteriales* etc. [22, 30].

In addition to exploration of new hydrocarbon reservoirs, a more recent focus has been set on another aspect: the reservoirs' storage capacity itself. According to the International Panel on Climate Change (IPCC), there is a need to massively reduce emissions of greenhouse gases like CO₂ [24]. One currently discussed option is Carbon Capture and Storage (CCS), a technique that requires large-scale storage capacities which are assumed for depleted gas or oil reservoirs and saline aquifers [23]. Besides geochemical storage mechanisms, biologically controlled carbonate precipitation is considered to secure storage safety and capacity of CO₂ in suitable reservoirs [32]. However, only little is known about the specific temporal and spatial variations of the physiologically active community responsible for the organic matter degradation in deep reservoir systems. Thereby, also an approximation of microbially mediated or enhanced CO₂ storage mechanisms will remain insecure until the microbial population is closer examined and functions are better understood.

The reservoir massif Schneeren (part of the Schneeren-Husum reservoir; Lower Saxony, Germany) studied for this report originated from the Upper Carboniferous [20, 27] with a temperature range of 60- 90°C for the gas bearing sandstone horizons. The whole reservoir is technically and geologically rated feasible for CCS applications [23], but no injection of CO₂ has been conducted so far. In a previous report considering the archaeal community of this model gas reservoir, differences in the community structure of two wells (Z2 and Z3) have been detected revealing the predominance of either *Methanobus* spp. or *Methanoculleus* spp. in the produced formation brines [11].

This study presents a detailed long-term monitoring of the microbial community in the formation brines of the Schneeren reservoir, thereby evaluating the metabolic versatility of the community and the extent of temporal variations in its activity and structure. Furthermore, this study includes the examination of deep reservoir fluids directly sampled *in situ* from the gas producing horizon. This allows for the first time a comparison for this reservoir of its specific reservoir community in produced and deep reservoir fluids. Our overall aim was to distinguish the indigenous biosphere and its microbial key-players from possible contaminants or enrichments in the production system. Further, to evaluate potential effects of technical interventions on the microbial biosphere. Our results will broaden the knowledge of the deep biosphere dynamics which is an important parameter for prospective applications like CCS in such deep geological reservoir formations.

4.1.2. Methods

Sampling procedure

Between 2008 and 2011, six sampling sessions were conducted at the gas formation plant Schneeren-Husum in the North German plain (Lower Saxony, Germany). A detailed description of the site and sampling procedure was given by Ehinger *et al.* [11]. Two well heads, Z2 and Z3, were chosen for the microbiological survey. Samples of produced fluids (PF used in the figures and tables) were sampled in May 2008, November 2008, April 2009, July 2009, and May 2010. The samples were taken at the well-head facility from the gas/water-separation system (temperature 38-45°C). The sample of May 2008 was used

only for activity measurements while an additional sample for further molecular biological analysis was taken in March 2011. In July 2009, deep reservoir fluids (DR) were sampled directly at the perforations zones with constant influx of the reservoir water into the tubing system (down-hole sampling; about 2500 m depth). The deep reservoir fluids were sampled with an iron bailer of approximately two litre volume that was flushed twice with sterile Milli-Q water before sampling and when changing the bore hole. All fluid samples were immediately transported to the lab for further analysis.

Water chemistry

For the investigation of the water chemistry, the fluids were immediately upon arrival filtered (0.45 to 0.22 μm depending on the analysis) and stored at 4°C or frozen (-20°C). Total inorganic carbon (TIC) and dissolved organic carbon (DOC; 0.45 μm filtered) were measured using catalytic high temperature combustion with a Shimadzu TOC-VCPN carbon analyser (Shimadzu, Japan). Anions were determined by ion chromatography with a DX-500 ion chromatograph system (Dionex, Germany). Ammonium was detected by flow injection analysis according to DIN EN ISO 11732 and cations were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES; Spectro Analytical Instruments GmbH & Co. KG, Germany).

The low molecular weight organic acids (LMWOA) from C1-C4 were measured using a high performance liquid chromatograph (Agilent 1200 series) equipped with a guard column (Eclipse Plus C8 Guard; 4.6 x 12.5 mm; 5 μm), a silica-based separation column (Eclipse Plus C8 4.6 x 150 mm; 5 μm ; temperature set to 20°C), and a diode array detector (G1315D) set at 220 nm. Gradient flow rate of mobile phase was 1 ml min⁻¹ using 0.05 M sulphuric acid and methanol (gradient: 30 sec 10 vol% methanol; 9 min 40%). The filtered (0.22 μm) and thawed fluid samples were amended with 50 mM sulphuric acid prior to the measurement (injection volume 10 μl).

The stable isotopic signature of CH₄ and CO₂ was measured using Thermo Fisher MAT252 GC-IRMS. The $\delta^{13}\text{C}$ values are expressed as ‰ vs. “Vienna Pee Dee Belemnite”

(VPDB) while δD values were measured against “standard ocean mean water” (SMOW) [42].

Determination of microbial activities in the reservoir fluids

For activity measurements, the fluids were filled into heat sterilised serum bottles under strict anaerobic conditions (Mecaplex anaerobic chamber systems). All microcosms were sealed with butyl stoppers and repeatedly flushed with N_2 to remove O_2 . The experiments were conducted in triplicates and incubated in the dark at 30°C (up to 80°C). Various amendments were supplemented in 10 mM final concentration (unless stated otherwise) to stimulate the microbial activity: H_2/CO_2 [20/5% (vol/vol) of the headspace], formate, acetate, benzoate, lactate, pyruvate, succinate, fructose, ethanol, methanol [5-10 mM]. Hydrogen, acetate, and methanol were amended on regular basis for the activity monitoring while the other substrates were applied only to fluids of one to two sampling sessions.

For better comparison, production fluids of both wells, Z2 and Z3, were repeatedly amended to reach 20 mM sulphate since the fluids differed considerably in their intrinsic sulphate concentration. Additionally, fluids were not amended (“no additions”) to measure the indigenous activity of the microbial community (control). Sterilised controls were prepared by adding sodium azide (0.02 g per 100 ml fluid) or formaldehyde (2% vol/vol) to differ between microbial mineralization activity and non-biogenic degassing.

Headspace gas samples were taken weekly to analyse the methane and CO_2 concentration using a GC 14B gas chromatograph (Shimadzu) with a flame ionization detector and a methanizer (SRI Instruments Europe GmbH) to quantify the CO_2 [35]. Sulphate reduction was followed via the production of copper sulphide from dissolved sulphide (HS^-) [4].

Microbial activities are given in $nmol\ mL^{-1}\ day\ [d]^{-1}$ with the standard deviation (SD) of two to three replicates. In substrate amended microcosms, the activity was classified as induced if values were significantly increased above values of the controls (p-value <0.05; student’s t-test using SPSS, Chicago, USA). Due to the sulphate content of the original

formation fluids and the presence of FeS precipitates in the samples, the sulphate reduction rates have to be interpreted as bulk sulphate turnover [21]. However, in the following document, the complex bulk sulphate turnover was seen convenient to determine the significantly substrate-induced microbial sulphate reduction.

Nuclein acid extraction and quantitative PCR

The molecular biological samples were filtered immediately upon arrival onto a 0.22 µm polycarbonate filter and stored at -20°C. Volumes between 100 to 500 ml were used for DNA extraction without pre-filtration as previously described by Ehinger *et al.* [11] to avoid the exclusion of particle associated microorganisms (please refer to Figure 18). The DNA was extracted using phenol-chloroform extraction protocol (16) performed with modifications that are described in the supporting information in detail.

For quantitative PCR (qPCR), specific standards of each target gene were prepared from pure cultures (obtained from the German Collection of Microorganisms and Cellcultures DSMZ) with defined copy numbers according to the processing described by Schippers *et al.* [44]. A complete list of the tested qPCR assays and processing is given in the supporting information (Table 5; including the publication sources).

Community analyses using T-RFLP and 454-pyrosequencing

For terminal restriction fragment length polymorphism (T-RFLP), bacterial 16S *rRNA* gene amplicons were prepared (Ba27f /907r (17)) following the conditions described by Piloni *et al.* [40]. The primer Ba27f was 5'-FAM labelled to determine the specific fragment size after digestion with *MspI* (*Bacteria*) [28, 40]. The distribution and abundance of T-RFs was determined using the T-REX platform after denoising the fingerprint profiles and aligning the T-RFs according to Culman *et al.* [6].

Already existing clone libraries and a T-RF database of Z3 fluids (published by Ehinger *et al.* [11]) were extended with a 454-amplicon pyrosequencing approach. The new analyses were performed with samples from July 2009 (produced and deep reservoir fluids) and March 2011(produced fluids) to compare the formation fluids for spatial and temporal differences. The preparation of bacterial amplicon libraries (bidirectional with

Ba27f and 519r) has been described in detail by Pilloni *et al.* [40, 41]. The demultiplexed reads were quality-clipped with Greengenes using an average Q_{20} cutoff over a moving window of 50 bp [9]. Trimmed reads were classified and diversity indices were inferred using the RDP pyrosequencing pipeline [45]. This workflow has been shown to yield robust and highly reproducible read abundances for dominating taxa across replicate samples [41]. For linking pyrotag with T-RFLP datasets, amplicon pools of matching forward- and reverse-reads were assembled into contigs with the SEQMAN II software (©2013 DNASTar Inc.). Assembly thresholds were at least 98% sequence similarity over a 50-bp match window. Only contigs containing at least one forward and one reverse read were considered for analyses which resulted in a substantial further denoising of our dataset and a focussing on dominating read amplicon pools. The next relative for each contig was identified using the RDP integrated seqMatch tool [28, 40, 41, 45].

The terminal fragment length of contigs was predicted *in silico* (using TRIFLe [25]) and linked with the abundant T-RFs in the T-RFLP data matrix. Accordingly, the microbes represented by abundant T-RFs were identified [40, 46] representing between 83 - 94% of the overall microbial community. In addition, a number of enrichment cultures from the activity measurements (using production fluids in April 2009) were analysed by T-RFLP fingerprinting. A more detailed description and results are included in the supplement (Figure S3 including the identification of T-RFs).

4.1.3. Results

Fluid chemistry

The arithmetic mean of important geochemical parameters was calculated for each well head (Z2 and Z3 in Table 1) using the dataset obtained from 2008 to 2011. The produced fluids of Z2 and Z3 showed in direct comparison some considerable differences. For example, a higher salinity of Z2 fluids (TDS at $65 \pm 2.2 \text{ g L}^{-1}$), but higher sulphate concentrations of Z3 fluids ($984 \pm 32 \text{ mg L}^{-1}$). The stable isotopic signatures of the dissolved methane was $\delta^{13}\text{C} (\text{CH}_4) -24.5 \pm 3.2\text{‰}$ and $\delta\text{D} -194.9 \pm 45.5\text{‰}$ confirming a thermogenic origin of the produced gas [47]. Likewise, the CO_2 , accounting for about 3%

of the produced gas [11], did originate from geochemical reactions ($\delta^{13}\text{C}$ (CO_2) $-4.9 \pm 0.6\text{‰}$).

Produced and deep reservoir fluids of well head Z3 differed in Fe(II) concentrations (Table 1), i.e. $2.6 \pm 1 \text{ mg l}^{-1}$ Fe(II) in the produced fluids, while the deep reservoir fluid contained about ten times more Fe(II) ($31 \pm 7 \text{ mg l}^{-1}$). The produced Z3 fluids were rich in black Fe(II)-sulphide precipitates indicating a higher sulphide concentration which decreased the aqueous ferrous iron content.

Table 1: Variation of physiochemical characteristics of collected fluids produced during 2008-2011 in comparison to a previously published dataset.

Well head	Z2		Z3	
	PF*	DR*	PF*	DR*
EC [mS cm^{-1}]	89.2 ± 2.1	85.6 ± 4.6	43.1 ± 0.5	43.8 ± 1.9
salinity [g L^{-1}]**	65-68	68	27-29	28
pH	6.9 ± 0.5	6.5 ± 0.3	7.6 ± 0.5	7.2 ± 0.6
Cl^- [mg L^{-1}]	35785 ± 1731	37981 ± 409	15301 ± 716	15810 ± 918
SO_4^{2-} [mg L^{-1}]	297 ± 13	325 ± 11.5	984 ± 32	1010 ± 33
Ca^{2+} [mg L^{-1}]	946 ± 76	985 ± 8	143 ± 37	165 ± 15
Fe^{2+} [mg L^{-1}]	38 ± 9	38 ± 6	2.6 ± 1	31 ± 7
Na^+ [mg L^{-1}]	22704 ± 1160	23546 ± 319	10773 ± 1624	11165 ± 467
NH_4^+ [mg L^{-1}]	47 ± 2	48 ± 1	18 ± 13	19 ± 1
DOC [mg L^{-1}]	65 ± 5	69 ± 7	85 ± 14	n.d.
TIC [mg L^{-1}]	190 ± 1	240 ± 39	401 ± 7.5	n.d.
Acetate [mM]	2.3 ± 0.5	2.4 ± 0.5	2.6 ± 0.2	2.7 ± 0.3
Isotopic signature of produced gas (both well heads)				
$\Delta^{13}\text{C}$ (CH_4)	-24.5 ± 3.2			
ΔD	-194.9 ± 45.5			
$\Delta^{13}\text{C}$ (CO_2)	-4.9 ± 0.6			

*arithmetic mean with standard deviation for each parameter of sampled fluids separately for each well head (PF: produced fluids: \pm SD for six sampling campaigns during 2008-2011; DR: deep reservoir fluids: \pm SD of two separate samples per well head in July 2009).

**manual (handheld) optical refractometer used at the sampling site giving a first indication of the quality of the sampled fluid (no standard deviation calculated)

n.d. not determined.

However, in summary only minor temporal geochemical variations were detected over the course of this study for the produced fluids (SD <10%) and spatially in comparison with the *in situ* sampled deep reservoir fluids (Table 1). Thus, the produced fluids likely originated from the gas-producing sandstone horizon and did not comprise condensates from the tubing system.

Long-term monitoring of activity and gene abundance in produced fluids

Although the isotopic origin of the produced natural gas did not indicate a biogenic origin of the natural gas, the methanogenic community of both well heads has been well documented in previous publications [11]. For a further assessment of the origin of the microbial population, it was important to understand the metabolic and physiologic adaptation of the methanogenic and sulphate-reducing community.

Rather high methanogenic activities were detected under ambient incubation conditions (30°C and atmospheric pressure). The rates were by a factor of four higher in produced fluids of Z2 than in fluids of Z3 (Figure 14). The amendment of different methanogenic substrates revealed a pronounced induction of methylotrophic methanogens (methanol) while hydrogenotrophic or acetoclastic methanogens were not significantly stimulated.

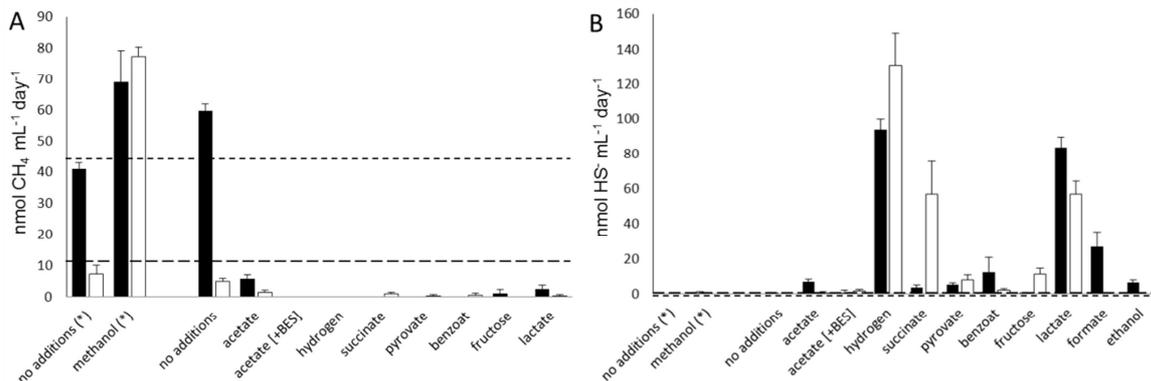


Figure 14: Activity pattern of potential methanogenesis (A) and sulphate reduction rates (B) in produced fluids of Z2 (black) and Z3 (white). The upper limits of microbial activity in the control setup (no addition) were indicated as dashed lines (short dash for Z2 and long dash for Z3) to illustrate the induction potential of various the substrates. Profiles were obtained with fluids in May (*) and November 2008. Error bars represent standard deviation of three replicated microcosms per substrate.

However, increasing the incubation temperature revealed a pronounced temperature dependency of the methanogenic community as no activity was detected above 45°C (Figure 15A). Further, the temporal variability of the methanogenic and sulphate-reducing activity was quantified from amended and unamended microcosms at several time-points (Figure 19 in the supporting information). From 2008 to 2010, the methanogenic activity

strongly decreased and no significant stimulation using methanol amendment was observed after April 2009 ($p > 0.1$).

The sulphate reduction rates were low for both fluids in unamended microcosms (at 30°C). However, several different substrates significantly stimulated sulphate-reducing prokaryotes. A strong stimulation was documented for H_2/CO_2 in both fluids (Figure 14). Additionally, albeit with lower stimulation potential, low molecular weight organic acids (LMWOA; e.g. lactate, acetate) induced sulphate reduction.

For microbial sulphate reduction no temperature dependency was apparent as documented for the methanogenesis activity (see Figure 15B). Furthermore, the potential activity and the stimulation of the sulphate-reducing community by hydrogen (significance of the induction between $p: 0.001$ to 0.02) were stable over the study from 2008 to 2010 (Figure 19 in the supporting information).

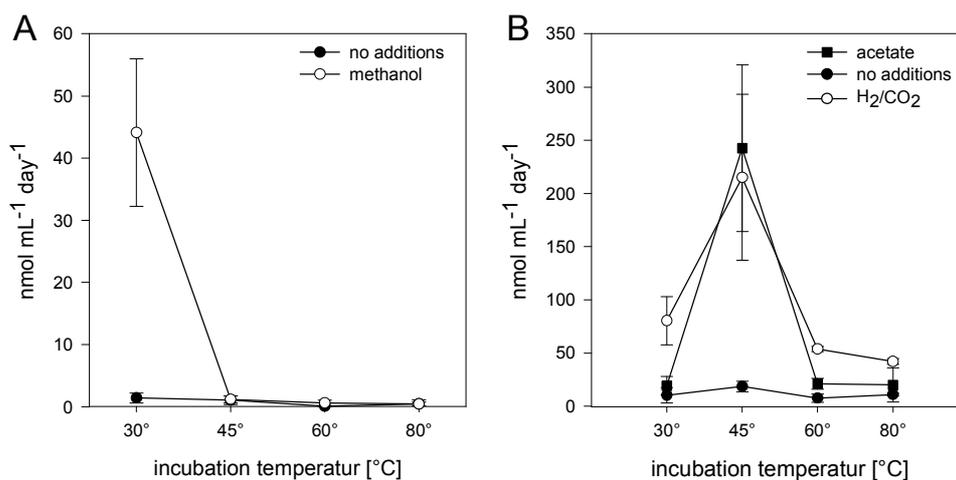


Figure 15: Microbial methane production (A) and sulphate reduction (B) activities for produced Z3 fluids (April 2009) over a temperature gradient. Error bars represent the standard deviation of triplicate microcosms.

Despite, the reduced activity of the methanogenic community, the sulphate-reducing population seemed to be highly active over the whole study. This result was also confirmed by the monitoring of bacterial and archaeal gene markers in the produced fluids (Figure 16). The archaeal gene copies for 16S *rRNA* and *methyl Coenzym M reductase* (*mcr* subunit A) were about two to three orders of magnitude higher in Z2 fluids than in Z3 fluids, likewise mimicking the higher methanogenesis activity of the Z2 originated fluids.

However, by the end of this study, the *mcrA* gene copy numbers decreased considerably corresponding to the declining methanogenesis activity for both well heads.

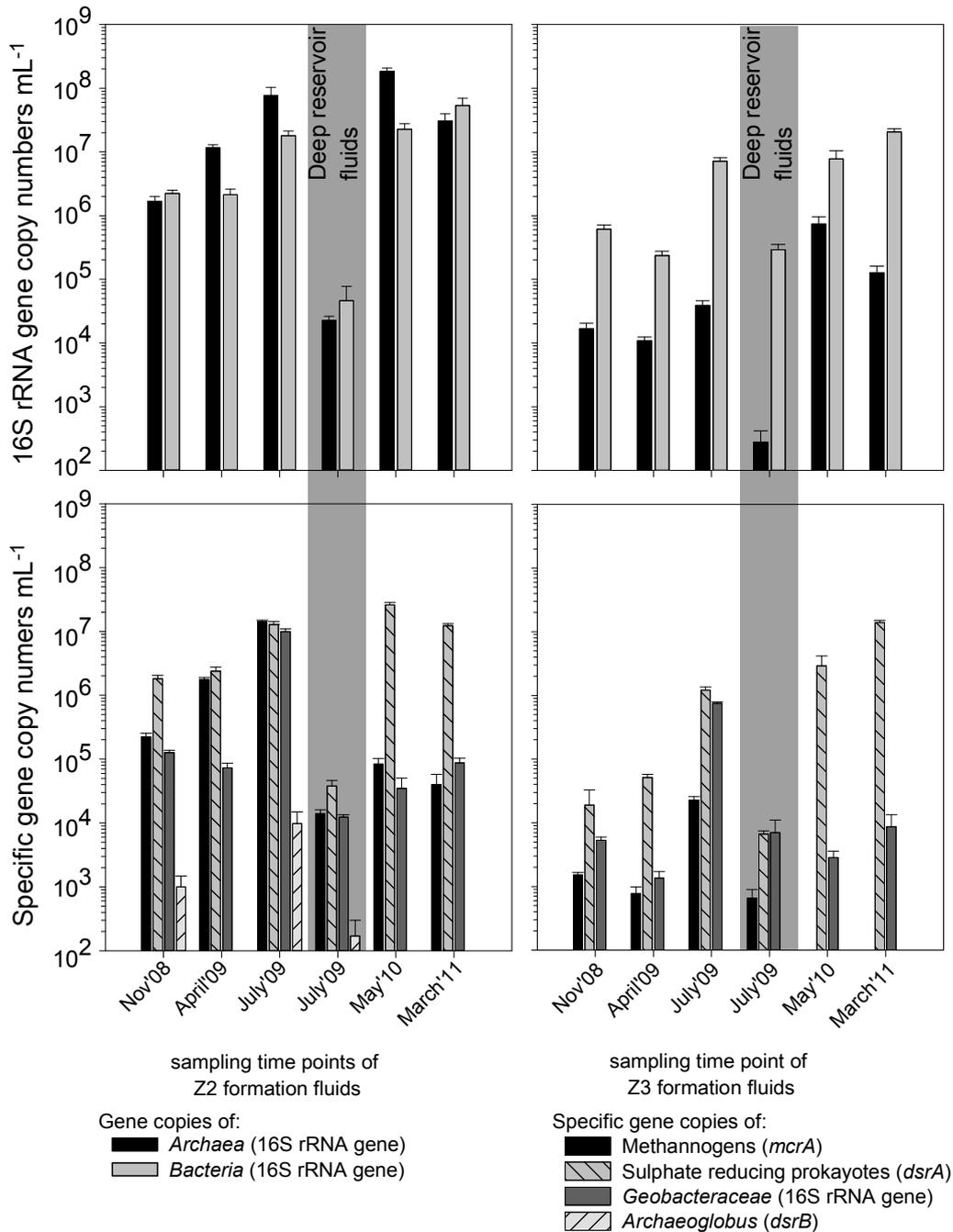


Figure 16: Temporal variations in the community size and composition as detected by quantitative PCR in produced and deep reservoir fluids of Z2 (left) and Z3 (right). Universal quantification of 16S *rRNA* genes for *Bacteria* and *Archaea* (upper panels) and the selective quantification of group specific and functional genes (lower panels) as determined with assays listed in Table 5 (supporting information).

In contrast, the high abundance of *dissimilatory sulfide reductase* (*dsr* subunit A) correlated with the time-dependent increase of the sulphate reduction activity. Both fluids

showed high *dsrA* copy numbers that increased by one to three orders of magnitude, e.g. $1.4 \pm 0.1 \cdot 10^7$ *dsrA* gene copies were detected in produced fluids of Z3 in March 2011. To identify the fraction of archaeal sulphate reducers detected with the *dsrA* assay, the *Archaeoglobus* specific *dsrB* gene was used. However, *Archaeoglobus* specific *dsrB* genes were only detected in low numbers in Z2 fluids (November 2008 and July 2009) and were below the detection limit for Z3 fluids. Thus, indicating a sulphate-reducing community dominated by *Bacteria*. Interestingly, the estimation of *Geobacteraceae* specific 16S *rRNA* genes revealed a strong increase of these organisms in July 2009 ($1 \pm 0.1 \cdot 10^7$ gene copies mL⁻¹) and a decrease afterwards back to the level of November 2008 (Figure 3).

Microbial activity and gene marker distribution for the deep reservoir fluids

For the first time, also *in situ* sampled deep reservoir fluids were included in the microbiological characterization of the Schneeren reservoir. The comparison of the microbial activities and gene marker abundances of deep reservoir fluids with those of the accompanied produced fluids can give further important information for the distribution of the microbial population within the reservoir.

Both deep reservoir fluids (Z2 and Z3) showed rather high sulphate reduction rates without substrate additions (e.g. 14.8 ± 2.6 nmol mL⁻¹ day⁻¹ for Z3 at 50°C), but no indigenous methanogenic activity which is in accordance to the isotopic signature of the produced gas (Table 2).

Table 2: Induction potential of selected amendments that significantly stimulate activities in produced (PF) and deep reservoir (DR) fluids in July 2009.

Fluid samples	Methanogenesis rates in nmol mL ⁻¹ day ⁻¹		Sulphate reduction rates in nmol mL ⁻¹ day ⁻¹		
	no additions	methanol	no additions	H ₂ /CO ₂	fructose
Z2 PF	14.3 ± 1.0	33.3 ± 3.1	3.2 ± 2.7	11.3 ± 4.6	(-)
Z2 DR	(-)	(-)	21.8 ± 5.4	31.3 ± 2.2	(-)
Z3 PF.	0.9 ± 0.4	30.5 ± 9.0	0.4 ± 0.04	73.4 ± 1.5	10.5 ± 0.9
Z3 DR	(-)	(-)	14.8 ± 2.6	44.8 ± 0.4	(-)

(-) not detected

Furthermore, sulphate reduction was significantly stimulated (e.g. $44.8 \pm 0.4 \text{ nmol ml}^{-1} \text{ day}^{-1}$ Z3 at 50°C) in deep reservoir fluids that were amended with hydrogen, while methanogenesis was not stimulated by any amendment.

The gene abundance of 16S *rRNA* gene markers was considerably reduced for the deep reservoir fluids of both well heads (Figure 16). However, the archaeal population was stronger affected showing a decrease by three orders of magnitude in the deep reservoir fluids compared to the produced fluids. The distribution of specific gene markers in the deep reservoir fluids mirrored the abundance profile of the produced fluids. In correspondence to the distribution of 16S *rRNA* gene copies, the numbers of the functional and specific gene markers were comparably reduced.

In summary, the microbiological characteristics of the deep reservoir fluids showed significant similarities with the accompanied produced fluids. The abundance and activity of sulphate-reducing bacteria was seen as important proxy for the *in situ* importance of these organisms in the reservoir formation. For a further characterization of the microbial key players, the bacterial community was analysed in detail.

Microbial diversity analyses

The bacterial community composition, its diversity, and temporal and spatial dynamics were analysed with a combination of T-RFLP and 454-amplicon pyrosequencing (Table 3). The utilised workflow has recently been shown to allow a highly reproducible and semi-quantitative measure of microbial community structure and *rRNA* gene ratios in subsurface samples [41]. In July 2009, produced and deep reservoir fluids' amplicons were affiliated to *Proteobacteria* (16-72%), *Bacteroidetes* (2-18%), *Acidobacteria* (max 6.25% in deep reservoir fluids of Z2) and *Firmicutes* (2-54%).

The evaluated community structure showed distinct differences between both well heads as already indicated by our other results. In Z2s' deep reservoir and produced fluids, *Deltaproteobacteria*, namely the taxa *Desulfovibrio* and *Desulfuromonadaceae* were highly abundant. In contrast, the produced Z3 fluids were mainly dominated with *Alphaproteobacteria* (e.g. *Sphingomonadales* 6.8%), *Gammaproteobacteria*

(*Alteromonadales* 2.2%) and with *Clostridiales* of the genus *Acetobacterium* (54%). The bacterial amplicons of Z3 deep reservoir fluids appeared to be largely affiliated to *Gammaproteobacteria* of the genus *Pseudomonas* (see Table 3) and, with lower abundance, to the alphaproteobacterial orders *Rhizobiales* (4.5%) and *Sphingomonadales* (1.9%) and the family *Erythrobacteraceae* (1.2%). However, due to the low quality of 454-amplicon reads of the produced Z3 fluid (July 2009), further analyses were not conducted (i.e. identification of T-RFs) with this dataset.

Table 3: Phylogenetic affiliation of partial bacterial 16S *rRNA* gene of 454-amplicons pyrosequencing analysis and diversity estimations of deep reservoir (DR) and produced fluids (PF) in July 2009 and March 2011.

	July 2009				March 2010	
	Z2_PF	Z2_DR	Z3_PF	Z3_DR	Z2_PF	Z3_PF
Bacteria (numb. of reads)	2019	3804	250	4684	5222	4222
<u>Relative abundance of taxa (in %)</u>						
Unclassified <i>Bacteria</i>	16.98	17.84	4	4.65	1.09	2.23
<i>Proteobacteria</i>	46.98	34.98	16	71.95	10.84	2.87
<i>Deltaproteobacteria</i>	36.04	14.40	4	0.75	10.11	2.63
<i>Desulfovibrio</i>	10.69	7.62	(-)	0.02	4.21	1.97
<i>Desulfuromonadaceae</i>	23.56	2.84	3.6	0.6	2.25	0.02
<i>Geobacteraceae</i>	8.27	1.94	3.6	0.06	(-)	0.02
<i>Alphaproteobacteria</i>	0.99	8.38	7.2	8.03	(-)	(-)
<i>Gammaproteobacteria</i>	9.46	5.41	4.4	60.42	0.71	0.19
<i>Pseudomonadaceae</i>	(-)	0.91	(-)	57.5	0.19	0.18
<i>Alteromonadaceae</i>	8.76	2.96	2.2	0.94	0.48	(-)
<i>Betaproteobacteria</i>	(-)	5.65	(-)	1.94	(-)	0.02
<i>Clostridia</i>	14.46	11.77	54	2.22	83.90	87.38
Unclassified <i>Clostridiales</i>	11.09	3.83	0.4	0.04	10.81	45.81
<i>Acetobacterium</i>	(-)	0.02	48.4	1.24	(-)	(-)
<i>Thermoanaerobacteraceae</i>	(-)	0.02	1.6	0.04	(-)	4.85
<i>Caminicella</i>	(-)	0.05	(-)	(-)	51.02	4.52
<i>Desulfotomaculum</i>	(-)	1.48	0.8	0.04	3.6	1.44
Bacilli	(-)	6.86	(-)	4.65	(-)	(-)
<i>Bacteroidetes</i>	18.32	3.23	4.4	2.35	1.65	5.99
<i>Synergistetes</i>	(-)	(-)	1.2	(-)	1.8	0.04
<i>Actinobacteria</i>	0.40	6.91	(-)	6.85	0.04	(-)
<i>Acidobacteria</i>	(-)	6.25	(-)	3.27	0.02	(-)
<i>Thermotogae</i>	(-)	2.10	2	(-)	(-)	1.03
<i>Nitrospira</i>	(-)	2.63	(-)	0.41	(-)	(-)
<u>Diversity estimations (3% distance level)</u>						
OTUs*	262	1140	ND	761	138	140
Chao1	272	1557	ND	1101	149	150
Shannon Index (H')	4.62	6.12	ND	3.78	3.33	3.37
Evenness (E)	0.83	0.87	ND	0.57	0.67	0.68
Assembled contigs ¹	46	78	ND	34	33	38

*OUT, operational taxonomic unit (3% divergence); ¹Numbers of contigs passing the quality control, i.e., at least one forward and reverse read per contig must be assembled (at 3 % distance level) resembling an at least 470bp long consensus sequence. (-), not detected in the amplicon libraries; ND, not determined.

Reads of produced fluids from March 2011 showed a strong temporal increase of *Clostridia* for both well heads while the abundance of *Proteobacteria* was drastically decreased (Table 3). Amplicons from produced Z2 fluids were affiliated to members of the genera *Caminicella* and *Desulfotomaculum*. Reads of Z3 fluids were mainly identified as unclassified *Clostridiales* (45.8%) ,the family *Lachnospiraceae* (16.9%) and as members of the phylum *Bacteroidetes* (6%).

In addition to 454-amplicon pyrosequencing, the fluid samples from July 2009 and March 2011 were analysed with T-RFLP to evaluate more specifically the distribution of microorganisms on species level (Figure 17 and Table 4). For July 2009, T-RF 66, identified as *Arthobacter* sp., was present in varying abundance in all fluid samples and were slightly increased in the produced fluids. Corresponding to the read abundance,

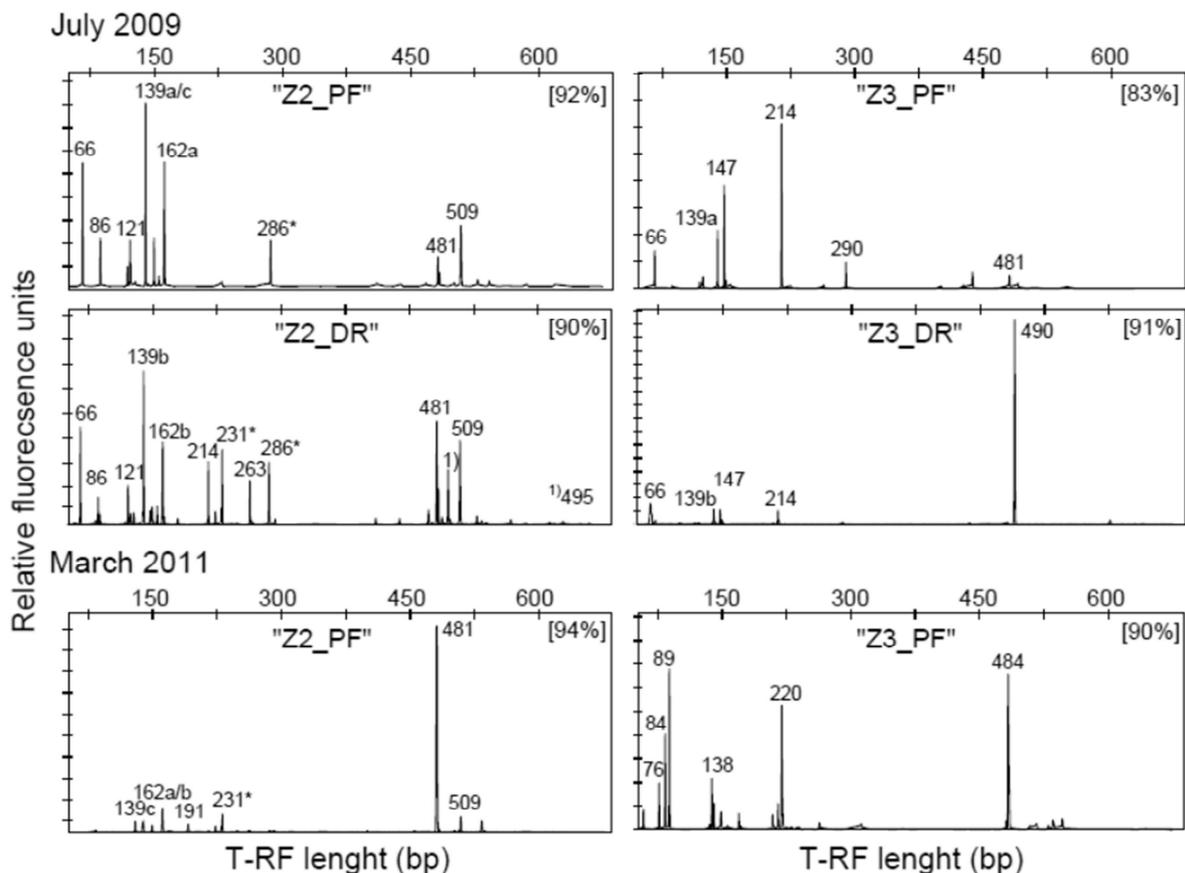


Figure 17: Comparison of bacterial community structure using T-RFLP with produced (PF) and deep reservoir fluids (DR) in July 2009 and March 2011. The identified TRFs (length in bp) corresponded to 83-94% of the total community as indicated in parentheses. The T-RFs were identified using constructed contigs of 454-amplicons with the predicted TRF as listed in Table 4. Fragment patterns were obtained with restriction enzyme *MspI*.

several T-RFs of the Z2 fluids were affiliated with *Deltaproteobacteria*, i.e. T-RF 121 and 509 which were affiliated to an unclassified *Desulfuromonadales* bacterium and *Desulfovibrio* sp., respectively.

In the deep reservoir fluids of Z3, an uncultured *Pseudomonadaceae* (T-RF 490) was highly abundant which was already indicated by the 454-amplicon classification results. For the produced Z3 fluid, T-RF 214 affiliated to *Acetobacterium* sp., and T-RF 147 identified as uncultured *Novosphingobium* (*Sphingomonadales*) were increased compared to the deep reservoir fluid.

Like the amplicon libraries, also the T-RFLP fingerprint profiles indicated a pronounced community change in March 2011 (Figure 17 and Table 4). T-RF 66 (*Arthobacter* sp.) and several others were reduced or even completely absent in the produced fluids of Z2 and replaced by one dominant fragment identified as *Clostridiales* of the genus *Caminicella* (T-RF 481). For Z3, several new T-RFs appeared replacing former, e.g. T-RF 220 identified as *Clostridium boliviensis* and T-RF 484 identified as *Clostridium caminithermale*.

Table 4: Identification of abundant T-RFs corresponding to Figure 17. The next relatives [GenBank accession number] were assembled with the contigs from the 454-amplicons using the SeqMatch tool (RDP platform; %ID represents the sequence similarity of the closest relative).

T-RFs	%ID	Next relative [Accession N°]	T-RFs	%ID	Next relative [Accession N°]
66	100	<i>Arthobacter</i> sp. [AB496410]	214	99.6	<i>Acetobacterium carbinolicum</i> [AB546237]
86	98.9	<i>Marinilabilia salmonicolor</i> [M62422]	220	99.8	<i>Clostridium boliviensis</i> [AY943862]
89	98.8	<i>Bacteroides</i> sp. [AY695838]	231	99.8	<i>Desulfotomaculum geothermicum</i> [AJ621886]
121	100	<i>Desulfuromonadales</i> sp. [AB260047]	263	99.6	uncultured <i>Petrogoga</i> [EU999020]
130	98.9	<i>Desulfobotulus</i> sp. [U85470]	481	97.5	<i>Caminicella sporogenes</i> [AJ320233]
139a	99.6	uncultured <i>Marinobacter</i> [EU999012]	484	97.9	<i>Clostridium caminithermale</i> [AF458779]
139b	99.2	<i>Geoalkalibacter subterraneus</i> [EU182247]	490	99.8	uncultured <i>Pseudomonadaceae</i> [JN030548]
139c	98.7	<i>Prolixibacter bellariivorans</i> [AB541983]	495	99.5	<i>Clostridium beijerinckii</i> [CP000721]
138	99.7	uncultured <i>Desulfotomaculum</i> sp. [EU999019]	509	99.6	<i>Desulfovibrio</i> sp. [U85475]
147	97.1	uncultured <i>Novosphingobium</i> sp. [EU36028]	286*	second restriction site of TRF 162b	
162a	97.6	<i>Pelobacter carbinolicus</i> [CP000142]	231*	second restriction site of TRF 121	
162b	99.4	<i>Desulfovibrio indonesiensis</i> [AB54625]	472*	second restriction site of TRF 66	
191	95.6	uncultured <i>Thermovirga</i> sp. [DQ647105]	76/84/290	Not in the 454-amplicon libraries	

4.1.4. Discussion

Fluid geochemical monitoring

The combined dataset of Z2 and Z3 (Table 1) documented a pronounced heterogeneity between both well heads although located within the same compartment of the formation (i.e. reservoir block), e.g. the values of the salinity and sulphate concentration. The formation fluids in the North German Basin generally originated from marine pore water that was buried with the sediment during the sandstone genesis [20]. The salinity of 28 and 65 g L⁻¹ (total dissolved salts) was within the lower range of 40-230 g L⁻¹ for formation fluids in this area [20, 27]. The fluids of Z2 and Z3 may migrate from pools of deeply buried ancient meteoric waters into the production horizons of the wells. The immixing of recent meteoric waters into ancient marine pore waters, thereby reducing its salinity, was excluded for the reservoir from isotopic analysis [27].

The presence of Fe(II)-sulfide precipitates in the sampled fluids and the typically present “rotten-egg” odor indicated the presence of sulphate-reducing prokaryotes. In conclusion, the geochemical profile revealed an ecosystem of sulphate rich fluids providing an electron acceptor and organic carbon concentrations comparable to other natural gas associated saline formation fluids (see 4; 63 mg L⁻¹). The organic carbon was likely received from the interaction of formation fluids and coal- and shale-bearing layers within the sandstone matrix [27]. The presence of sulphate in ancient meteoric waters was connected to the overlying Husum salt diapir of marine evaporites and Permian Zechstein salts which supplied the electron acceptor in millimolar concentrations [20]. Combining our results with those previously published by Ehinger *et al.* [11], the geochemical parameters of the well heads Z2 and Z3 were documented over almost six years showing only minor variations.

Sulphate reduction as prominent metabolic process in the reservoir

As indicated by the geochemical data, the reservoir provided a suitable habitat for sulphate reducing prokaryotes, which became further evident in the measurement of

activity profiles and abundances of metabolic key-genes (*dsrA*). The sulphate-reducing activity under high temperature conditions in produced and deep reservoir fluid confirmed the importance of sulphate reducing prokaryotes for the actual *in situ* sandstone horizons. The sulphate reducing community showed a pronounced metabolic versatility as different substrates, like LMWOA, saccharides, and alcohols, were utilised. The capability of the microbial population to mineralise these substrates is well documented for oil and gas reservoirs [3, 5, 15]. T-RFLP fingerprints of substrate stimulated microcosms from Z2 and Z3 fluids additionally indicated a dominance of sulphate reducers (Supporting information Figure 20).

In contrast to sulphate reduction, methanogenesis was induced only by methanol. Albeit, the methanogenesis activity exceeded previously reported rates for natural gas field reservoirs [12, 33]. According to Ehinger *et al.* [11], the archaeal community of the gas field Schneeren-Husum was dominated with the potential methylotrophic and hydrogenotrophic genera *Methanolobus* and *Methanoculleus*, respectively [11]. Despite, the detection of these taxa for high temperature environments [37] and the detection of *mcrA* genes in the deep reservoir fluids, no methanogenesis was detected under increased temperature constraints (Figure 15).

Thus indicating an enrichment of methanogenic *Archaea* within the tubing system, as the temperature decreases gradually during the production process. In the water separation system the temperature was 38-45°C mimicking the temperature range methanogenesis was detected in the microcosm incubations. Moreover, the enrichment of *Methanosarcinales* under well-head conditions is in good agreement with reports of other low-temperature environments [14]. In conclusion, our results provide evidences for an active sulphate reducing population under reservoir and well head conditions while the ecological *in situ* importance of the predominantly detected *Methanosarcinales* [11] remains unclear.

Sulphur transforming microorganisms in the community

The majority of the identified bacterial taxa in both deep reservoir and produced fluids were related to organisms metabolising various sulfur redox states. The detected *Desulfovibrio* spp. and *Desulfuromonadales* have been repeatedly reported for different hydrocarbon associated environments reducing several sulfur redox states with a variety of electron donors including alcohols, fatty acids and carboxylic acids [e.g. 29, 34, 37]. *Pelobacter corbinolicus* and *Geoalkalibacter subterraneus*, found in Z2, were able to reduce elemental sulfur next to Fe(III) and used a variety of hydrocarbons as electron donor in different reservoir systems [16, 26].

The *Acetobacterium* sp., identified here, has been documented as major cultivable hydrogen utilising species in oil field fluids [10]. *Acetobacterium* produces acetate via the reductive acetyl-CoA pathway and was documented to live in syntrophic partnerships [13]. Despite putative thermophilic taxa, the detected *Desulfobutulus* sp. was isolated from a hydrocarbon-fed bioreactor run at 25°C. The organism was capable to utilise acetate, propionate, and butyrate amended to oil field formation fluids [17].

A member of the *Pseudomonadaceae* was very abundant in the Z3 deep reservoir fluids and was related to an uncultured bacterium clone (GenBank JN030548) from rock fissure fluids collected from deep subsurface bore holes. The detected alphaproteobacterial *Sphingomonadales*, an uncultured *Novosphingobium* (EU36028), has been isolated from deep saline aquifer fluids as well. Interestingly, for both taxa hydrocarbon mineralization under anaerobic conditions has been described in connection to denitrification and fermentation [1, 19].

The detected *Petrotoga* sp. was already described by Ehinger *et al.* [11] and seems to represented a persistent member of the microbial community of the formation fluids. For the cultivated representatives of the genus *Petrotoga*, e.g. *Petrotoga halophila*, the fermentation of mainly carbohydrates (mono- and oligosaccharides) was described besides the reduction of sulfur and thiosulphate [31]. In general the fluids showed a quite

diverse sulfur utilising community that can use a broad spectrum of substrates as indicated with the stimulated microcosm experiments.

Community shift towards a high abundance of Clostridiales

Surprisingly, the community analysis revealed a strong increase of the thermophilic spore-forming *Clostridiales* by March 2011 (Table 3 and 4). The community shift showed a pronounced enrichment of two *Clostridium* spp, *Desulfotomaculum* spp., and *Caminicella sporogenes* for the production fluids. These taxa were also traced in the deep reservoir fluids of July 2009 but became increasingly abundant by March 2011. Especially, the detected genus *Desulfotomaculum* has been repeatedly reported for high temperature reservoir formations reducing sulphate, sulfite and elemental sulfur by oxidation of e.g. n-alcohols, LMWOA and monosaccharides [36, 43]. Cultured representatives of *Caminicella* were described as fermentative heterotrophs from high temperature environments [2]. Further, *Thermovirga* species (only present in minor abundance in Z2) have been described as amino-acid fermenting organisms, although strains were also reported to reduce elemental sulfur [7]. Interestingly, the enriched *Clostridiales* have been repeatedly postulated as indigenous community members for high temperature reservoirs [8, 22, 37].

Since, the Schneeren-Husum reservoir is economically exploited, technical measures were constantly implemented to increase or stabilise its productivity. These measures changed the fluid production either permanently or temporarily. For example during July 2009, a new production horizon was perforated into the reservoir rock system, opening the unique opportunity for down-hole fluid sampling. However, scaling prevention connected to the perforation included an acidification to solve mineral scales within the tubing system (personal communication with the industrial company).

A sequence of technical measures was started in early Mai 2009 and which is likely the cause of the detected quantitative and qualitative community shift. The increase of *Geobacteraceae* in July 2009 indicated an environmental advantage for these organisms under the temporally acidified conditions during the scaling prevention, which likely

provided increasing ferrous iron concentrations in the fluids. In long term, the applications might lead to an environmental selection of spore-forming *Clostridiales*.

Likely, the industrial interventions temporally shifted the redox potential, the pH and also possibly oxygen was introduced during the perforation. Further the perforation might lead to the influx of so far unproduced formation fluids from the newly opened gas-bearing sandstone layers. Therefore, the presence of the thermophilic spore-forming sulphate reducers either represents a gradual outcompeting of the primary *Proteobacteria* clade by better adapted *Clostridiales* or the influx of new reservoir formation fluids that inhabited a more indigenous community. Since fluid samples in November 2011 and April 2012 indicated the “reappearance” of *Proteobacteria* in the production fluids (Frerichs unpublished data), the second scenario seems more probable.

In final conclusion, our results indicated a general functional advantage sulfur metabolism for the Schneeren-Husum formation. The long-term monitoring showed a highly dynamic microbial community, i.e. in its size, activity potential and structure, which was furthermore affected by the technical interventions to increase gas-productivity. The detected thermophilic spore-forming *Clostridiales* and other thermophilic taxa likely represent indigenous inhabitants of the reservoir biosphere. This study emphasises the need of a detailed microbial community monitoring and the environmental control mechanisms acting on the deep biosphere. These parameters will be important for prospective industrial applications like CCS in deep geological systems in future.

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4.1.5. Supporting Information

Nucleic acid extraction of saline reservoir formation fluids

For DNA extraction varying formation fluid volumes were filtered on polycarbonate filters (0.22 µm; Nuclepore® Track etched membrane; Millipore Inc.) and stored at -20°C until extraction was proceed. The filter retarded cells were lyzed in phosphate buffer (100 mM; pH 8.5) using a bead-mill (FastPrep instrument; MP Biomedical Inc.; 20 sec. set at 4.5), followed by an enzymatic lysis (Lysozym [50mg/L]; ProteinaseK [10 mg/L]; 15 min at 37°C). The DNA was purified from the aqueous phase with phenol-chloroform-isoamyl alcohol mixture (Roth® PCI; 25:24:1) and washed twice with chloroform-isoamyl alcohol (Roth®; 24:1). DNA was precipitated from the aqueous phase in 1:1 volume 2-propanol and 0.3 M sodium acetate at -20°C for 8-12 hours. The precipitated DNA was washed twice with 70% ethanol, dried, and dissolved in ultrapure PCR water (Fluka) and stored at -20°C for further use.

The quantitative real-time PCR was performed using either SYBRGreen I (Power SYBR®; Invitrogen) or a combination of primer and FAM/Tamra labeled probes (using TaqMan® universal master mix II, Applied Biosystems). Melting curves (68 -95°C) were measured after each run using SYBRGreen I-specific fluorescence as detection method. Each environmental sample was measured in triplicate with two to three different dilutions to check for PCR inhibition. For Archaea and Bacteria , the detection limit was 10² to 10³ 16s rRNA gene copies while the other assays detected <50 gene copies (please refer to publication sources for detailed description). The results were calculated as gene copies mL⁻¹ of fluid sample with a standard error mean of the replicated measurements (SEM with n = 5 - 6). The site specific variability was tested using two to three separate extractions of fluid samples from November 2008 and April 2009 showing only minor variations in the SEM range (data not shown) between the parallel extractions.

Short description of processing and results of the microbial community in substrate amended microcosms

Additionally to the community description in the original fluids T-RFLP fingerprints of substrate amended microcosms were conducted to analyse the induced community. The DNA of the enrichments was extracted after 60 days of incubation under ambient conditions (not pressurised at 30°C). The enrichment culture material was pooled from the triplicates to increase the total sample volume (min. 50-70 ml). The fluorochrom labeled amplicons were restricted with *AluI* (Ehinger *et al.* (11)) to analyse the enrichments amended with acetate, H₂/CO₂ and methanol from April 2009.

The abundance levels of the identified T-RFs was determined using the constructed data matrix of the T-Rex platform corresponding to the processing of the original fluids . Since the appearance of T-RFs in the fingerprint was not seen as good indicator for a significant stimulation of the corresponding organisms the constructed data matrix was used for quantitative comparison (Culman *et al.* (6)). The relative abundance levels of T-RFs were used to virtually form distribution profiles (Figure S3). The given identified T-RFs represented the significantly stimulated organisms for each substrate with the abundance level given in percentage of the total community.

In accordance to the original fluid community analyses, the substrate stimulated microcosms showed considerable similarities in the distribution of the proteobacterial clades and *Clostridiales*. Although the enrichments were conducted with produced fluids of April 2009, the generated amplicon libraries were sufficient for the identification of the stimulated community indicating the agreement of the different time points (Figure 20).

The unamended microcosms of Z2 showed a rather diverse fingerprint with T-RF 338 affiliated with *Desulfotomaculum* sp. (10% of the community), T-RF 138 as *Petrotoga* sp. (11%), two T-RFs 72/244 identified as *Thermoanaerobacter* spp. (25% and 8%, respectively), and T-RF 234 identified as an uncultured *Marinobacter* spp. (20%) that was also previously identified by Ehinger *et al.* (11). The different incubation setups with Z2 fluid induced under methanol two T-RFs identified as *Desulfovibrio* spp. (8% and 26%,

respectively), T-RF 138 *Petrotoga* sp. (26%) and an unknown T-RF at 286bp that could not be identified. Only one T-RF was induced at 141 bp with acetate that was assigned as putative *Sphingomonadaceae*, an uncultured *Novosphingobium* (58%). Hydrogen induced T-RF 181 assigned as *Desulfovibrio* sp. (T-RF 181; 79%) while T-RF 138 was decreased (*Petrotoga* sp. 6.5%).

The unamended microcosms of Z3 were dominated by one T-RF at 141 that was identified as *Sphingomonadaceae* (93% of the community) and with lower abundance the T-RF of the *Petrotoga* sp. (T-RF 138; 7%). The supplemented substrates induced a much higher diversity showing for acetate the stimulation of the already for Z2 detected T-RFs. For example T-RF 234 of the uncultured *Marinobacter* (18.7%), T-RF 181 of *Desulfovibrio* sp. (6.8%), two T-RFs at 249 and 338 bp identified as *Desulfotomaculum* spp. (6% and 3%, respectively), and T-RF 244 a *Thermoanaerobacter* sp. (14%). In hydrogen amended microcosms, T-RF 66 identified as further *Desulfovibrio* sp. (45%) and the T-RF of *Petrotoga* sp. (T-RF 138; 28%) were dominant. For the methanol amended incubation, the T-RF of the putative *Sphingomonadaceae* (61%) was highly abundant.

Supplemented Tables and Figures

Table 5: Overview about quantitative PCR (qPCR) assays used in this study.

Target Organism	& gene	Primer	Sequence (5'-3')	Annealing	Reference
<i>Bacteria</i>	16S rRNA	Bac331F Bac797R Bac	TCCTACGGGAGGCAGCAGT GACTACCAGGGTATCTAATCCTGTT CGTATTACCGCGGCTGCTGGCAC	60°C	(1)
<i>Archaea</i>	16S rRNA	Arch341F Arch806R Arch516	G(C/T)GCA(G/C)CAG(G/T)CGA(C)GAA(A/T) GGACTAC(A/G/C)(G/C)GGGTATCTAAT TG(C/T)CAGCCGCCGCGGTAA(A/C/T)ACC(A/G/C)GC	59°C	(2)
SRP ¹	<i>dsrA</i> [‡]	DSR-1F+ DSR-4R	AC(G/C)CACTGGAAGCACGGCGG GTGG(A/C)(A/G)CCGTGCA(G/T)(A/G)TTGG	60°C	(3)
<i>Archaeoglobus</i>	<i>dsrB</i>	Arch1830F DSR4R	TGCTGTC(A/G/C/T)AACATG GTGTAGCAGTTACCGCA	56°C	(4)
Methanogens	<i>mcrA</i> [†]	ME 1F ME 3R	GC(A/C)ATGCA(A/G)AT(A/C/T)GG(A/T)ATGTC TGTGTGAA((G/C)CC(G/T)AC(A/G/T)CCACC	54°C	(5)
<i>Geobacteraceae</i>	16S rRNA	GEO494F GEO825R	AGGAAGCACCGGCTAACTCC TACCCGC(A/G)ACACCTAGT	60°C	(6)

¹SRP; sulphate-reducing prokaryotes targeting the [‡]dissimilatory sulphite reductase subunit A (*dsrA*) while subunit B was specifically detected for *Archaeoglobus*.

[†]methanogens were detected using methyl Coenzym-M subunit A

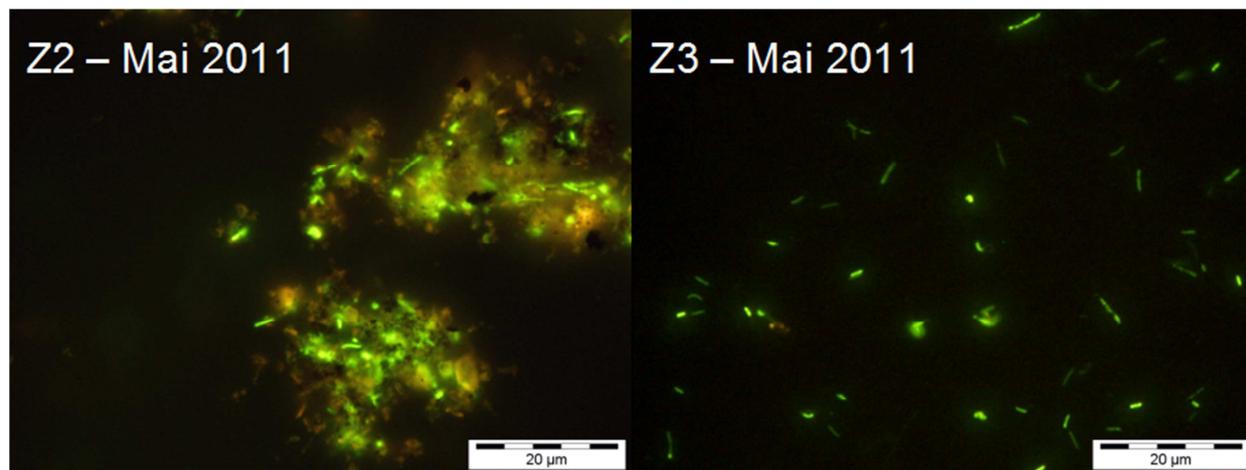


Figure 18: Microscopic pictures of produced fluids in May 2011 showing the particle associated microorganisms in Z2 in comparison with the “free living” cells in Z3 formation fluids.

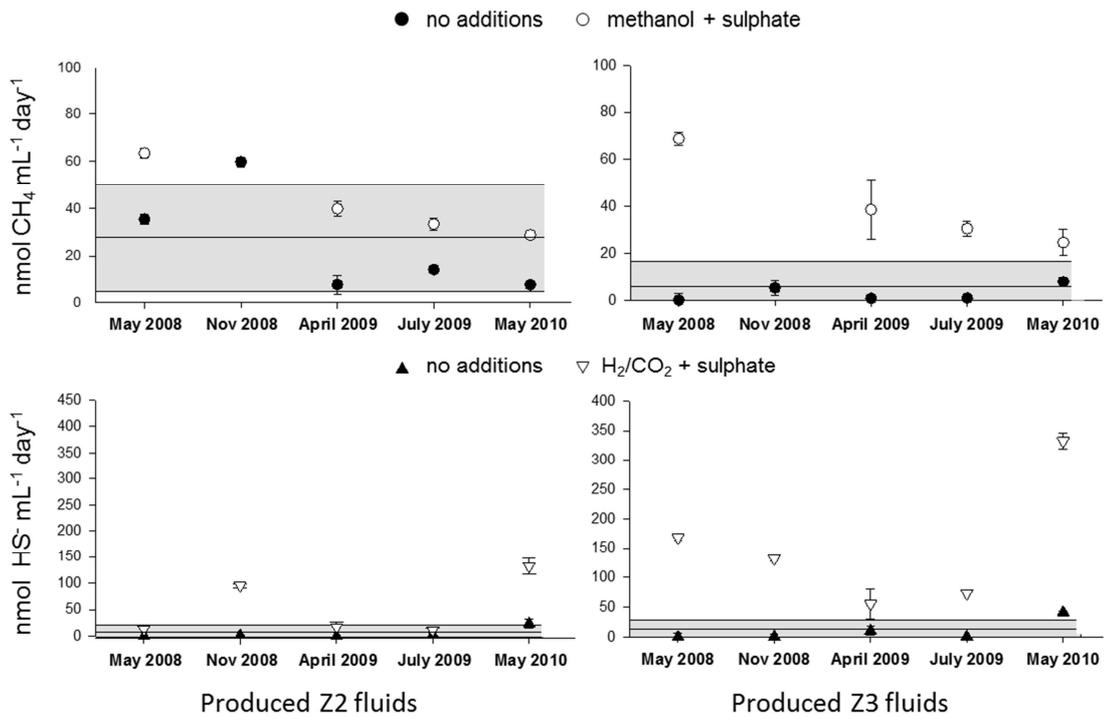
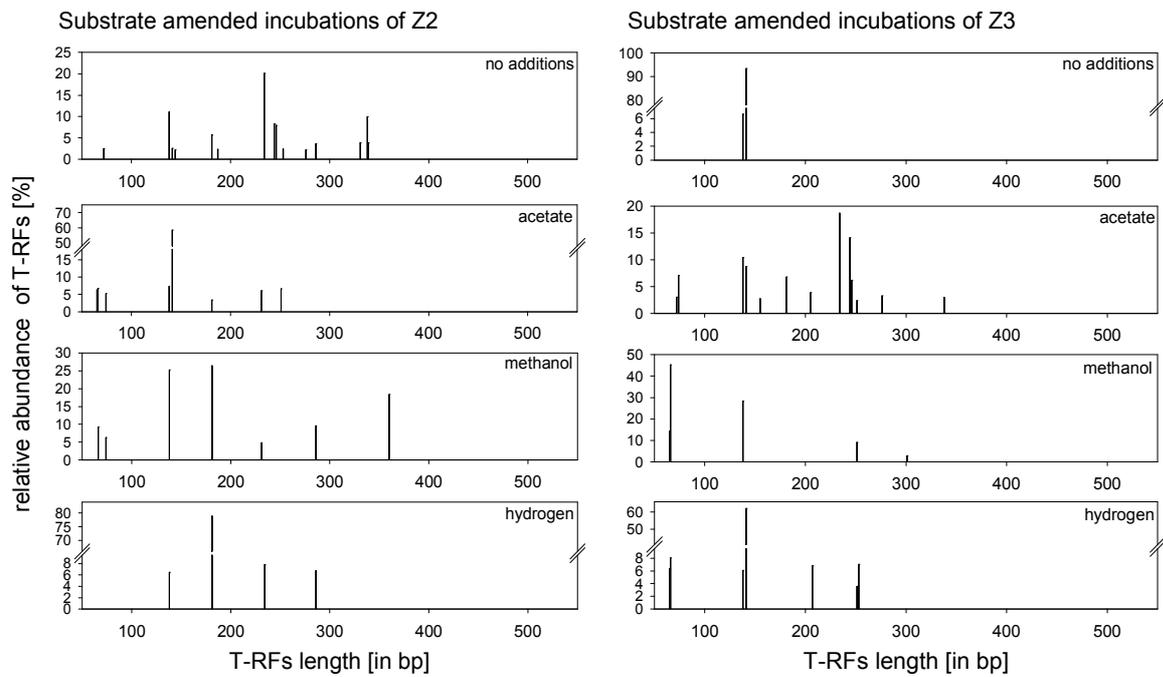


Figure 19: Variation of microbial methane production (upper panels) and sulphate reduction (lower panels) over two years in production fluids of Z2 and Z3 in substrate amended microcosms (open symbols) or in controls (closed symbols) showing the indigenous activity. Error bars represent the standard deviation of three to six replicates. The grey shaded area illustrates the arithmetic mean (solid line) of the indigenous activity with its standard deviation (n=21; outlines of the grey area). No methanol amended microcosms were prepared in November 2008.



T-RF	%ID	Next relative [Acc]	T-RF	%ID	Next relative [Acc]
65/66	97	<i>Desulfovibrio dechloracetivorans</i> [AB546253]	234	99.6	uncultured <i>Marinobacter</i> [EU999012]
73	97.6	uncultured <i>Pelobacter</i> sp. [DQ647155]	236	97.6	<i>Prolixibacter bellariivorans</i> [AB541983]
138	98.9	<i>Petrogala halophila</i> [AY800102]	244	99.6	<i>Thermoanaerobacter</i> sp. [GU561619]
141	97.1	uncultured <i>Novosphingobium</i> sp. [EU360289]	246	99	<i>Desulfotomaculum geothermicum</i> [AJ294428]
155	95	uncultured <i>Clostridiaceae</i> 4 (not further assigned)	251/252	97.6	<i>Pelobacter carbinolicus</i> [CP000142]
181	97.8	<i>Desulfovibrio longus</i> [AY359867]	276	97.6	<i>Geoalkalibacter</i> sp. [AB260047]
187	98.8	<i>Thermoanaerobacter acetoethylicus</i> [X69336]	286	92.6	uncultured <i>Acetivibrio</i> [JN448695]
205	98.1	<i>Sphingomonas</i> sp. [AB288317]	301	97.6	uncultured <i>Thermovirga</i> [DQ647105]
207	99.8	<i>Desulfovibrio gabonensis</i> [U31080]	338	99.8	uncult. <i>Desulfotomaculum</i> [EU999019]
231	98.9	<i>Desulfobotulus</i> sp. [U85470]	360		Not in the 454-amplicon libraries

Figure 20: Comparison of bacterial community structure and relative TRF abundance (representing cumulative 80-95% of the community) in enrichment cultures of produced fluids (April 2009). The TRFs were assigned using the 454-amplicon libraries of constructed contigs with the corresponding predicted TRF length and the already existing TRF database of the site (Ehinger *et al.* 2009) (%ID represents the similarity of the closest relative using SeqMatch). Fragment patterns were obtained with restriction enzyme AluI.

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4.2. Viability and adaptation potential of indigenous microorganisms from natural gas field fluids in high pressure incubations with supercritical CO₂

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Keywords: CCS, dissimilatory sulphate reduction, spore-forming Clostridiales, T-RFLP, qPCR, natural gas field

Running Title: Survival of indigenous microorganisms stressed with supercritical CO₂

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Abstract

Carbon Capture and Storage (CCS) is currently under debate as large-scale solution to reduce the emissions of the climate affecting greenhouse gas CO₂. Depleted gas or oil reservoirs and saline aquifers are considered as suitable reservoirs providing sufficient storage capacity. We investigated the direct influence of high CO₂ concentrations on the indigenous microbial population in the saline formation fluids of a natural gas field. In incubation experiments with formation fluids under near *in situ* high pressure and temperatures the microbial community changes were closely examined at elevated CO₂ concentrations.

Conditions in the reactor systems simulated reservoir fluids, (i) close to the injection point saturated with CO₂ and ii), closer to the outer boundaries of the dissolution gradient. While total cell number showed only minor variations, no sulphate reduction was detected during the incubations with CO₂. After transfer to ambient conditions an actively growing sulphate-reducing community was re-established. The predominance of spore-forming *Clostridiales* during and after the CO₂ stress provided evidence for the resilience of this taxon for the antimicrobial influences of supercritical (sc)CO₂. The viability potential of fermentative and sulphate-reducing bacteria has to be considered in the selection, design, and operation of CCS sites.

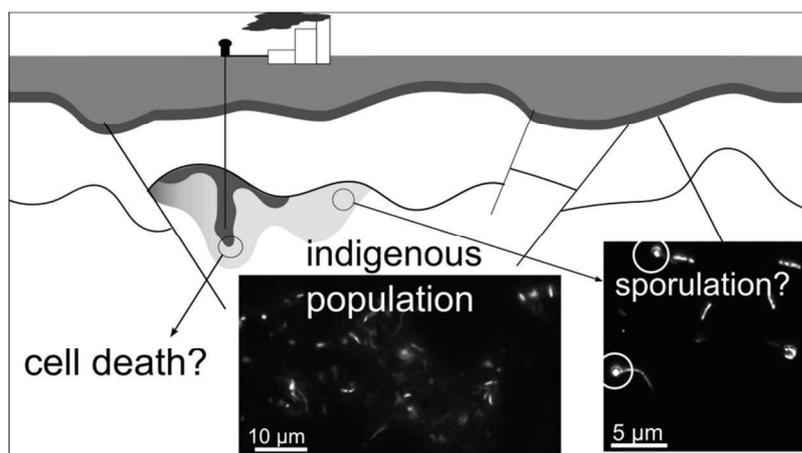


Figure 21: Description of the experimental and scientific setup of the high pressure reactor incubations mimicking different locations within an evolving CO₂ concentration gradient.

4.2.1. Introduction

The increasing atmospheric CO₂ concentration during the last 150 years was related to the anthropogenic usage of the fossil fuels as energy source [21]. One currently discussed technique to reduce these emissions, Carbon Capture and Storage (CCS), suggested the separation of the produced CO₂ and the subsequent injection into suitable storage reservoirs [18]. Potential storage sites are deep saline aquifers and depleted gas or oil reservoirs that meet the criteria in storage capacity and cap rock integrity to prevent the upward-migration of stored CO₂ [20]. Furthermore, only storage reservoirs below 0.8 km depth are providing the physical conditions to store CO₂ in its supercritical state in order to minimise the necessary injection volume [20]. The injection involved the accumulation of supercritical (sc)CO₂ at the top boundaries of the reservoir due the density differences of the scCO₂-phase and the formation fluids (physical trapping) [29]. With the successive dissolution of the CO₂ (solubility trapping) in the formation fluids the pH will be acidified [23] leading to mineral dissolution and finally to the long-term mineralisation of the CO₂ as carbonates (mineral trapping) [22; 23; 29]. Altogether, the trapping mechanisms will lead to the formation of concentration gradients comparable with contaminant plums in groundwater with the highest dissolved CO₂ concentrations (saturation) at the injection point and the top boundaries [28; 32].

The discussed storage sites, especially the hydrocarbon reservoirs, often represent hotspots within the terrestrial and marine deep biosphere with elevated population sizes and mineralisation activities [19; 27]. The influence of these organisms on the geochemical conditions of their environment could enhance the mineralisation trapping mechanism for the injected CO₂ in the reservoir [31]. Kirk *et al.* [24] reported that acetate-dependent iron(III)-reducing bacteria were able to gain even more energy under CCS conditions, while the energy availability for sulphate-reducing bacteria and methanogenic archaea was unaffected. Urea degrading cultures of *Sporosarcina pasteurii* decreased the porosity of the incubated sandstone core due to biofilm formation and precipitation of

calcium-carbonate [36] which was postulated as possible mechanisms to seal leakage pathways in the sandstone matrix.

However, recently severe problems were reported, like the inhibition of urease activity under anoxic conditions [28]. Instead of bioaugmentation of organisms and substrates [36] the indigenous microbial population presumably can also be stimulated by dissolved organic compounds like acetate which accumulated in formation fluids after mobilization from the sandstone matrix by scCO₂ [22; 40].

Also antimicrobial effects of scCO₂ are documented, like the reduction of the germination indexes of fruit juices and meat products [16]. Supercritical CO₂ permeabilised the cell membranes, decreased intracellular pH, inhibited protein synthesis [1], and finally lead to cell death [16]. The negative effects were shown to act stronger on planktonic cells than on sessile biofilms [30], and spore-forming organisms seem to sustain the CO₂ stress due to their Gram-positive cell wall structure [42]. However, these studies were conducted on pure cultures or defined mixed cultures, so the knowledge about the effects of scCO₂ on an indigenous reservoir community is still scarce [32]. Particularly, the adaptation potential of a complex environmental microbial community stressed with scCO₂ is not known.

Consequently, this study aimed at investigating the potential of indigenous microorganisms in saline formation fluids of a natural gas field (North German Plain, Germany) to survive during and after simulated injections of scCO₂. The experiments mimicked the environmental conditions in pressure and temperature in the reservoir for different compartments of the CO₂ gradient [17; 23]. These zones can be defined as: (i) fully CO₂ saturated fluids close to the injection point and (ii) lower CO₂ concentration in more distance to the injection point with non-limiting energy resources. The identification of resistant and sensitive community members, potential mineralisation activities and the recovery potential after release of the scCO₂ allows us to understand the adaptation potential of the reservoir biosphere, thus possibly affecting the application of CCS.

4.2.2. Methods

General processing of formation fluids and microbiological characteristics of the formation

At the well head facility of the gas reservoir produced fluid was sampled from the surface gas/water-separation system. For each experiment 10 litre fluid was sterile sampled and immediately transported to the lab [27]. To determine the total cell counts (TCC), formation fluids were fixed overnight in 2% (v/v) formaldehyde in PBS (130 mM NaCl, 5 mM Na₂HPO₄, 5 mM NaH₂PO₄) at 4°C. The fixed material was sonicated (20 sec at 20% in 2 cycles), filtered in appropriate volumes onto 0.22 µm black polycarbonate filters (GTBP, Millipore) and stained with SybrGreen[®] (Invitrogen; embedded with Moviol/glycerol [26]). At least 800 cells per replicate filter were counted and calculated as cells ml⁻¹ (SD; n=2-3). Samples for water chemistry were filtered (0.45 µm pore size) and stored at 4°C. The concentrations sulphate and other mayor ions were determined by ion chromatography with a DX-500 system (Dionex, Germany).

Ahead of this study, the gas field formation fluids, that is, the water chemistry, the microbial activity, and the reservoir community, were monitored over almost 6 years (Frerichs unpublished data and Ehinger *et al.* [12]). The produced fluids of the respective well head contained about 984 ± 32 mg L⁻¹ sulphate (10 mM), 2200 mg L⁻¹ HCO₃⁻, considerable amounts of dissolved organic material (acetate, alcohols etc.) and about 28 g L⁻¹ total dissolved solids (TDS – with 0.39 mol NaCl dissolved) [12]. The microbial biosphere in the formation fluids comprised of methylotrophic archaea [12] and a diversity of sulphur utilising and fermentative bacterial species including *Desulfovibrio*, *Desulfomonadales*, *Desufotomaculum*, *Thermoanaerobacter* and *Petrotokea* (Frerichs unpublished data). Especially sulphate-reducing bacteria were active, using a variety of organic substrates and showed mineralisation activity also at the estimated reservoir temperature of about 80°C. Additional high pressure incubations with high CO₂ concentrations (up to 40 bar pure CO₂) were conducted in advance of this study. These experiments used enrichment cultures obtained from the same reservoir growing on fructose. The incubation revealed only very minor reactivation potential of the enriched

organisms. A short description of the method and the results is given in the supporting information (with Figure 25 and Figure 26).

Preparation of fluid samples for pressure incubations

Two separate experiments were conducted with freshly sampled formation fluids. The experiments represented the assumed environmental conditions in different compartments of the CO₂ gradient that would evolve after the injection of scCO₂ in the reservoir system [17; 23] (refer to Figure 22): a) fully CO₂ saturated fluid close to the injection point with CO₂ saturated formation fluid (saturation experiment) and b) in distant to the injection point with a CO₂ undersaturated formation fluid that got amended with amiable energy resources (stimulation experiment).

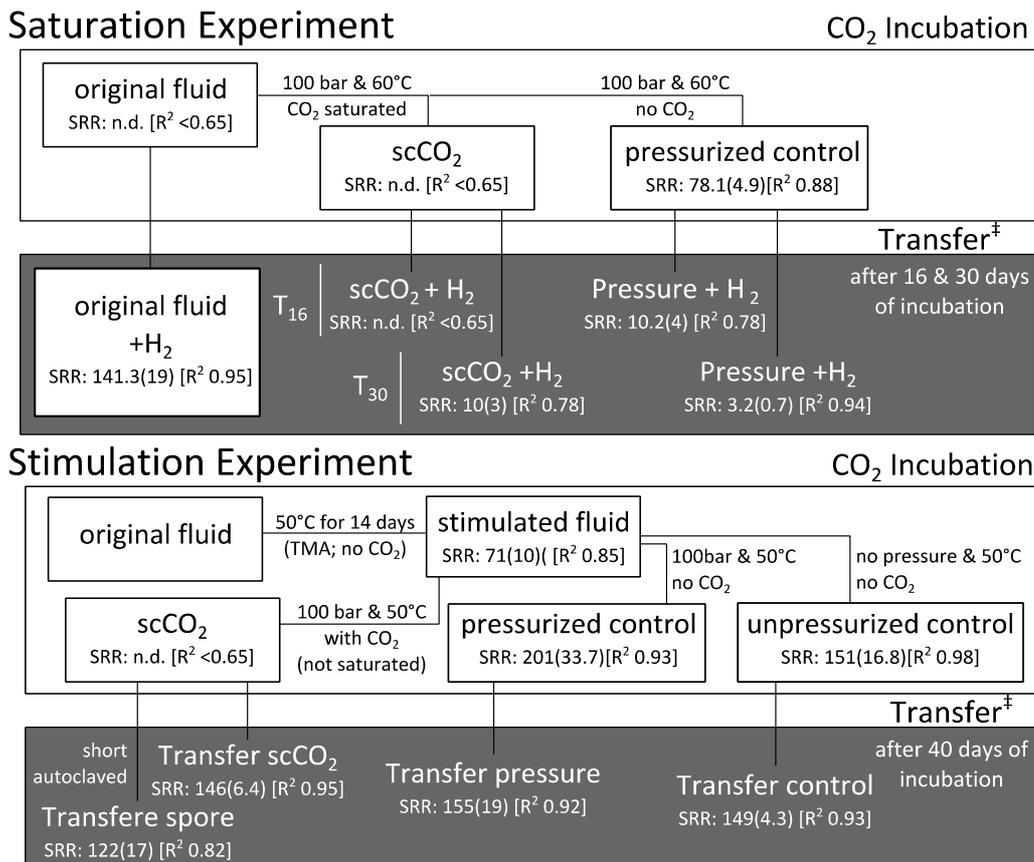


Figure 22: Experimental setup (design and conditions) of saturation and stimulation experiment with both experimental phase, CO₂ incubation and transfers for the reactivation approach. The sulphate reduction rates (SRR) were given for each incubation set-up in nmol mL⁻¹ day⁻¹ (± SD n=2-3) with the correlation coefficient of the linear regression (R²). ‡Transfers were conducted with sample material from the respective incubation and incubated under ambient pressure at 50 or 60°C.

For the saturation experiment the formation fluid was immediately upon arrival subdivided into two setups: (i) pressurised control (10 replicates of 10 ml each), and (ii) one experimental batch for the incubation with scCO₂ (80 ml).

For the stimulation experiment the production fluid was stimulated for two weeks prior to the actual experiment to enhance microbial mineralisation activity before starting the experiment. For stimulation the fluid was filled into heat sterilised glass bottles within an anoxic glove chamber (Mecaplex), sealed with butyl stoppers, and repeatedly flushed with N₂ to remove residual O₂. Microcosms were amended with sulphate (final 20 mM) and trimethylamine (TMA; 5 mM) and incubated for 14 days at 50°C. At the beginning of the high pressure incubation the batch culture was subdivided anaerobically into the following setups (see Scheme 1): (i) non-pressurised control (3 replicates of 30 mL each), (ii) pressurised control (10 replicates of 10 ml each), and (iii) one experimental batch for the incubation with scCO₂ (100 ml). TMA was chosen to prevent the application of gaseous substance other than CO₂ in the system.

For both experiments the starting point was defined by sub-sampling the fluid for DNA extraction (community analyses), total cell counts (TCC), sulphate measurement and pH-values. In case of the saturation experiment the starting point equals the original fluid since no delay in the experimental setup was conducted.

Pressure incubation systems and analytical procedure during the experiments

The pressurised controls without application of CO₂ were incubated using a hydrostatic pressure systems [33]. Each replicate consisted of a completely filled and sealed vial (10 mL), connected via a hypodermic needle with a sterile syringes (5 ml). Each syringe was filled with sterilised formation fluid to transmit the external hydrostatic pressure onto the vial microcosm. Thus prepared microcosms were placed into a cylindrical pressure-proven steel vessel that was filled with demineralised water and pressurised to 60 bar using an air-driven hydraulic pump system (Dustec GmbH) [33]. The pressurised system was heated to 50-60°C whereby the pressure increased to a pressure of 100 to 110 bar. During the experiment the pressurised control was sampled at selected time points to

minimise the negative effect of repeated decompression and pressurisation [15]. For DNA extraction, total cell counts, sulphate concentration and pH-measurement 2-3 replicates were withdrawn from the system. At the same time points samples were taken from the unpressurised control kept at ambient pressure at 50°C.

Supercritical CO₂ incubations were conducted in a cylindrically shaped gold bag of about 100 ml volume closed with a titanium cap [44]. Prior to use, the complete assemblage was heat sterilised to prevent contamination problems and to oxidise the titanium surface (5h at 400°C). The gold bag was filled with the sample in an anoxic glove chamber (Mecaplex) and sealed with the titanium cap. The closed assembly was placed into a commercial available pressure vessel (Parr Instrument), filled with demineralised water and heated to 50-60°C. The system was pressurised after equilibration of the temperature with a syringe pump (Teledyne ISCO, Inc. US) holding the pressure continuously at 100 bar. Gaseous CO₂ was pressurised to 110 bar to reach supercritical state before dispersing it into the fluid within the gold bag. The CO₂ solubility in the Z3-brine at the given temperature, pressure and salinity was calculated using the online available NaCl-Saline-CO₂ algorithms [10].

Temperature and pressure were constantly recorded showing less than 1% variance over both experiments (data not shown). Samples were extracted via sampling ports directly from the formation fluid phase within the gold bag without pressure loss or temperature changes. At each sampling point the pH was measured at ambient pressure and room temperature (VWR, pH100), while samples for total cell counts and DNA extraction (sample volume between 1-3 ml) were prepared. To minimise the necessary sample volume from the scCO₂ reactor, a dilution was prepared for the sulphate measurement using 1% HNO₃. For the exclusion of dilution errors the measured ion concentrations were normalised against the sodium concentrations measured in the undiluted, original fluid sample (performed as duplicates). Microbial sulphate reduction was followed via the decrease of sulphate during the incubation and calculated as nmol mL⁻¹ day [d]⁻¹. The rate was evaluated using the linear regression of sulphate concentration over time while only rates with a significant correlation coefficient were

considered (significance level $R^2 > 0.65$). The geochemical modeling software PHREEQC Version 2.18 [35] was used to calculate saturation indices, species distributions and activities and estimate the pH under the in situ and within the reactor systems.

Recovery & viability assessment

The activity of surviving organisms and possible recovery of the microbial community was verified by transfers of fluid material after and during the incubation with scCO₂ (see Figure 22) into anaerobic and sterilised formation fluid (0.1 µm filtered). The effective sterilization of this media was proven as no cells, activity, or extractable DNA was observed in the sterilised media with or without added substrates (data not shown). Fluid samples of the saturation experiment were transferred at day T₀, T₁₆ and T₃₀ and amended with sulphate (20 mM) and hydrogen (H₂/CO₂ 80/20 Vol%), since hydrogen has been shown to possess the highest stimulation potential (Frerichs unpublished data). The activity was compared to original fluid samples that were directly prepared after the sampling approach. These control microcosms of original fluid were stimulated with the same substrate combination to verify and quantify the activity potential of the transferred sample material from the pressurised systems. All transfers and control microcosms were incubated at 60°C for 40 days (ambient pressure) and then prepared for DNA extraction by pooling the triplicate incubation.

From the stimulation experiment the final time point after releasing CO₂ and pressure (T₄₀) was transferred and amended with TMA and sulphate (5 and 20 mM, respectively). All transfers were prepared in triplicates from unpressurised and pressurised controls and from the scCO₂ reactor. Additionally, the proportion of thermophilic spore formers was tested for the scCO₂ incubation by transferring a second sample after short autoclaving at 95°C for 15 min. In the following description this sample is further referred to as “spore-transfer”. All prepared microcosms were incubated for at 50°C for 60 days (ambient pressure) and then prepared for DNA extraction to compare the community re-established in the transfers (see Figure 22).

Microbial sulphate reduction activity was measured by the precipitation of copper sulphide from continuously accumulating dissolved sulphide (HS^-) in the media [4]. Rates were calculated as $\text{nmol ml}^{-1} \text{ day}^{-1}$ (SD of $n=2-3$ microcosms; $R^2>0.65$). Possible methane accumulation in the headspace was monitored weekly (gas chromatographically [33]) but no methanogenesis activity was detected at any time point (data not shown).

Molecular biological analyses

For DNA extraction fluid samples of the original formation fluid (200-300 ml), of several time points during the incubation (about 1-3 ml), and from the pooled transfer microcosms (40-60 ml) were filtered onto a $0.22 \mu\text{m}$ polycarbonate filter (GTTP, Millipore) and stored at -20°C until further processing. The filter-retarded cells were lysed in phosphate buffer (100 mM; pH 8.5) using bead-mill (FastPrep instrument; MP Biomedicals 10 sec. and 4.5), followed by an enzymatic lysis (Lysozym [50mg/L]; ProteinaseK [10 mg/L]; 15 min at 37°C) and purifying the DNA from the aqueous phase with phenol-chloroform-isoamyl alcohol mixture (Roth® PCI; 24:23:1). Before DNA precipitation with 2-propanol (-20°C for about 12 hours), the extract was washed twice with chloroform-isoamyl alcohol (Roth®;24:1) to remove the phenol residues. The resulting DNA was dissolved in 100 μl ultrapure PCR water (Fluka) and stored at -20°C .

Since, sulphate reduction was the predominant mineralisation activity sulphate-reducing prokaryotes were quantified targeting the *dissimilatory sulphite reductase* operon (subunit a; *dsrA*) in a quantitative PCR (qPCR) approach [41] using the primers DSR1F+/DSR-R [25]. The specifically amplified *dsrA* gene of *Desulfobacterium autotrophicum* DSM 3382 was used as internal standard and each run was finalised with a melting curve to check for unspecific by-products or primer dimers. The specific detection limit of the fluid was calculated according to Bach *et al.* [2] whereas, unspecific PCR products in non-template controls (here $C_t \Rightarrow 34$) were reported to cover the specific signal from the samples. Including the dilution factor necessary to overcome co-extracted inhibitors and the extraction volume of the formation fluid about $300-400 \text{ cells mL}^{-1}$ were calculated as sample specific detection limit.

For terminal restriction fragment length polymorphism (T-RFLP) bacterial 16S rDNA amplicons were prepared with Ba27f*5'fluorochrom/907r using described PCR protocols [37]. Amplification of flouochrome marked amplicons was not successful for the day 16 of the saturation experiment due to poor DNA recovery (data not shown) and was excluded for day 1 to 37 for the stimulation experiment due to the apparent sedimentation of the cells (see Figure 1). The amplicons were digested with MspI [37] and the lengths of terminal fragments were determined. The T-RFLP profiles of each fluid sample were analysed using the t-REX software [5] for denoising the datasets and peak alignment into defined T-RF. The alignment was used to evaluate the sample specific abundance of each T-RF. Significantly changed T-RFs were identified using *in-silico* predicted T-RFs from established 454-amplicon libraries of the formation fluids (Frerichs submitted manuscript). The t-REX integrated MatLab tool was used to analyse Additive Main Effect and Multiple Interaction (AMMI) to produce two-dimensional interaction plots evaluation the Interacting Principal Components (IPCAnalysis; cumulative about >85% of the variation explained) as previously described for T-RFLP community analyses by Culman *et al.* [6]. For the variance model the following variables were defined: CO₂ presence/absence, pressurised/unpressurised, substrate addition (unamended, Hydrogen, TMA), transfer/original fluid, day of sampling.

4.2.3. Results and discussion

Conditions during the different incubation experiments

In the saturation experiment the CO₂ concentration was saturated at 0.95 CO₂ mol kg⁻¹ formation fluid [10] (44'000 mg kg⁻¹ brine) which resembled a 19-fold increase of the dissolved carbonate concentration. No additional energy or carbon source was amended other than the originally provided sulphate and organics (Ehinger *et al.* [12] and Frerichs unpublished data). The dissolution of CO₂ decreased the pH from circumneutral to 5.4 (Figure 23). The estimated pH would be somewhat lower (3.2) under pressure and temperature in the reactor system [35] as no significant mineral phase or substrate with buffering capacity was applied. Sulphate concentrations were stable over time and decreased slightly in the pressurised (no CO₂) system (Figure 23).

For the stimulation experiment the dissolved CO₂ concentration was about 7-fold increased (0.35 M CO₂) compared to the original concentration and the pH decreased from circumneutral pH to 6.3 within the first 6 hours after injection (Figure 23). After full equilibration the pH was lowered to 5.9± 0.1 (average pH between day T₇ and T₄₀). The estimated pH of this system (pH 4.2) was partially buffered by the added TMA. The sulphate concentration of the stimulated system was alike the saturation experiment stable over time, while the pressurised control reactor showed a considerable decrease over time (Figure 23).

Under the actual *in situ* conditions (80°C, 200 bar 0.39 M NaCl) about 1.04 mol L⁻¹ CO₂ would fully saturate the gas field fluids [10] slightly more than in the saturation experiments but the pH was likewise estimated at 3.2. However, this calculated estimation is much higher than previously measured values for reservoir formations with scCO₂ injection [13; 22]. Kihm *et al.* [23] modelled the dissolution and mineralisation processes including the fluid convection showing that only very localised around the injection point the fluid will be fully saturated with CO₂. Therefore, the CO₂ concentrations in our microbiological experiments simulated realistic conditions for the reservoir corresponding to different areas of the CO₂ injection plume: (i) in proximity to the interface of brine and

scCO₂ phase and (ii) in greater distance from the injection well or the formation trapped scCO₂ plum following the diffusion gradient. For the reservoir system a considerable buffering capacity could be expected including mineral dissolution processes [29] and the dissolution of organics like acetate [22; 40]. The temperature in the reactor systems (50 and 60°C) was an adaptation to the short termed experimental setup. At the estimated *in situ* temperatures of 80°C the bacterial activity was much slower than at the chosen experimental temperature (Frerichs unpublished data).

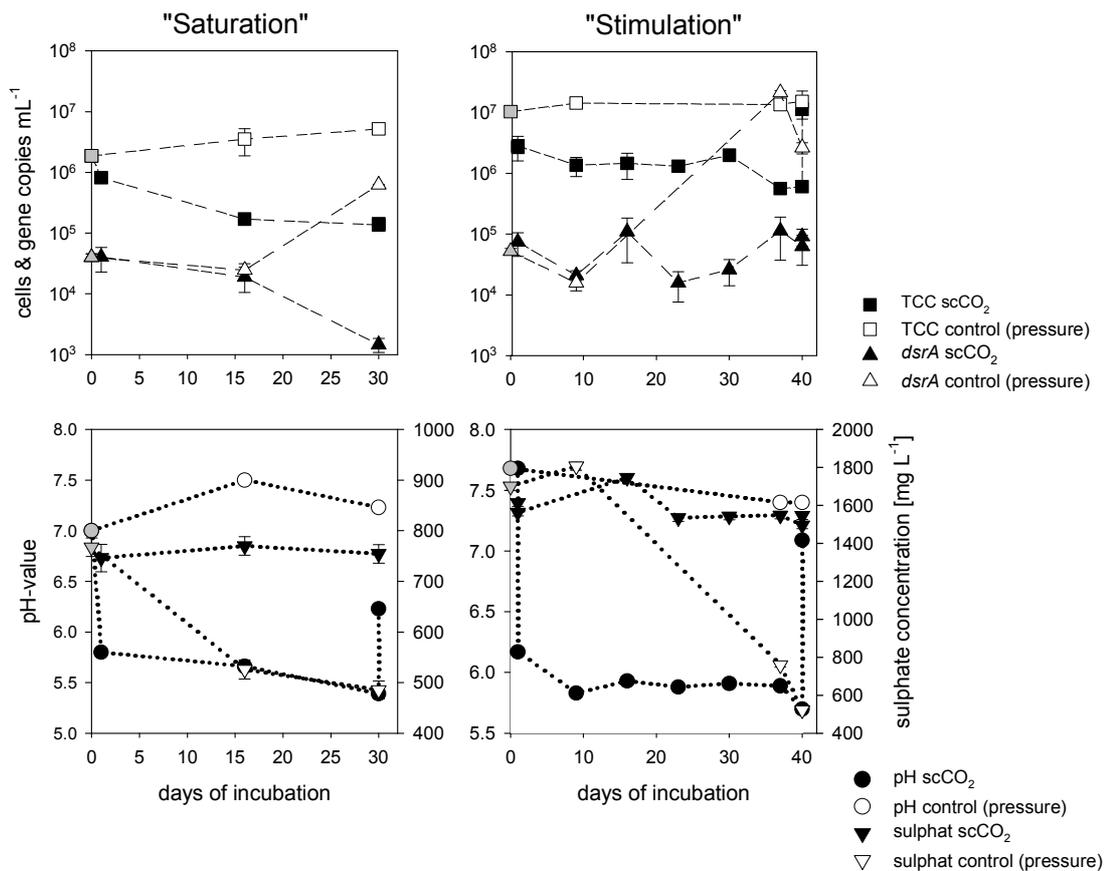


Figure 23: Development of microbiological and chemical parameters during saturation and stimulation experiment showing the pressurised control with open and the scCO₂ reactor with filled symbols. Upper panel: Total cell counts (TCC) and *dsrA* copy numbers; Lower Panel: pH-values and sulphate concentration. Values of the starting point were indicated in light grey for better visualization. At the end of the experiment (30 and 40 days, respectively) two samples were recovered from the scCO₂ reactor, before release of the CO₂ and after release from the homogenised residual fluid in the reactor. Error bars represent the standard deviation and were not shown if smaller than the symbol.

Effects of pressure and scCO₂ on the microbial sulphate reduction activity

The stable sulphate concentration during the saturation experiment showed that no microbial sulphate reduction was taking place (Figure 22; R² 0.5). In contrast, the sulphate concentration in the CO₂-free pressurised control replicates decreased resulting in a net

sulphate reduction of about $78. \pm 4.9 \text{ nmol mL}^{-1} \text{ day}^{-1}$. The unpressurised and unamended original fluid showed, similarly to the scCO_2 reactor set-up, no significant sulphate reduction (R^2 0.52).

During the stimulation experiment the sulphate concentrations in the scCO_2 -reactor were also not significantly changed (R^2 0.35), although some variations were detected (Figure 22). The control setups showed a net sulphate reduction of 201 ± 33.7 and $151 \pm 16.9 \text{ nmol mL}^{-1} \text{ day}^{-1}$ for CO_2 -free pressurised and unpressurised microcosms, respectively.

The rates of the pressurised controls of both experiments indicated an advantage of the sulphate-reducing community under pressure. However, the microbial population of both scCO_2 systems showed no sulphate reduction activity although pressurised. The negative effects of scCO_2 were correlated with a disturbance of the membrane potential [16]. Thereby, respiration processes using the proton-motive force like dissimilatory sulphate reduction could be completely inhibited by the high CO_2 concentration. However, fermentative organisms generating ATP by substrate-phosphorylation and microorganism using for example a sodium driven membrane gradient for ATP generation [38] could maybe survive the disturbance of a proton depended membrane gradient.

Influence of high CO_2 concentrations on cell numbers and *dsrA* gene abundance

The cell density at the starting point of the saturation experiment was $1.8 \pm 0.1 \cdot 10^6 \text{ cells mL}^{-1}$ and the *dsrA* abundance about $4 \pm 0.5 \cdot 10^4 \text{ gene copies mL}^{-1}$. During the incubation the total cell numbers and gene copy abundance of *dsrA* decreased considerably to about $1.5 \pm 0.4 \cdot 10^3 \text{ dsrA gene copies mL}^{-1}$ by the end of experiment in the system (Figure 23). In the CO_2 -free pressurised control the cell density was not changed while the *dsrA* abundance was increased to $6.2 \pm 0.06 \cdot 10^5 \text{ gene copies mL}^{-1}$. Considering the development of the *dsrA* abundance during the first 16 days, the “saturated” system and the pressurised control showed minor differences, while during the second half of the experiments the cell and copy numbers were increased in the control setup. This development indicated a certain time depended influence factor for the scCO_2 incubations.

Actually, the here documented experiments comprise the longest incubation experiment with a microbial population under scCO₂ stress that have been conducted so far. In general, the incubation time with scCO₂ cover 1-2 days [36; 42] or merely hours [1; 30] to investigate the effects on the microorganisms. Since, the estimation of cell numbers or gene copies does not directly indicate a survival of the organisms, the reactivation potential (see below) of the transfer is very important to actually verify the survival potential of the microorganisms.

The stimulation experiment started with a considerably higher cell density of the pre-enriched batch culture (Figure 23; $1 \pm 0.2 \cdot 10^7$ cells mL⁻¹). Considering the cell density, the non-stirred scCO₂ reactor system showed a considerable decrease after CO₂ application. However, the decrease was connected to the sedimentation of the cells. Thereby, these samples were also later excluded from the statistical comparison of the community as the fingerprints could not explain the community development during the incubation. After CO₂ degassing at the end of the experiment and resuspension of the residual fluid no significant decrease in the total cell numbers ($1.1 \pm 0.1 \cdot 10^7$ cells mL⁻¹) was detected. More importantly, the cell density was alike with the evaluated density of the pressurised control ($1.5 \pm 0.7 \cdot 10^7$ cells mL⁻¹). In comparison, the cell density of the unpressurised control was slightly increased ($3.9 \pm 0.4 \cdot 10^7$ cells mL⁻¹).

The gene abundance of sulphate-reducing prokaryotes (*dsrA*) was about $5.3 \pm 0.6 \cdot 10^4$ copies mL⁻¹ at the beginning of the stimulation experiment (Figure 23). Similarly to the sulphate concentrations, the *dsrA* abundance in the scCO₂ reactor was not changed during the incubation ($5.6 \pm 0.7 \cdot 10^4$ gene copies mL⁻¹). The *dsrA* abundance in the pressurised control system increased to $1.2 \pm 0.9 \cdot 10^7$ gene copies mL⁻¹ in correspondence with the high sulphate reduction rate. The *dsrA* gene abundance increased also in unpressurised control to $9.6 \pm 4 \cdot 10^6$ gene copies mL⁻¹ fitting to the slightly lower sulphate reduction rate compared to the pressurised control fluid.

In conclusion, the microbial monitoring (activities combined with cell/gene abundance) of the incubations with scCO₂ revealed a quite pronounced effect of the total CO₂ concentration and the acidification (modelled pH 3.2 and 4.3) of the fluid. Interestingly, the

decreasing pH due to the dissolution of CO₂ was shown to inhibit microbial growth and survival more effectively than acidification by acids (e.g. HCl) [46]. The inhibitory effects of high dissolved CO₂ concentrations [16] will lead to higher maintenance energy debit (pH homeostasis) for the cells preventing considerable cell growth or could lead under energy constrains to cell damages and death [30; 32; 42]. Likewise, the population size in the stimulation system was more stable as the TMA provided a considerable buffering capacity. Furthermore, the substrate could provide also an energy resource for fermenting organisms as sulphate reduction was not detectable.

Considering the CO₂ diffusion gradient, the buffering capacity of an potential storage reservoir [23], and the possibly mobilised organic material [22; 40] the present microbial community might better survive than previously postulated [32; 42]. In the term of CCS, these parameters have to be closely evaluate to actually define the proposed influence of the microbial population on the reservoir capacity [29; 31], especially in the outer areas of the diffusion plume. These results emphasise furthermore, the necessity to evaluate the community changes and reactivation potential of organisms after the contact with CO₂. This will help to verify the resilience and adaptation mechanisms of an indigenous community.

Reactivation potential after incubations with scCO₂

After ending the scCO₂ incubations, aliquots of the fluid from the pressurised control and the scCO₂ reactor systems (as well as from unpressurised control in case of the stimulation experiment) were transferred to evaluate the needed reactivation time of the sulphate-reducing community. Almost all transfers from the scCO₂ reactor incubations showed the reactivation of sulphate reduction. Methanogenesis was not detected in any transfer microcosms independent from the origin from controls or of scCO₂ systems under stimulated and saturated conditions (data not shown).

The transfers from saturation experiment, from the pressurised control and scCO₂ reactor, were compared with the prepared microcosms of original fluid. These control incubations with original fluid showed a pronounced sulphate reduction activity with

hydrogen amendment ($141.3 \pm 19 \text{ nmol mL}^{-1} \text{ day}^{-1}$). However, the transfer of the pressurised control reduced only about 10.2 ± 4 and $3.2 \pm 0.7 \text{ nmol sulphate mL}^{-1} \text{ day}^{-1}$ (with hydrogen) after incubation under pressure for 16 and 30 days, respectively. In the scCO₂ transfers no activity was detected after 16 days under CO₂ saturated conditions (R^2 0.5). After 30 days under fully CO₂ saturated conditions about $9.9 \pm 3 \text{ nmol sulphate mL}^{-1} \text{ day}^{-1}$ was reduced in the transfer microcosms of the scCO₂ reactor. The inactivity of the transfer after 16 days under CO₂ influence indicated that the detected cells (see Figure 23) merely represent cell debris and/or dead cells. The used fluorescence dye (SybrGreen) did not differ between living and dead cells. Thereby, the cells did not necessarily survive the incubations or represent cells with damages beyond repair [16].

However, the transfer microcosms of the reactor systems, pressurised control and scCO₂ reactor (after 30 days), were considerably less active than implicated by the activity potential of the original fluid (+hydrogen). Presumably, the proportion of transferred dead cells was equally increased for both systems. Since the rates of the pressurised control were decreased with time a certain time related stress factor seems likely (starvation). Anyway, the reactivation potential for sulphate reduction activity even after 30 days under CO₂ saturated conditions was very surprising, especially, in consideration of the described germicide effect of scCO₂. The similarities of the sulphate reduction rates in the scCO₂ transfer microcosms and the pressurised control transfer might indicate that also similar organisms survived the incubation. For a more detailed explanation the organisms that were presumably active in the transfer microcosms need to be identified.

The stimulation experiment showed no significant differences in the reactivated sulphate reduction rate for the different incubation setups (Figure 22 and Figure 26), e.g. about 146 ± 5.8 and $122 \pm 16.9 \text{ nmol sulphate mL}^{-1} \text{ day}^{-1}$ was reduced in the scCO₂ transfer and the spore transfer, respectively. The detected microbial activity proved that sulphate reducers survived the scCO₂ incubation in the stimulated experiment for 40 days.

The microbial community of the saturated experiment during and after incubation with scCO₂

The cell density and sulphate reduction activity detected in the transfer microcosms indicated viable cells in the fluid after the incubation under CO₂ saturated conditions. Therefore, the community structure was evaluated at the starting point, by the end of the reactor incubation, and in the afterwards conducted transfer microcosms to reveal which community members were able to survive under the CO₂ stress. The community structure of the saturation experiment showed only minor variations of the pressurised control and scCO₂ community after the reactor incubations were finished (after 30 days of incubation). However, a relatively pronounced shift occurred in comparison to the original fluid at the starting point of the experiment (Figure 24 and Table 6; supporting information: Figure 28 showing the relative abundance T-RFs). The structural likeness indicated that also within the pressurised system the community became altered (Figure 24 and in the supporting information Figure 28) independently from the application of high (saturated) CO₂ concentrations. The viable organisms in the transfer might represent organisms capable to withstand a variety of environmental stress factors (e.g. substrate limitation, starvation periods, temperature increase etc.).

The community structure of the original fluid (before the experiment was started) was dominated by the T-RFs 490, 204 and 156/157 identified as an uncultured *γ-Proteobacteria*, *Peptococcaceae* (cluster 2) and uncultured *Bacteroidetes*, respectively. Next to these, the T-RFs 264 and 209 were abundant representing a *Petrotoga* sp. and *Desulfotomaculum* sp., respectively. The general diversity of the community and abundance of the taxa was comparable with the previously reported descriptions of the formation fluids [12] (and Frerichs unpublished data).

The community after 30 days under CO₂ saturated concentrations (Table 6 and Figure 28) showed a significant increase of T-RFs identified as putative spore-forming bacteria, e.g. two *Desulfotomaculum* spp. (T-RF 209 and 230/231). Also T-RF 488 related to a *Pseudomonas* sp. was relatively increased. Slightly increased in the scCO₂ reactor were T-RFs identified as *Thermoanaerobacter* spp. (T-RF 138 and 148) and *Geotoga* spp. (T-

RF 164/165 and 472). All taxa were also detected in the previous monitoring of the fluid community (Frerichs unpublished manuscript). Presumably inhibited organisms in the CO₂ saturated experiment were identified with T-RF 279/280 that was related to *Anaerobaculum* sp. Furthermore, the T-RF 264, which resembled *Petrotoga* sp., was decreased in comparison to the original fluid.

The pressurised control was very similar to the scCO₂ incubation. However, the *Thermoanaerobacter* sp. (T-RF 138), *Anaerobaculum* sp. (T-RF 279/280), and one *Geotoga* sp. (T-RF 472) was relatively increased compared to the applied original fluid and the CO₂ saturated incubation.

Alike to the community structure directly after the reactor incubations of pressurised control and scCO₂, also the transfer microcosms (with hydrogen) showed relative similarities between each other. Furthermore, a pronounced difference with the original fluid control microcosms with hydrogen supplementation was apparent (Figure 24 and in the supporting information Figure 28). In general, the hydrogen amended microcosms showed a relative diversity reduction, which was very pronounced for both reactor incubation transfers. The original fluid amended with hydrogen showed several taxa (e.g. T-RF 138) that were not detected in the transfers of the reactor setups explaining the relative distance in the IPCA plot. Since, the community of this amended microcosm was very different it facilitates no direct comparisons, e.g. T-RF 76 resembling a sulphate-reducing enrichment (Acc. AB518055 not included in Table 6) represented about 25% of the community but did not appear in any transfer microcosms.

Two T-RFs were predominantly enriched in the scCO₂ transfer representing together more than 93% of the community and both identified as thermophilic spore-forming *Clostridiales* [34; 39], representing *Desulfotomaculum* spp. (T-RF 209) and an uncultured *Peptococcaceae* 2 (T-RF 204). Additionally, the T-RF of the *Petrotoga* sp. (T-RF 264) was enriched for the pressurised control transfer, but still not detected in the scCO₂. The organism seems to be inhibited during the first phase of the experiment in the CO₂ saturated system and did not recover in the transfer with scCO₂ influenced fluids.

In general, the community of the reactor samples after the first phase of the experiment and the “recovered” community in the transfers showed surprisingly high similarities between both reactor incubations. The relative community richness directly after the experiment likely represents the DNA of organisms that were either already dead (DNA still intact) or the cells were alive but damaged beyond repair [16] and thus not viable in the reactivation approach. For example, *Petrotoga sp.* and *Anaerobaculum sp.* could be interpreted as significant negatively affected by the high CO₂ concentrations. The increased abundance of thermophile spore-forming *Clostridiales* was highly significant for the scCO₂ incubation. Furthermore, in the both transfers the most abundant organism could be related to the same putative sulphate-reducing *Desulfotomaculum sp.* which corresponded well with the similar microbial activity in the reactivation approach.

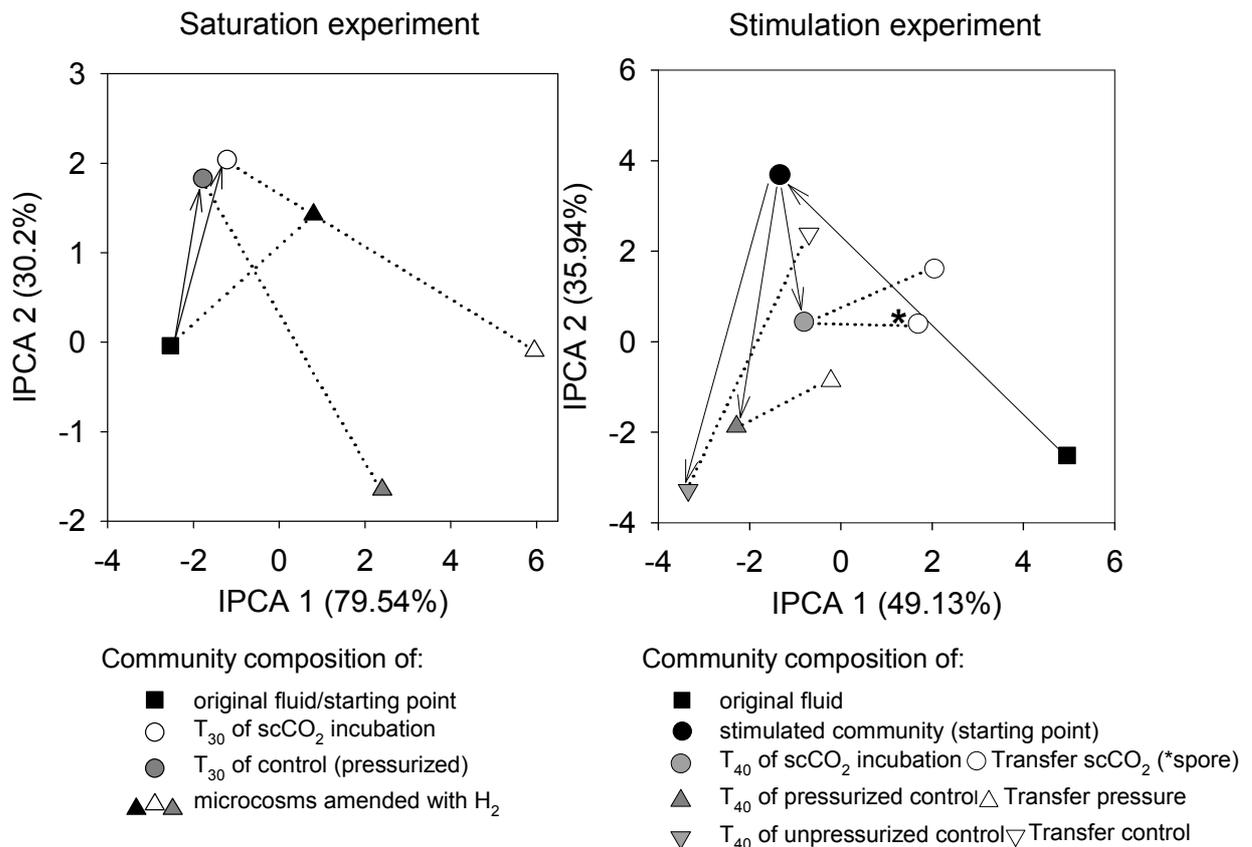


Figure 24: Statistical evaluation of the community changes during incubations with scCO₂, in the control setups and in the reactivation transfers of the saturation (left) and stimulation (right) experiment. The t-REX integrated MatLab tool was used to analyse Additive Main Effect and Multiple Interaction (AMMI) to produce two-dimensional interaction plots evaluation the Interacting Principal Components (IPCAAnalysis; cumulative about >85% of the variation explained) as previously described for T-RFLP community analyses by Culman *et al.* [6].

In conclusion, the viability of the *Clostridiales* showed their capability to withstand severe environmental changes. To survive the very high CO₂ concentrations represents an environmental advantage of these organisms that was likely connected to their Gram-positive cell wall structure and the capability to form endospores [9; 45].

Surviving organisms of an actively growing community under CO₂ constrain

In contrast to the saturation experiment, the stimulation experiment used a sulphate-reducing enrichment directly obtained for the formation fluids for the experiment. Thus, the community of the starting point was already changed in comparison to the original fluid due to the stimulation phase in advance of the experiment. However, the stimulated organisms were already present in the original fluid, albeit at lower abundance levels confirming that these microorganisms represent indigenous habitants of the reservoir (in the supporting information Figure 29).

The different setups of the stimulation experiment (Figure 24) showed the strongest shift for the microbial community of the unpressurised control sample, while the population of the pressurised control was closer to the starting community (Figure 24 and in the supporting information Figure 29). The microbial community from the scCO₂ incubation was closest to the starting culture possibly reflecting the inactivity of the microorganisms during the incubation as also no significant cell growth or sulphate reduction activity was detected (Figure 23). The developed communities in the transfers were quite different from the community structure directly after the end of the first phase of the stimulation experiment. However, the transfers of the scCO₂ and the shortly autoclaved spore transfer (Figure 24) developed quite similarly and showed a closer relative distance to the pressurised than to the unpressurised control. Thus, the pressurisation effected the development of the community also beyond the actual incubation under pressure (transfers incubated under ambient conditions).

Significantly increased for the scCO₂ reactor was T-RF 64, 132, and 138, which were affiliated to *Thermovirga* sp., *Desulfobutulus* sp. and *Thermoanaerobacter* sp., respectively (Table 6). The detected T-RFs 209, 230/231 and 204 were not affected and

corresponded to several *Desulfotomaculum spp.* and the uncultured *Peptococcaceae* already detected in the saturation experiment. Interestingly, the T-RF representing *Petrotoga spp.* (T-RF 264) was decreased alike to the development of the saturation experiment.

In contrast to the saturation experiment the community richness increased in the transfer incubations. In both scCO₂ transfers several T-RFs (re-)appeared, e.g. TRF 164/165 identified as *Geotoga sp.* Furthermore, the already mentioned T-RFs 224 and 230/231 both identified as spore-forming *Desulfotomaculum spp.* were increased. As listed in Table 6 almost all identified T-RFs were affiliated to organisms isolated or documented for high temperature environments and are likely indigenous for the indigenous subsurface biosphere of the natural gas field [10; 13; 36]. The T-RFs in the scCO₂ incubations were predominantly associated to thermophilic spore-forming *Clostridiales* including putative sulphate-reducing organisms, e.g. several *Desulfotomaculum spp.* or *Thermovirga sp.* (sulphur reducing *Synergistaceae*) [3; 7; 8; 11; 14; 34; 39]. The latter organism was enriched in all transfers and related to an isolate (*Thermovirga lienii* 95.5% sequence similarity) that was cultivated from a high temperature oil field and likely possessed some heat tolerance. *Thermovirga lienii* is described to ferment also amine compounds and some amino acids. Furthermore, the organism was clustered into a group of *Clostridiales* that could all be related to the fermentation of amino acids [7]. The here detected organisms (about 95% similarity; family level) was already increased in the course of the reactor experimental phase and further in the transfer incubations. The pronounced enrichment in the reactivation approach indicated a fermentative degradation of the tri-methylated amine (TMA) added as substrate by the organism.

The synthesis of saturation and stimulation experiments revealed a pronounced dominance of putative spore-forming *Clostridiales*, especially for the reactivation approach representing the organisms that were still viable. Previously, it was shown that spores of *Firmicutes* compromise a relative high resilience for scCO₂ [45; 46]. The thick spore coat

and cortex structure was shown to slow the diffusion of even small molecules considerably and the low water content could reduce the acidification [43]. The resistance of spores was mainly evaluated on pure cultures (tested for food-borne pathogens) showing that sterilization was only effective in combination with temperature increase [45; 46]. Since, the here surviving organisms were predominantly associated to thermophilic taxa the resistance also under increase temperature constrains seems obvious. Furthermore, also the vegetative cells of spore-forming *Firmicutes* were shown to have a higher resilience for high CO₂ concentration [42]. The thick peptidoglycan layer of the Gram-positive cell wall presumably also reduces the diffusion of CO₂ into the membrane and cytoplasm where it disturbs the membrane integrity and intercellular pH, respectively [16; 45; 46].

In consequence, the selection of Clostridiales by the effects of scCO₂ on the community will influence their distribution in the potential CO₂ storing reservoir structures and could possibly even effect the storage capacity [31; 36]. The “recovery” of sulphate reducers was previously also shown by Morazova *et al.* [32] albeit no sulphate reduction was documented and the identity and origin of these organisms remained unclear, i.e the possible contaminant origin or enrichment on organics supplemented by the drilling mud. In conclusion, our experiments with an indigenous microbial population derived from a high-temperature hydrocarbon reservoir revealed for the first time a pronounced resistivity for spore-forming *Clostridiales*. These results actually emphasis to study the indigenous microbial community more closely to actually interpret the biological control mechanism that was previously postulated to affect CCS [31].

Table 6: Community development in the different incubation set-ups of the saturation and stimulation experiment giving the relative abundance [%] and identity of significantly changed T-RF.

TRF	Closest affiliated species (Acc. No.) with sequence similarity ¹ and isolation source ²	Saturation experiment [%]			Stimulation experiment [%]					
		original	pressure	seCO ₂	start	control	pressure	seCO ₂	spore	
64	<i>Thermovirga lenii</i> (CP003096)	95.5	58°C; oil reservoir	(-)	(-)	(-)	0.6	0.87	1.1	1.9
132	<i>Desulfobolus</i> sp. BG14 (U85470)	98.6	salt marsh sediment	1.7	0.6	(-)	(-)	(-)	(-)	4.5
138	<i>Thermoanaerobacter</i> sp. HL-3 (GU561619)	99.6	60°C; oil reservoir	2	12.1	4.9	18.3	18.2	15.8	30
148	<i>Thermoanaerobacter acetohydrolicus</i> (L09163)	98.6	65°C; thermal spring	0.9	(-)	3.6	0.71	1.6	1.3	2.51
156/157	uncult. <i>Peptococcaceae</i> 1 (AB478004)	96	50°C; geothermal fluids	10	0.9	5.8	0.9	0.6	1	3
164/165	<i>Geotoga petraea</i> (L10658)	99.3	50°C; oil field brines	3.5	(-)	1.5	0.5	11	(-)	(-)
204	uncult. <i>Peptococcaceae</i> 2 (FN356238)	96	50-80°C; oil reservoir	10.5	(-)	11	(-)	3.6	2.9	(-)
209	<i>Desulfotomaculum kuznetsovii</i> (AY036904)	99.8	60°C; geothermal fluid	7.9	9.2	12.1	(-)	0.6	1.6	(-)
224	<i>D. geothermicum</i> (AJ294428)	99.2	60°C; geothermal fluid	2.3	4.3	0.7	13.8	5.3	5.1	6.3
230/231	<i>D. geothermicum</i> (AJ621886)	99.8	60°C; geothermal fluid	2	9.2	3.8	29.8	15.7	15.5	27.1
264	<i>Petrogala halophila</i> (AY800102)	99.5	60°C; oil reservoir	15.8	2	4.5	7	23.8	30.9	(-)
279/280	<i>Anaerobaculum</i> sp. OSI (FJ862996)	98.6	50°C; oil reservoir	(-)	12.5	0.8	0.9	(-)	(-)	(-)
472	<i>Geotoga subterranea</i> (L10659)	96.2	50°C; oil field brines	0.5	21.9	1.28	(-)	1.9	2	(-)
488	<i>Pseudomonas</i> sp. OCR2 (AB240201)	99.6	oil reservoir	1.9	(-)	18.4	(-)	(-)	(-)	0.4
490	uncult. <i>Pseudomonaceae</i> (JN030548)	98.6	deep rock fissure fluids	24.5	(-)	(-)	(-)	(-)	(-)	(-)
Established ("recovered") taxa in transfer microcosms³										
64	<i>Thermovirga lenii</i> (CP003096)	95.5	58°C; oil reservoir	(-)	(-)	(-)	6.3	11.7	18.3	21.2
138	<i>Thermoanaerobacter</i> sp. HL-3 (GU561619)	99.6	60°C; oil reservoir	8.3	(-)	(-)	6.9	27.6	9.8	17
140	<i>Thermoacteogentium phaeum</i> (AB020336)	97	58°C; waste water	(-)	(-)	(-)	1.7	1.3	3.4	4.3
148/149	<i>T. acetohydrolicus</i> (L09163)	98.6	65°C; thermal spring	2	0.3	(-)	1.4	1.6	2.2	2.4
156/157	uncult. <i>Peptococcaceae</i> 1 (AB478004)	96	50°C; geothermal fluids	1.9	(-)	(-)	1.5	(-)	16.37	17.8
164/165	<i>Geotoga petraea</i> (L10658)	99.3	50°C; oil field brines	0.6	(-)	(-)	0.4	(-)	3.8	4
204	uncult. <i>Peptococcaceae</i> 2 (FN356238)	96	50-80°C; oil reservoir	(-)	15.8	15.1	0.2	2.2	1.2	(-)
209	<i>D. kuznetsovii</i> (AY036904)	99.8	60°C; geothermal fluid	11	47.1	79.8	1.9	(-)	4.2	1.4
215	uncult. <i>Peptococcaceae</i> 2 (AB478010)	96	44°C; geothermal fluid	6.7	0.8	(-)	14.9	3.5	6.1	5.7
230/231	<i>D. geothermicum</i> (AJ621886)	99.8	60°C; geothermal fluid	0.3	0.4	(-)	5.1	18.5	8.3	11.5
264	<i>Petrogala halophila</i> (AY800102)	99.5	60°C; geothermal fluid	2.47	21.6	(-)	14.4	6.9	5.8	(-)

¹relative sequence similarity of cultivated species with max. 5% divergence (family level) towards the here detected sequence; in case of similarities <95% the closest uncultivated relative is given; (-) not detected

²Environmental conditions of the habitat, here temperature optimum and isolation source, provided by the NCBI GenBank entry or the taxonomic description.

³Transfer microcosms were conducted following Scheme 1; hydrogen amended transfers of day 30 included for saturation experiment; TMA amended transfers for stimulation experiment.

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4.2.4. Supporting Information

Description of incubation method and results for hydrostatic/gaseous CO₂ incubation experiment

An enrichment culture was grown on mineral media with 28 g L⁻¹ salts and additive vitamins, trace elements and selenite-tungstate solution described earlier [2,3]. The culture was obtained in dilution series from May 2010 production fluids of the natural gas field growing with fructose and sulphate additions (10 mM each). The culture was freshly transferred in advance of the experiment amending fructose and sulphate (10 mM each). After incubation over night at 30°C the batch culture was sampled for total cell counts (TCC) and subdivided into the following setups: (i) unpressurised control (triplicates with 50 ml each), (ii) pressurised control (10 replicates with 10 ml each), (iii) CO₂ incubation (12 replicates with 25 mL each). The replicates of the pressurised control were incubated and prepared as described for the control setup of the supercritical CO₂ experiments. The CO₂ influenced microcosms were prepared anaerobically in 50 mL glass bottles giving a 50% headspace/fluid ratio. Headspaces were replaced with pure CO₂ purging the bottle for 5 min. The pressure-safe reactor used for the incubation was similar to the hydrostatic pressure system [1] except for a second inlet valve for gaseous media (Figure 25). All glass vessels were interconnected to the gas valve (valve C) in the reactor lid. The prepared microcosms were placed in the 5 L steel cylinder that was completely filled with demineralised water. The reactor was securely sealed and the partial pressure of CO₂ was applied valve C into the microcosms directly from a connected CO₂ gas cylinder (controlled via the pre-pressure valve of the CO₂ cylinder). The maximum final pressure equals the fill pressure of the CO₂ cylinder (~50 bar). To secure the integrity of the glass bottles the microcosms' intern-pressure (p_{in}) was transmitted to the outer media (p_{ex} water filled reactor) via the piston of an empty syringe that was connected to each glass vessel. The whole system was placed into a climate chamber heated to 30°C.

The cultures were used to test the effect of high CO₂ concentrations on enriched organisms from the reservoir (Figure 25) prior to the experiments reported in the main

text. During the incubations at 30°C with 40 bar pure CO₂ (gaseous phase) the cell numbers were slightly decreased and no sulphate reduction was detected in comparison to the control setups including pressurised and unpressurised grown enrichments (Figure 26). The incapability of the enrichments to grow after the release of the CO₂ indicated some inhibition or cell damage. However, the used autoclave system allowed only minor headspace pressures up to 45 bar (gaseous CO₂), were restricted to temperatures <50°C, and most importantly could not be sampled under stable pressure and temperature conditions. The limitations of these experiments lead to the changed experimental design. Furthermore, the usage of original formation fluids in the “saturation” experiment was more appropriate to answer the question about possible in situ community changes for the reservoir, while the “stimulation” experiment represent an actively mineralising community stimulated by the substrate addition.

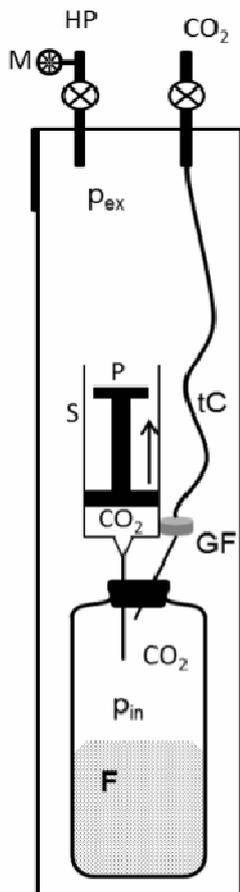


Figure 25: Schematic description of hydrostatic/gaseous pressure-proof autoclave systems. The fluid (F) was placed in the water filled system and connected with the CO₂ valve (CO₂) via a flexible Teflon tube (tC) and a gas filter (HPLC filter with PVDF membrane of 0.2 μm). The internal pressure increase (p_{in}) was transmitted by means of piston (P) of an connected syringe(S) into the outer media (p_{ex}). The pressure transmission was controlled at the pressure manometer (M) at the second valve that was connected with the hydrostatic pump (HP). Each incubation tube was connected as described above with the CO₂ valve and equipped with the syringe to secure an adequately pressure transmission.

Literature Supporting information:

1. Nauhaus K, Boetius A, Krüger M, Widdel F (2002). In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environmental Microbiology* 4: 296-305.
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Supplemented Figures for the main text body:

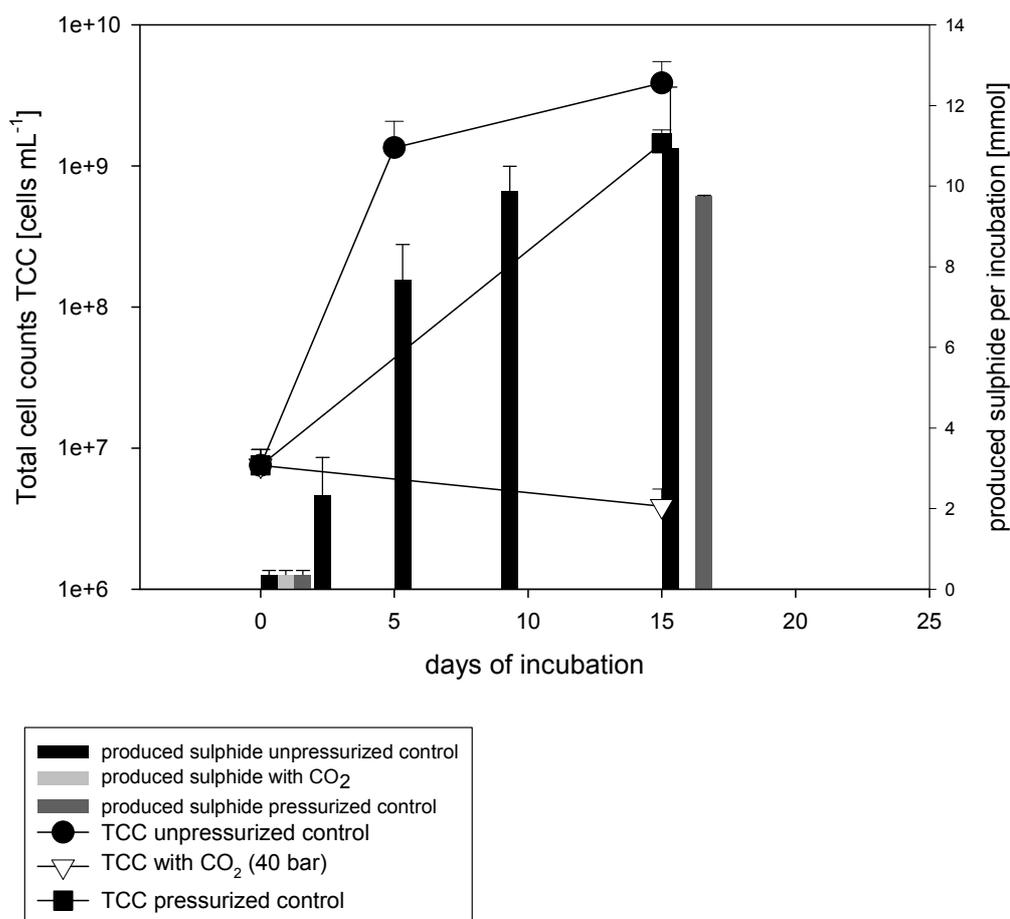


Figure 26: Development of total cell counts and sulphide concentration in incubations with fructose fermenting/sulphate-reducing enrichment culture. The CO₂ partial pressure was increase to 40 bar at 30°C incubation temperature.

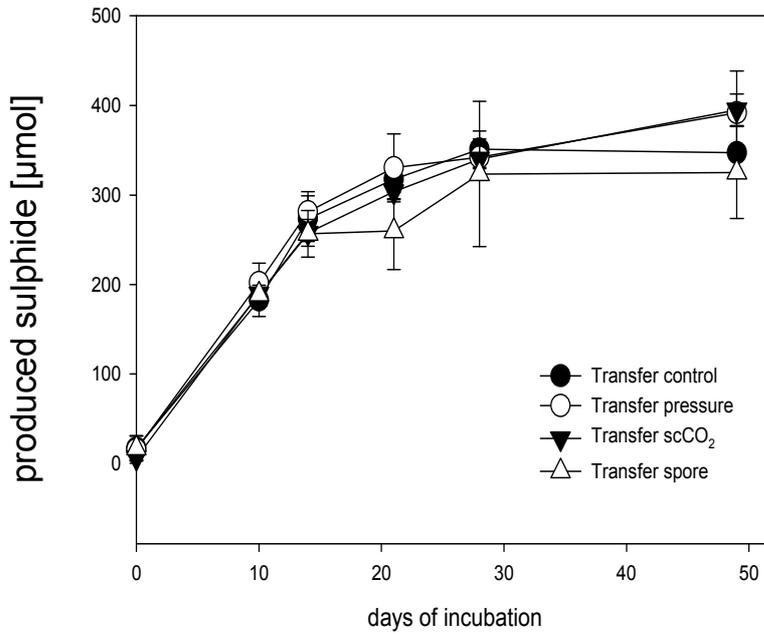


Figure 27: Sulphide production (normalised for incubation volumes) of the transfer microcosm from the stimulation experiment.

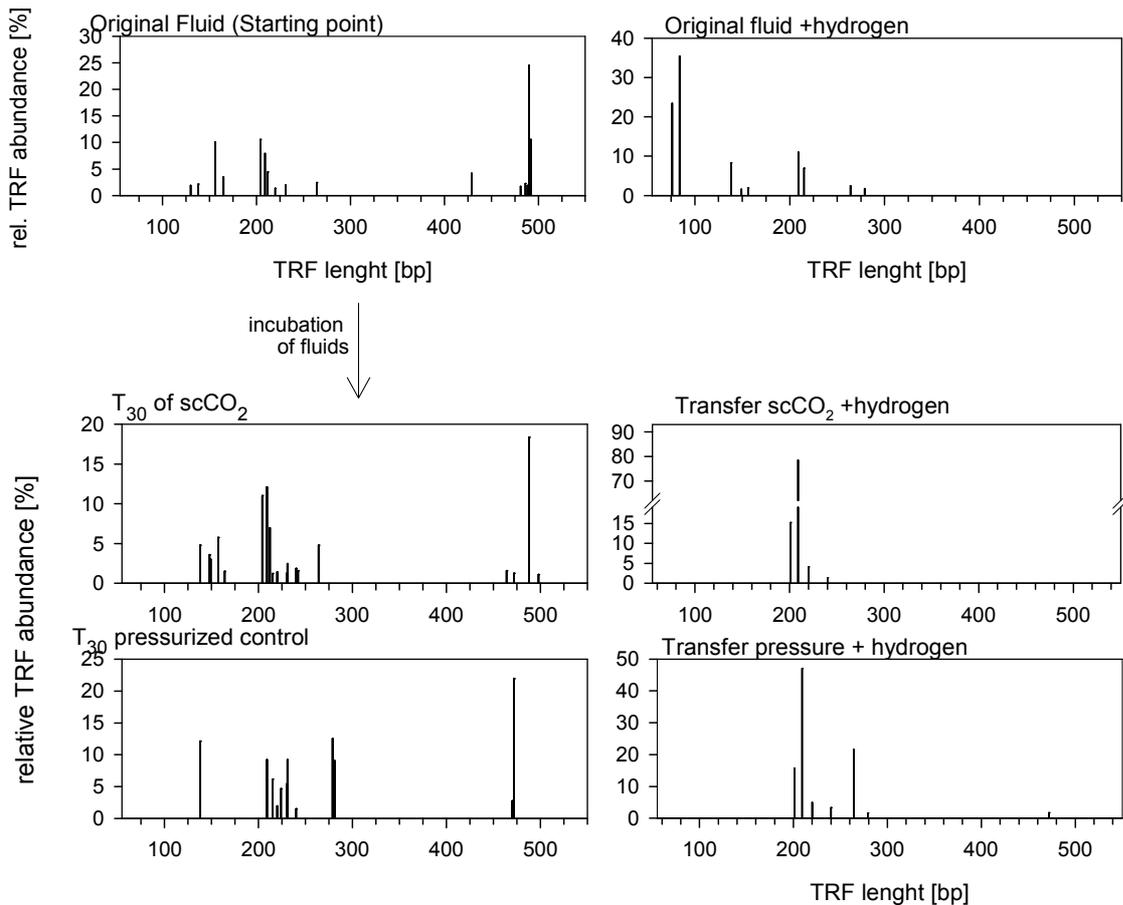


Figure 28: Comparison of bacterial community structure giving the relative TRF abundance during and after (transfer) incubations of the saturation experiment. Microbial community structure was analysed with the web-based software T-Rex (Culman et al. #[5] in the main text) using the fragment pattern after restriction with the enzyme MspI.

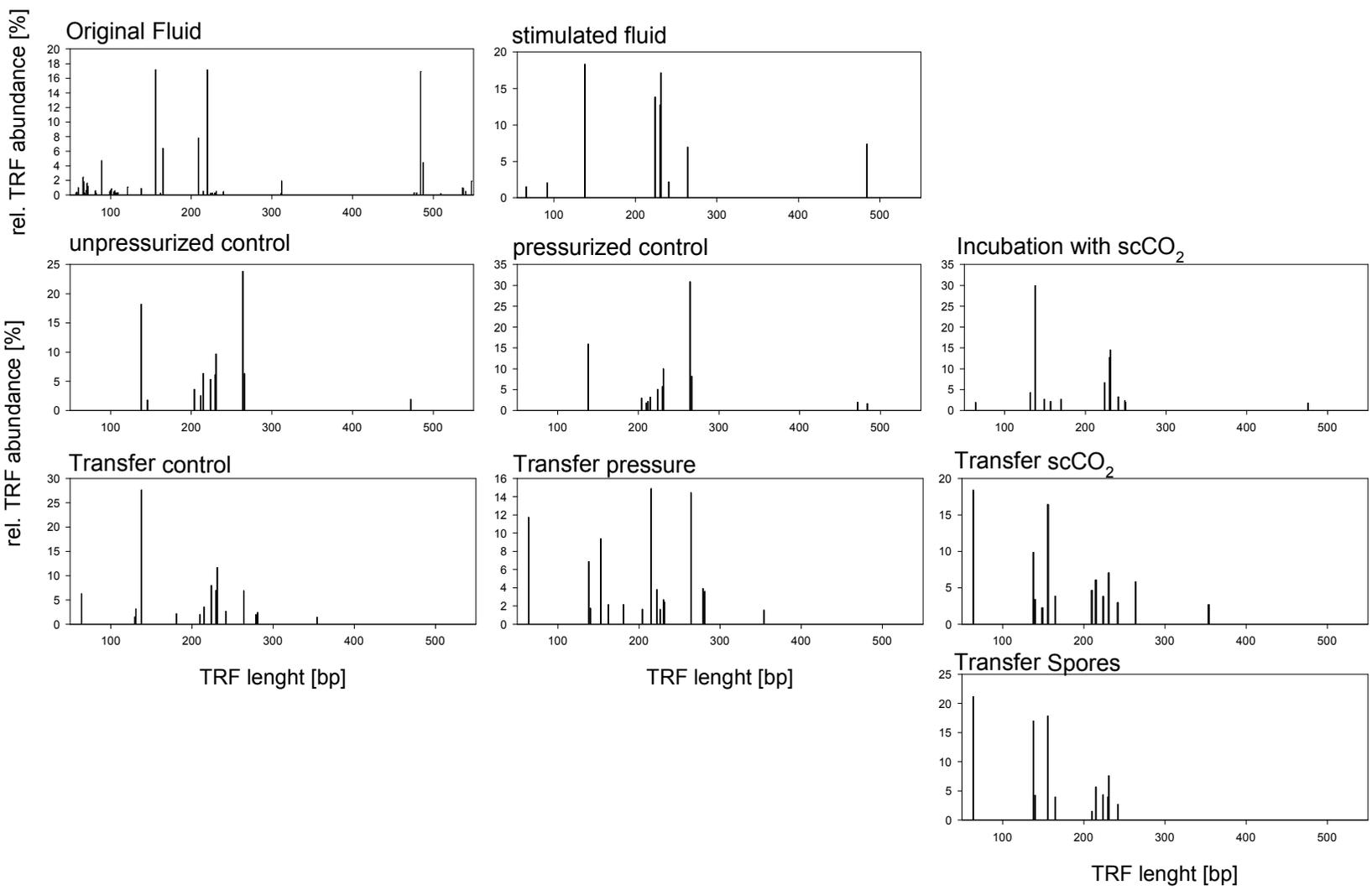


Figure 29: Comparison of bacterial community structure giving the relative TRF abundance during and after (transfer) incubations of the stimulation experiment. Microbial community structure was analysed with the web-based software T-Rex (Culman et al. #[5] in the main text) using the fragment pattern after restriction with the enzyme MspI.

4.3. Effects of elevated CO₂ concentrations on the vegetation and microbial populations at a terrestrial CO₂ vent at Laacher See, Germany

Krüger M^{a,*}, Jones D^b, Frerichs J^a, Oppermann B^c, West J^b, Coombs P^b, Green K^b, Barlow T^b, Lister R^b, Shaw R^b, Strutt M^b and Möller I^a (2011).

“Effects of Elevated Co₂ Concentrations on the Vegetation and Microbial Populations at a Terrestrial Co₂ Vent at Laacher See, Germany.” *International Journal of Greenhouse Gas Control* **5**: 1093-1098.

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Keywords: CCS; CO₂; microbiology; methanogenesis; denitrification; acidification; Laacher See

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Abstract

CO₂ capture and geological storage offers an option for reducing man-made greenhouse gas emissions. But one major concern related to geological CO₂ storage is the possibility of leakage from the reservoir and subsequent effects on the environment, which cannot completely be excluded. This study aims at investigating the environmental impact of CO₂ release from reservoirs into near surface terrestrial environments. To understand the effect of CO₂ leakage on such an ecosystem, detailed knowledge on the abundance and diversity of plants and microorganisms is essential. Therefore, an ecosystem study has been conducted within the Network of Excellence "CO₂GeoNet" on a natural CO₂ vent at the Laacher See, Germany. Near surface CO₂ conditions and CO₂ fluxes of the venting area were described by means of conventional soil gas measurement equipment, and brought up the difference between the CO₂ anomalies and their surroundings.

A comparison of the soil columns between control sites and the centre of the venting area showed a small but significant change in the soil pH below 10 cm. The botanical survey revealed some remarkable vegetation changes like the investigation of important soil microbial communities showed significant differences between the CO₂-rich sites (up to 90% and more of soil gas), medium CO₂ sites (~20%), and control locations with background CO₂ concentrations. The ecosystem appears to be adapted to the different conditions through species substitution or adaptation, showing a shift towards anaerobic and acidotolerant to acidophilic species under elevated CO₂ concentrations. At the end, this ongoing study should identify possible candidates in the botanical and microbial kingdoms, whose presence or absence provide easily detectable indicators for the leakage of CO₂ from deep reservoirs into near surface terrestrial ecosystems.

4.3.1. Introduction

The fourth IPCC report on global warming states once again that the rise in average global temperatures observed over the last century is most likely due to the release of anthropogenic greenhouse gases (IPCC, 2007). It turns out that large-scale solutions are needed immediately to quickly reduce greenhouse gas emissions and to mitigate their subsequent environmental effects. CO₂ capture and geological storage in deep saline aquifers or depleted gas and oil reservoirs offers a new option for reducing greenhouse gas emissions in large quantities. To proceed with a responsible large-scale deployment of this technology, all potential risks should have been studied, understood and, finally, minimised to exclude harm to the environment including humans. For this, it is a priori important to assess the potential risks associated with the unlikely leakage of significant volumes of CO₂ from the reservoir into the near surface environment (West *et al.*, 2005; West *et al.*, 2006). Although several studies have been published regarding the effect of increased atmospheric CO₂ concentrations on ecosystems (Jossi *et al.*, 2006), there are only very few that have examined the effects of increasing CO₂ concentrations in the soil column due to upwardly migrating gas. These include a detailed study of a terrestrial CO₂ vent at LATERA, Italy (Beaubien *et al.*, 2008; Oppermann *et al.*, 2010), a survey at Mammoth Mountain (California, USA) for the influence of volcanic CO₂ on soil chemistry and mineralogy (Stephens and Hering, 2004) and research at Stavesinci (NE Slovenia) for the influence of high soil-gas concentrations of geothermal CO₂ on plants (Macek *et al.*, 2005; Pfanzen *et al.*, 2007).

In order to address some of the above-mentioned issues, this study investigates the potential environmental impact of CO₂ release from deep reservoirs on near surface terrestrial environments. Particularly the effect of CO₂ leakage on the abundance and diversity of plants and microorganisms is investigated in an ecosystem study conducted as a joint activity within the European network "CO₂GeoNet" at a natural CO₂ vent at Laacher See, Germany.

The Laacher See volcanic centre is located in the core of the East Eifel volcanic field, and comprises of about 100 eruptive centres that cover an area of approximately 330 km². The East Eifel volcanic field is located west of the Rhine River in the still uplifting Paleozoic Rhenish Massif. The Laacher See eruption is the only known large explosive eruption that took place in central Europe during late Quaternary time (~12,900-12,880 yrs BP; e.g. Boogard and Schmincke, 1985). The Laacher See volcanic centre is morphologically characterised by a basin filled by a lake (Laacher See), with 3.3 km² area, surrounded by a steep ringwall rising 90 to 240 m above the basin. The ringwall, which can be classified as an extinct volcanic caldera, is made up by different basanitic/tephritic cinder cones and the tephra deposits of the Laacher See eruption. The internal structure of the Laacher See basin is dominated by an East northeast-West southwest striking thrust and four other geological lineaments: 2 running more or less North-South, the other Northeast-Southwest. CO₂ is produced below the caldera, it emerges from degassings of the upper earth mantle and migrates along faults and fractures to the surface (Möller (ed.), 2009). Release to the atmosphere typically occurs from gas vents, characterised by a small core of elevated gas flux.

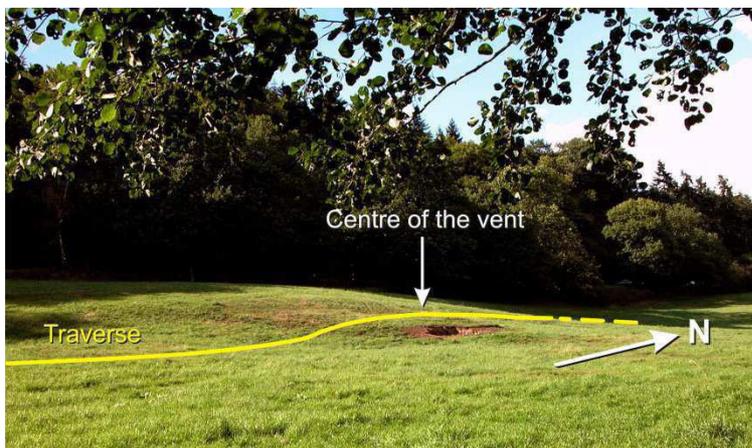


Figure 30: Sketch of the investigated CO₂ vent close to the western shore of the lake Laacher See; note the vegetation change from closed, green grassland to patchy *Polygonum arenastrum*-mats towards the centre of the vent.

One defined gas vent was chosen for this study, located in an almost naturally-vegetated pasture field on the western side of the lake (Figure 29). The vent is situated in an area which became dry land very recently. As a consequence of two tunnel constructions in 1164 and 1844, the water level of the lake was artificially lowered by about 10 plus 5 m. The terrestrial development of the studied site is therefore very young.

Some organic material from former lake deposits is still to be found in deeper soil horizons. The vent is clearly visible due to a 5 m wide core of nearly bare soil surrounded by an approximately 40 m wide area of variably-impacted vegetation.

4.3.2. Methods and materials

Surveys were conducted in September 2007 and July 2008 along a 60 m long transect across the vent (Figure 29), providing a spectrum of different CO₂ flux rates, soil gas concentrations and compositions. In addition to the detailed survey of these conditions (soil gas concentrations and gas fluxes), intensive botanical studies and sampling for microbiological, mineralogical and geochemical analysis were performed at the same time.

Characterisation of the near surface CO₂ conditions

The applied techniques are potential methods for the near surface monitoring of geological CO₂ storage sites; they were used during both field campaigns in 2007 and 2008. First, a rapid surveying of the whole study area was undertaken by means of a newly developed, mobile open path laser system which was mounted at about 30 cm above the ground on a quad bike (for details see Jones *et al.*, 2009). The system detected already known CO₂ vents, and confirmed and discovered suspicious or unknown degassing sites.

Afterwards, the 60 m long traverse across the strongest vent was intensively investigated with conventional soil gas concentration and flux measurement equipment: Steel probes and handheld infrared gas sensors (Li-Cor and Dräger instruments) for soil CO₂ concentration, and commercial and custom-made accumulation chambers for the CO₂ flux quantifications. The measurements were carried out in 0.5 m intervals. Some additional gas samples taken along the traverse for comparative laboratory analyses were also used for the determination of carbon isotope ratios ($\delta^{13}\text{C}_{\text{CO}_2}$; by means of a Thermo Delta plus XL mass spectrometer) which give hints on the origin of the CO₂.

Botanical impact survey

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

Microbiological analyses

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

Determination of microbial activities:The collected soil samples were first mixed 1:1 with artificial mineral medium to obtain homogenous slurries (Widdel & Bak 1992). Subsequently, 9 ml of medium were added to 3 ml of soil slurry into sterile glass tubes (20 ml) which were afterwards sealed with butyl-rubber stoppers and screw caps. The headspace was either flushed with N₂ for methane and anaerobic CO₂ production as well as sulphate reduction measurements, with air for aerobic CO₂ production or with air and 2% CH₄ for aerobic methane oxidation.

As important indicators of the gross mineralisation in the soil the CO₂ production (CPR; under aerobic and anaerobic conditions), the anaerobic methane production (MPR), and the sulphate reduction rates (SRR) were quantified. The potential aerobic oxidation of methane rates in the soil samples were determined in vitro as described previously by Krüger *et al.* (2002). Triplicate tubes were incubated horizontally at 20°C and gently shaken once per day to ensure an even distribution of gases or sulphate within the microcosms. The rates were calculated per gram of dry weight (g_{dw}) as determined after drying at 80°C for 48 h and deviations are expressed as 95% confidence intervals unless

stated otherwise. The sulphide content was determined using the formation of copper sulphide after Cord-Ruwisch (1985). Methane and CO₂ were determined using a GC 14B gas chromatography (Shimadzu) as described in Nauhaus *et al.* (2002), which was additionally equipped with a methaniser to quantify the CO₂.

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

DNA standards for quantitative real time PCR (qPCR) were prepared as described previously by Engelen *et al.* (2008). Specific fluorescent probes were used targeting the ubiquitous 16S *rRNA* genes of bacterial or archaeal organisms (Takai & Horikoshi 2000, Nadkarni *et al.* 2002). The assays were carried out using the TaqMan PCR Master Mix (Applied Biosystems). Each DNA extract was measured in triplicate and in two to three dilutions to check for PCR inhibition. Conversion factors for DNA copy numbers to cell numbers were: 4.1 for *Bacteria*, and 1.5 for *Archaea* (Lee *et al.* 2009). The detection limits for qPCR analyses were 10³ DNA copies g⁻¹ dry weight for the assays specific for *Bacteria* and 10¹ DNA copies g⁻¹ dry weight for the assays specific for *Archaea*.

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

4.3.3. Results and discussion

Gas monitoring and soil chemistry

The results of the gas surveys are generally very similar from year to year. The soil CO₂ concentration and flux data series of the two years are very homogeneous and well correlated as shown by their coefficients of determination (see Table 7).

Hence, the spatial patterns observed in 2007 could be confirmed in their shapes in 2008. Figure 30 illustrates the soil CO₂ concentrations in 15 and 60 cm depth while Figure 30 shows the CO₂ fluxes from the underground to the atmosphere for the different years.

Table 7: Correlation matrix (coefficients of determination, r^2) of soil CO₂ concentrations in 15 and 60 cm depth and CO₂ fluxes for 2007 and 2008.

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

Three main zones of higher CO₂ concentrations and fluxes could be identified along the traverse: Between locations 11-17 m, 21-28 m, and 30-42 m, the latter representing the so-called centre of the vent where peak concentration values of more than 90 vol% CO₂ were registered in 60 cm depth (Figure 30). But already in a very short distance from these distinct anomalies the CO₂ concentrations and fluxes drop back to background values; demonstrating the limited size of natural CO₂ vents. Furthermore, particularly the figures of the CO₂ concentrations show also the relatively high small scale variability which could be quite marked between adjacent measurement points.

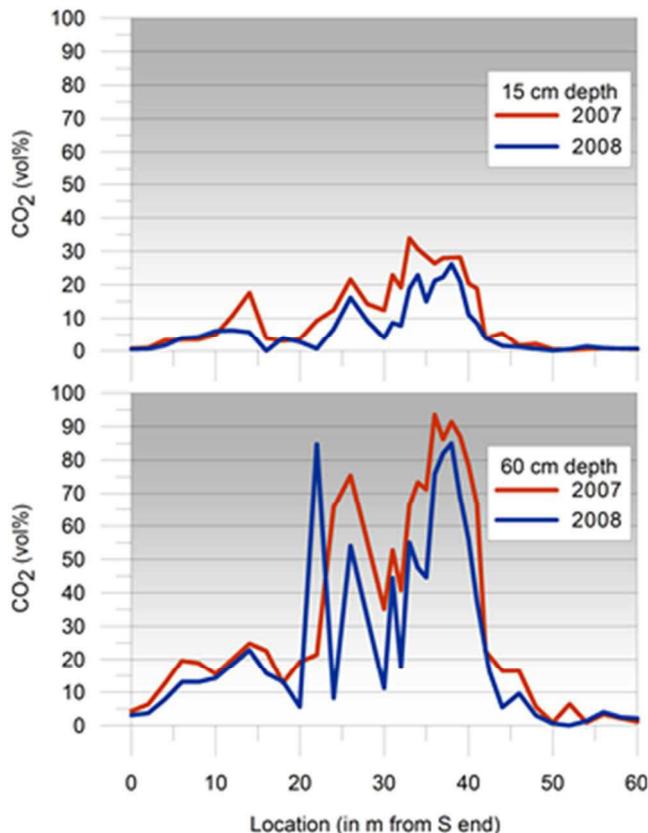


Figure 31: Comparison of 2007 and 2008 CO₂ concentrations in soil gas along the traverse across the studied vent (September 2007 and July 2008, using only points measured in both years).

There certainly are also some differences in detail between the two years, but this is to be expected given that the sampling locations will not precisely match within a few centimetres, and there could be changes in the migration pathways of gas to the surface owing to changing underground conditions. One clear difference between the two years are the generally higher gas concentrations and fluxes in 2007 (see Figures 2 and 3). Data correlations with meteorological parameter suggest that factors such as lower atmospheric pressure and higher wind speed drew slightly larger amounts of gas from the ground in the autumn and overrode any impeding effect of higher soil moisture.

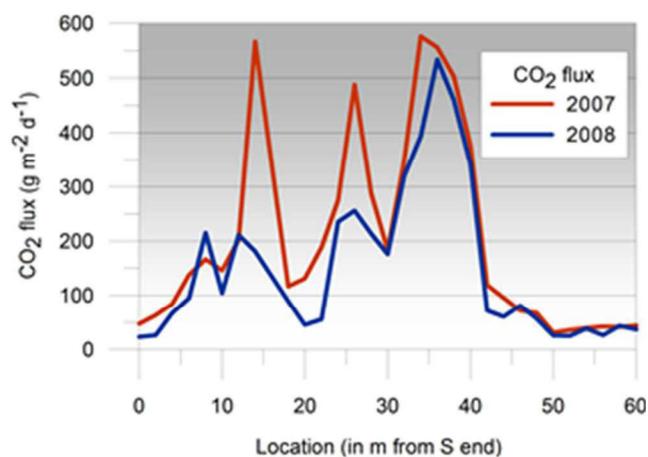


Figure 32: Comparison of 2007 and 2008 CO₂ flux data for the traverse across the vent.

Carbon isotope analyses ($\delta^{13}\text{C}_{\text{CO}_2}$) were helpful for the characterisation of the venting areas since the isotope ratios differ from CO₂ rich sites (-4.1 to -2.7 ‰ PDB) to those with medium (-1.7 to -0.2 ‰) to low concentrations (-1.0 to 0.8 ‰). The $\delta^{13}\text{C}_{\text{CO}_2}$ values for the CO₂ rich sites point directly to the upper earth mantle and/or lower earth crust as origin of the CO₂. Contrastingly, the CO₂ gas from medium to low concentration sites is already affected by mixing processes and isotope fractionation, probably under the influence of some underground carbonate levels.

In terms of bulk mineralogical compositions and soil chemistry, the analysed samples from the centre of the vent (35 m) and the control site (55 m) were relatively similar in the top 70 centimetres. Going deeper into the soil, a small but significant change in the soil pH was observed below 10 cm (Figure 31). This might influence the activity and composition

of the microbial communities, as well as the soil mineralogy as also seen at Latera (Beaubien *et al.*, 2008).

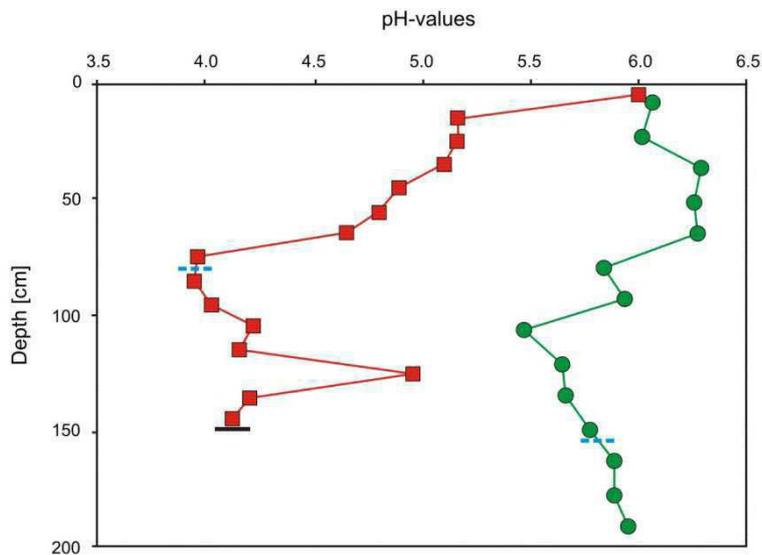


Figure 33: Acidity of two sediment cores (pH profiles); in red = area of highest CO₂-seepage, in green = control site; the blue lines show the top of the water table, the black the rock base.

Botanical investigations

The botanical survey showed that CO₂ soil gas concentrations influence vegetation types with grasses predominating below 20% CO₂. Above this concentration two predominant dicotyledonous plant species were observed and could be used as bioindicators of high CO₂ soil gas concentrations.

Main results of the botanical survey are summarised in Figure 32, which shows the percentage coverage for total moss, total grass (monocotyledonous plants), *Polygonatum arenastrum* and 'other' dicotyledonous flowering plants. *P. arenastrum* is the only observed dicotyledonous plant between 25 and 50 m along the transect where CO₂ concentrations are between ~10-35% at 15 cm depth and ~35-90% at 60 cm depth. Where CO₂ concentrations are below 20% at 15 cm depth, grasses predominate and *P. arenastrum* is not observed (0-25 m and 40-60 m) although other dicotyledonous plants are present. These results can be compared to observations from another natural CO₂ gas vent site at Latera, Italy where only grasses were observed when concentrations of CO₂ were between 5-40% at 10 cm depth (Beaubien *et al.*, 2008). Indeed, dicotyledonous plants did not appear to be able to tolerate CO₂ concentrations over 5% at this site. Other observations at a controlled injection site in an English pasture also suggested that

grasses were more tolerant to higher concentrations of CO₂ than dicotyledonous plants (West *et al.*, 2008). The observation of a dicotyledonous plant as a bioindicator of increased soil gas CO₂ is therefore unexpected and also demonstrates that botanical changes are site specific, depending also on other factors such as soil moisture, pH influencing plant ecology, etc. However, monocotyledonous plants appear, in general, to be more tolerant to increased soil gas CO₂.

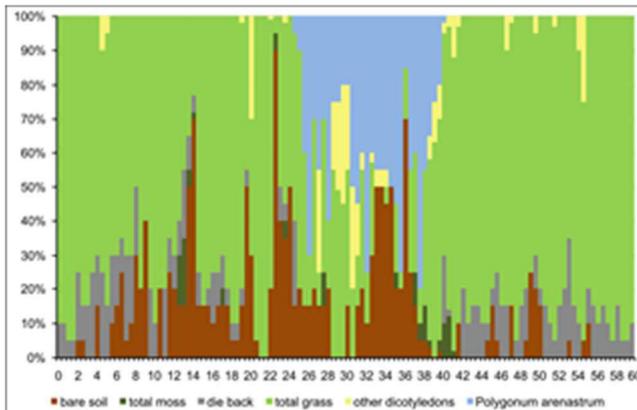


Figure 34: Effect of CO₂ emissions on the distribution of different botanical groups/species along the transect across the CO₂ vent (centre at approx. 30-35 m); x-axis: location in m from S end, y-axis: coverage.

Microbial community composition and activities

The determination of environmentally important microbial activities in the soil samples showed significant differences between the CO₂-rich sites (>90 % of soil gas), medium CO₂ sites (20%) and control locations with background CO₂ concentrations. To get some more detailed information, potential sulphate reduction rates as well as methane production and oxidation were determined in sediment samples from the different sites. These measurements with samples from vent and non-vent sites should also provide first information on the influence of elevated carbon dioxide concentrations on selected microbial populations (Figure 34).

Gross CO₂ production was under aerobic conditions about 100-fold higher than under anaerobic conditions. Under anaerobic conditions CO₂ production was similar at the vent and the control site, with 4.34 ± 0.25 and 1.03 ± 0.32 $\mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$. In contrast, aerobic rates were with 432 ± 57 $\mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$ significantly higher at the control site than in the vent centre with 121 $\mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$.

Potential methane production rates without substrate addition in the sediment samples from 10-20 cm depth were at about $0.33 \pm 0.007 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ d}^{-1}$ and therefore much higher than at the vent centre (location 36 m) than in the control site samples (55 m) where they reached $0.12 \pm 0.002 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ d}^{-1}$. Data for methane oxidation under aerobic conditions showed the opposite picture: Higher rates of $5.4 \pm 0.42 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ d}^{-1}$ at the control site compared to $2.2 \pm 0.35 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ d}^{-1}$ in the intermediate CO_2 positions (14 m) and $0.4 \pm 0.11 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ d}^{-1}$ at the centre of the vent. Finally, a remarkable aerobic methane oxidation activity was found even in sediment samples down to 1.5 m depths with an identical pattern (data not shown). This points towards a methane supply for the methanotrophic bacteria from deeper sources present in the deepest oxygen-poor, organic deposits of the soil column and tracing back to the limnic evolution of the study site.

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

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

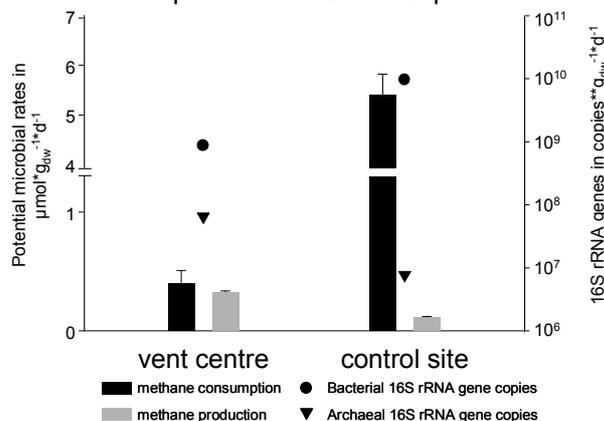


Figure 35: Differences in microbial activity and 16S rRNA gene copies at 10 to 20 cm depth

In accordance with the microbial activities, total numbers of microorganisms showed also significant differences between the individual sites. Cell numbers of *Bacteria* were determined using quantitative PCR (qPCR, [17]): They were highest at the control site and substantially lower towards the vent centre; the values decreased from 9.6×10^9 to 8.7×10^8 gene copies $\text{g}_{\text{dw}}^{-1}$ of soil. For *Archaea* in contrast, the values increased from control site towards the centre, with 7.7×10^6 and 6.5×10^7 gene copies $\text{g}_{\text{dw}}^{-1}$ of soil.

One explanation for the observed changes in the community composition might be the replacement of oxygen in the soil gas with CO₂, leading to first microaerobic and then to anaerobic conditions. This would thus favour e.g. methane-producing *Archaea* or sulphate-reducing bacteria. To analyse this in more detail, group-specific qPCR assays are carried out currently to reveal, whether certain functional groups, like the methane oxidising or sulphate reducing bacteria, were absent or stimulated at the CO₂-rich sites.

Another implication for strong changes in the microbial community came from the lipid biomarker studies. Although the cell numbers of bacteria decreased, the biomarker studies showed that in the CO₂ vent, bacterial non isoprenoidical tetraethers lipids were contained in higher quantities than at the control site (e.g. in 50-60cm depth $503 \mu\text{g g}^{-1}$ TOC at the CO₂ vent site and $302 \mu\text{g g}^{-1}$ TOC at the control site). Even though the source organisms of bacterial tetraethers are not known yet, they most likely derive from anaerobic bacteria (see Oppermann *et al.*, 2010 and references therein). This finding is of special interest since ether lipids are more stable than ester lipids that are commonly found in bacteria. Bacteria able to synthesis etherlipids are therefore probably better adapted to the low pH conditions found at the CO₂ vent (Figure 31).

4.3.4. Conclusions

CO₂ gas fluxes into the Laacher See are roughly estimated in the range of about 5,000 tons of CO₂ per year (Aeschbach-Hertig *et al.*, 1996). Additional CO₂ gas seepages from the underground occur permanently at the fringes of the lake. Even if a CO₂ gas release of up to $600 \text{ g m}^{-2} \text{ d}^{-1}$ could be registered along the studied vent, our results indicate that the effects of the gas vents are spatially limited. Nevertheless, some significant effects of high

CO₂ concentrations on the terrestrial ecosystem were observed. The ecosystem appears to have adapted to the different conditions through species substitution or adaptation, showing a shift towards anaerobic and acidotolerant to acidophilic species under elevated CO₂ concentrations. The present results have stimulated future research activities which will include an extensive investigation campaign with gas, water and sediment sampling both for the Laacher See and carbonic springs nearby. At the end, this study should identify possible candidates in the botanical and microbial kingdoms, whose presence or absence provide easily detectable indicators for the leakage of CO₂ from deep reservoirs into near-surface terrestrial ecosystems.

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4.4. Microbial community changes at a terrestrial volcanic CO₂ vent induced by soil acidification and anaerobic microhabitats within the soil column

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„Microbial Community Changes at a Terrestrial Volcanic CO₂ Vent Induced by Soil Acidification and Anaerobic Microhabitats within the Soil Column.” *FEMS Microbiology Ecology* **84**: 60-74.

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Abstract

CO₂ capture and storage (CCS) in deep geological formations is one option currently evaluated to reduce greenhouse gas emissions. Consequently, the impact of a possible CO₂ leakage from a storage site into surface environments has to be evaluated. During such a hypothetical leakage event, the CO₂ migrates upwards along fractures entering surface soils, a scenario similar to naturally occurring CO₂ vents. Therefore, such a natural analogue site at the Laacher See was chosen for an ecosystem study on the effects of high CO₂ concentrations on soil chemistry and microbiology. The microbial activities revealed differences in their spatial distribution and temporal variability for CO₂-rich and reference soils. Furthermore, the abundance of several functional and group specific gene markers revealed further differences, e.g. a decrease of *Geobacteraceae* and an increase of sulphate-reducing prokaryotes in the vent centre. Molecular biological fingerprinting of the microbial communities with DGGE indicated a shift in the environmental conditions within the Laacher See soil column leading to anaerobic and potentially acidic microenvironments. Furthermore, the distribution and phylogenetic affiliation of the archaeal 16S *rRNA* genes, the presence of ammonia-oxidising *Archaea*, and the biomarker analysis revealed a predominance of *Thaumarchaeota* as possible indicator organisms for elevated CO₂ concentrations in soils.

4.4.1. Introduction

The reduction of the potential greenhouse gases CO₂ and CH₄ in the atmosphere becomes obligatory in order to mitigate the effects of global warming (IPCC, 2007). One option globally discussed which might offer the necessary large scale applicability is carbon capture and storage (CCS) (Haszeldine, 2009). However, before starting CO₂ storage in the deep geological underground, the risks and consequences of an unlikely but possible leakage from storage sites into near surface ecosystems have to be assessed. The geological setting of volcanic CO₂ vents resembles the closest natural analogue for possible CCS leakage scenarios, with the CO₂ migrating upwards through fractures or cleavages into near surface ecosystems (West, *et al.*, 2005). This then leads to significantly elevated CO₂ concentrations, however, as expected also for a leakage scenario only within a very small and defined area.

Several studies reported geochemical changes in soil induced by upwards migrating CO₂, like acidification, weathering effects on minerals, and higher soil humidity (Farrar, *et al.*, 1995, Stephens & Hering, 2004, Beaubien, *et al.*, 2008), with consequently decreased oxygen availability in surface soil horizons (Oppermann, *et al.*, 2010). Negative effects of high CO₂ concentrations in soil atmosphere were documented on plant development and species richness, and also on microbial respiration rates (Macek, *et al.*, 2005, Pierce & Sjögersten, 2009). Nevertheless, in such CO₂-affected soil ecosystems still only little is known about the interactions between soil chemistry, microbial community structures and activities, which in turn have a significant influence on ecosystem health, plant communities and land use.

In the Rhenish Massif, located in Middle and Western Europe, dissolved carbon species and free CO₂ reach the surface at many places, though concentrated in the volcanic fields. As one volcanic field the East Eifel in Germany consists of about 100 former eruptive centres covering an area of approximately 330 km². The Laacher See caldera is situated in the centre of the East Eifel volcanic field in Germany with gaseous CO₂ discharge along the (north-) eastern border of the lake. The whole volcanic system of

the Laacher See is estimated to emit several thousand tons of CO₂ per year into the atmosphere (Aeschbach-Hertig, *et al.*, 1996). Release of CO₂ in this area typically occurs from gas vents characterised by a small core of elevated gas flux. The vents on the pastures around the lake are often visible due to a 5-6 m wide core of reduced vegetation or even bare soil surrounded by an area of variably-impacted vegetation (Krüger, *et al.*, 2011). The selected site at the western shore had undergone significant human modifications. Due to the man-made lowering of the lake's water level, a relatively large area of land was gained to the south and the west of the lake. Today this land is used for extensive agricultural purposes, mainly cattle farming. The selected CO₂ vent was sampled along a transect ranging from high CO₂ concentrations in the vent centre (>90% of the soil gas), over medium concentrations (20-30% CO₂) towards background CO₂ levels at the reference site (Jones, *et al.*, 2009).

Following the initial introductory description of the gas geochemical and botanical characteristics of this vent site (Krüger, *et al.*, 2011), our main objective in the present study was to describe in detail effects of elevated CO₂ on the ecosystem and the microbial community in up to 1.8 m long sediment cores. This should help to identify possible indicator species within the biosphere either sensitive or resistant to high CO₂ concentrations. For example, several soil ecosystem studies revealed the importance of *Crenarchaeota* (soil group 1.1c) or recently reclassified *Thaumarchaeota* (group 1.1b (Pester, *et al.*, 2011)) as possible ammonia oxidizers for acidic habitats (Kemnitz, *et al.*, 2007, Nicol, *et al.*, 2008, Gubry-Rangin, *et al.*, 2010).

Nevertheless, for CO₂ affected sites it still remained unclear which soil parameters (pH, humidity, aeration etc.) related to the increased CO₂ concentrations cause a differentiation within the biosphere. This work therefore concentrated on molecular and microbiological investigations in combination with geochemical surveys to identify potential controlling parameters for the microbial biosphere.

4.4.2. Methods

Site description

The studied vent system was located on a pastured field at the western side of the Laacher See, a typical maar lake in the East-Eifel, Germany (Krüger, *et al.*, 2011). Several vents were visible on the pasture due to 5-10 m wide areas with reduced vegetation and bare soil. Based on these observations, measurements of soil CO₂ concentration led to the selection and final layout of a 60 m long roughly north-to-south transect across one terrestrial CO₂ vent.

Soil samples and underlying sediments were collected during two campaigns (2007 and 2008) along this transect ranging from background to high CO₂ soil gas concentrations. Samples were taken at the maximum zone of CO₂ fluxes in the centre of the vegetation free zone (vent centre; location: 36 m, measured from the southern end of the transect), as well as in a zone with elevated CO₂ concentrations at the outer rim of the venting area (medium site; location: 14 m). The reference sample was taken in proximity of the CO₂ vent, in an area where no elevated gas concentration was observed (reference site; location: 55 m).

Sampling procedure

In September 2007 and July 2008 surface soils were sampled with three parallel push cores per site in direct proximity to each other. The top 10 cm of each core were divided into two parts (0-5 and 5-10 cm) and separately pooled from all cores. The sample represented the organic rich surface coverage with the main rooting zone within the soil column (mineralogical Ah-horizon) and CO₂ concentrations and flux measurements were also conducted below (at 15 cm depth). After homogenising the composite material, subsamples were taken for DNA extraction (stored at -20°C), activity measurements, and geochemical analysis (stored at 4°C). The two collected surface samples were separately analysed to describe the specific heterogeneity of the soil surface. Unfortunately, the

surface samples of 2007 for DNA extraction were discarded as the samples were thawed during transport and cross contaminated.

In addition to the surface sampling, two long soil cores were collected in 2007 using a steel probe with a “dutch” auger. The cores were sampled in intervals of 10 cm down to the maximum depth, i.e. 150 and 180 cm for vent centre and reference site, respectively. The humidity of the soil samples was measured from about 1g of each composite sample using the “Moisture analyzer” MA100 (Sartorius GmbH Germany). The averaged value for the surface was correlated to the CO₂ level using Pearson product-moment correlation with a probability-value of 0.05. The deeper core samples recovered in 2007 were situated below the water table and thus water saturated (Krüger, *et al.*, 2011).

Soil geochemistry

The geochemical analysis included an extensive soil gas survey and analysis of soil parameters (see below for details), like total organic carbon (TOC), for the reference and the vent centre. The soil gas measurements were carried out in 15 and 60 cm depth following 0.5 m intervals along the transect. Several additional gas samples were taken along the traverse for comparative laboratory analyses and for the determination of carbon isotope ratios ($\delta^{13}\text{C}_{\text{CO}_2}$; by means of a Thermo Delta plus XL mass spectrometer). The complete description of the methods and results for concentrations and gas flux measurements were recently published ((Krüger, *et al.*, 2011)/ supporting information with short description).

Total carbon (TC) and total nitrogen were measured with an elemental analyser (Carlo Erba Science 1500 CNS Analyser; Erba Science, Italy) after the soil samples were dried at 105°C and milled. For the quantification of total organic carbon (TOC), inorganic carbon was removed with HCl. Repeated measurements showed an error of less than $\pm 0.05\%$. An aliquot of the samples were taken for isotopic analyses of TIC and TOC. The $\delta^{13}\text{C}$ -values were determined using a Finnigan MAT 252 mass spectrometer after high-temperature combustion (1020°C) to CO₂ in a Carlo Erba NA-2500 elemental analyser (Erba Science, Italy). Sulphur was removed with Sulfix (WAKO Chemicals, Japan) after

combustion following a method by (Carlson, 1998). Based on replicate measurements of reference standards IAEA-CH-6 (IAEA, Austria) and IVA-Sediment (IVA, Germany), the analytical precision was better than 0.1‰. Duplicate measurements showed a mean deviation of < 0.2‰. Soil pH was determined using a solid body pH electrode in a slurry composed of a soil sub-sample suspended in a 0.01 M CaCl₂ solution. The method has UKAS and MCERTS accreditation and is validated for all soils with a pH between 3 - 10.

Determination of microbial activities

Each composite soil sample was converted into a homogenous slurry by the addition of artificial mineral medium (see supporting information) in a ratio of 1:1 and aliquoted into heat sterilised serum bottles according to Krüger *et al.* (2011). The anaerobic manipulations were performed under an atmosphere of N₂/CO₂ (90/10 [v/v]) in an anaerobic chamber (Mecaplex). The incubations were sealed with butyl stoppers and repeatedly flushed with N₂ to remove residual O₂. For aerobic setups the slurries were left open for several minutes to aerate (loosely covered). All incubations were conducted in three analytical replicates from each composite soil sample and incubated in the dark at 20°C. Headspace gas samples were taken daily (up to one week) after shaking of the microcosms. Methane and CO₂ were determined using a GC 14B gas chromatography (Shimadzu) as described by (Nauhaus, *et al.*, 2002), which was additionally equipped with a methaniser to quantify the CO₂.

The general microbial mineralization of organic material was quantified as the CO₂ production (CPR) in aerobic incubations. Potential methane oxidation rates (MOR) were measured following the depletion of methane in aerated soil samples amended with 5 Vol.% methane. To determine the initial potential methane production rates (MPR) three replicates of an anaerobic slurry from each soil sample were incubated without any amendment (Krüger, *et al.*, 2002).

The determination of potential sulphate reduction rates (SRR) was conducted with slurry incubations amended with 10 mM sulphate and set up as described above. The produced sulphide was analysed from a withdrawn subsample using hypodermic needles

and plastic syringes pre-flushed with N₂ via the formation of copper sulphide (Cord-Ruwisch, 1985).

All microbial activities were calculated via the product increase (or reactant decrease) over incubation time and expressed in $\mu\text{mol g}_{\text{dw}}^{-1} \text{day}^{-1}$ (g_{dw} : dry weight). The arithmetic mean was calculated from both composite soil samples with three analytical replicates ($n=6$; $\pm\text{SD}$) for each year. The linear regression model was calculated and tested for its probability using t-test (p -value <0.05). Statistical evaluation of significance of observed variance was conducted using factorial ANOVA analysis with a group assignment of CO₂ concentration and year in a 3x2 set up with a significance level of 1% (0.01) for the dataset (degrees of freedom_{Sum}: 35 ($n= 36$ data points for each rate)).

Community analysis using molecular-biological approaches and biomarker analysis

The community was analysed using 16S *rRNA* based methods, the abundance of functional gene markers, and specific lipid biomarkers. Extended descriptions of DNA analysis (primer and PCR specifications; Table 10 for quantitative PCR) and lipid extraction are given in the supporting information. In brief: the DNA was extracted from 1 to 0,5 g of each composite soil sample following the manufacturers manual of the FastDNA Spin Kit for Soil (Bio 101; MP Biomedicals) (Webster, *et al.*, 2003).

The gene abundances of both composite samples were quantified separately and averaged giving the mean and standard deviation (SD; 2 samples with 5-6 measurements each) to include site specific heterogeneity. The quantification results of the microbial community were given as gene copies g^{-1} of the wet soil samples. Differences in the abundance of the genes between the sampling sites were analysed using a paired Students t-test with a significance level of $p: <0.05$ (5%).

Sequencing of reamplified DGGE-bands was conducted at Seqlab GmbH (Göttingen, Germany) with the reverse primers. All sequences were classified using the RDP and Green Genes tools. The obtained DNA sequences were aligned with the ARB-SILVA aligner and assembled in the existing Parsimony tree of a recent reference database (SSURef 106, Feb2011) according to Pruesse *et al.* (2007). The next relatives were

retrieved and used to calculate a phylogenetic tree for the bacterial sequences with the ARB software package, while the archaeal tree was further examined regarding the environmental affiliation of the representative organisms using the dataset of Nicol *et al.* (2008). The tree (Figure 4) was constructed using the PHYLIP(DNA) package adding the obtained sequences into the resulting maximum likelihood tree without changing the overall topology. The exported tree was used for further analysis using FastUniFrac (Hamady, *et al.*, 2009) for statistical examination (Bonferroni corrected p-test) of the cluster and the principal coordinate analysis affecting the distribution within the soil column. The sequence specific sample factors were determined to distinguish the variation factors as: vegetation (bare/grass), CO₂ (concentration in percent), O₂ (concentration in percent), pH (acidic; slight acidic and neutral), sampling depth (surface/deep core). The sequences (56 bacterial and 34 archaeal partial 16S *rRNA* gene sequences) were submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov>) under accession numbers JF717665-JF717754.

The extraction, isolation and analysis of fatty acid biomarkers followed the protocol of Oppermann *et al.* (2010) with a few modifications for this study. In short, lyophilised and homogenised samples were hydrolysed with 6% KOH in methanol (2 h, 80°C) and extracted with *n*-hexane to release neutral lipids (e.g. alcohols). Alcohol fractions were obtained after separation from other neutral lipids via column chromatography (Merck silica gel 60, using dichloromethane and methanol). Further descriptions of the protocol are provided in the supporting information.

4.4.3. Results

Soil CO₂ conditions

The complete dataset of CO₂ concentrations and flux measurements has been published recently (Krüger, *et al.*, 2011). In brief, three main zones of elevated CO₂ concentrations existed along the traverse between 11-17 m, 21-28 m, and 30-42 m, the latter representing the centre of the vent.

In 2007 the peak CO₂ concentration in the soil gas in 60 cm depth reached more than 90 Vol. % at the vent centre, with flux rates of up to 550 g CO₂ m⁻² d⁻¹. The oxygen concentration at this site in 60 cm depth was below 3 Vol. %. Further upwards in 15 cm depth the CO₂ accounted for 30 Vol. % of the soil gas, while the oxygen level was between 16-18 Vol. %. The strongly decreased oxygen concentration showed that the aeration of the soil column at the vent centre was effectively suppressed.

The medium site was located at 11-17 m with moderately elevated CO₂ concentrations of 20 Vol. %, 16-18 Vol. % O₂ in 60 cm depth, and a flux rate of ~177 g CO₂ m⁻² d⁻¹. These values decreased to 4-6 Vol. % CO₂ and 20 Vol. % O₂ in 15 cm depth. Already at a relatively short distance from the vent centre and the medium site, the CO₂ concentrations dropped back to background values of 2 % CO₂ in 60 cm, and less than one per cent in 15 cm depth (reference site at 51 m). The oxygen level at the reference site was around 20 Vol. % at both depths.

The CO₂ concentrations as well as flux rates described above for 2007 were higher compared to those of the subsequent field campaign in 2008, which could be correlated to the weather conditions. During the autumn sampling in 2007, the weather documentation recorded a higher wind speed and lower atmospheric pressure than in 2008, both factors supporting the efflux of CO₂ (for details see Krüger *et al.* 2011). Weather conditions are important parameters for temporal and spatial variations of CO₂ flux rates (pumping effects) from vent systems (Beaubien, *et al.*, 2008).

The carbon isotopic signature of the CO₂ ($\delta^{13}\text{C}_{\text{CO}_2}$) collected from the soil gas showed different isotope ratios for CO₂ in the vent centre (-4.1 to -2.7 ‰ PDB) compared to medium (-1.7 to -0.2 ‰) and reference site (-1.0 to 0.8 ‰). The $\delta^{13}\text{C}_{\text{CO}_2}$ value measured in the vent centre point directly to the upper earth mantle and/or lower earth crust as origin of the CO₂. Contrastingly, the CO₂ collected at medium and reference sites was already affected by mixing processes with e.g. atmospheric and biogenic CO₂, and by isotope fractionation processes.

The water content in the surface soil samples was not significantly correlated to the increasing CO₂ concentrations for both years (p-value >0.1), probably influenced by the

preceding rain period in 2007. In this year, the soil humidity was 32 ± 3 % at the reference and 22 ± 2 % at the medium CO₂ site. The vent centre was almost water-saturated and highly inhomogeneous (48 ± 20 %) with depth. In 2008, the humidity was 10 ± 1 % in the reference and 19-20 % in medium and vent site, respectively. The soil pH in the top 10 cm was with 6 similar for CO₂-affected and unaffected sites, However, it changed drastically with depth. At the vent centre, the pH in the deeper soil samples was significantly lower, between 5.2-3.9, while it remained around 5.5 at the reference site (Krüger *et al.* 2011).

Soil carbon and nitrogen

The concentration of total organic carbon showed at both sampling sites strong variations with depth (Figure 35). The highest concentrations were detected at the reference site below 90 cm soil depth (14.0-18.4% wt. of dry soil). In the soil horizons above this zone the TOC-concentrations decreased up to 60 cm depth, followed by a strong increase between 50 and 60 cm. Further upwards, between 20 and 50 cm the concentrations of soil TOC are relatively low (1.1-1.4% wt. of dry soil). In the Ah-horizon the concentrations of soil TOC rose again (3.0-5.8% wt. of dry soil). The concentration of soil TOC in the vent centre followed the trend of three zones with high concentration observed at the reference site. In the centre, the TOC concentrations were overall higher in the upper part of the profile (0-70 cm; 4.5-10.8% wt. of dry soil), but decreased in the lower part of the profile (70-150 cm; 0.5-5.5% wt. of dry soil).

The $\delta^{13}\text{C}$ -values (Figure 35) of soil TOC in the Ah-horizon (0-20cm) showed only small differences between vent centre and reference site (-26.9 to -27.3‰ and -26.8 to -27.2‰ , respectively). Towards the first maximum in TOC concentrations in 60 cm depth, the $\delta^{13}\text{C}$ -values showed a ¹³C enrichment at both sampling sites with -24.0‰ and -22.1‰ at the CO₂ vent and the reference site, respectively. In the deeper soil horizons, the $\delta^{13}\text{C}$ -TOC-values remained relatively ¹²C-enriched in the vent centre (-25.0‰ and -25.6‰), whereas the $\delta^{13}\text{C}$ -TOC-values at the reference site showed an enrichment of ¹³C.

Inorganic carbon was not present in significant amounts in the studied soil samples. The concentrations of total nitrogen in soil at the reference site are by a factor of 8 to 13

smaller than the TOC concentrations (Figure 35). The steep increase of soil TOC concentration in 110-150 cm was not mimicked by the TN leading to a change of the C/N ratio in the depth profile. In contrast, at the vent centre the C/N ratios remained relatively stable in this zone, but showed a greater variability between 40 to 80 cm depth.

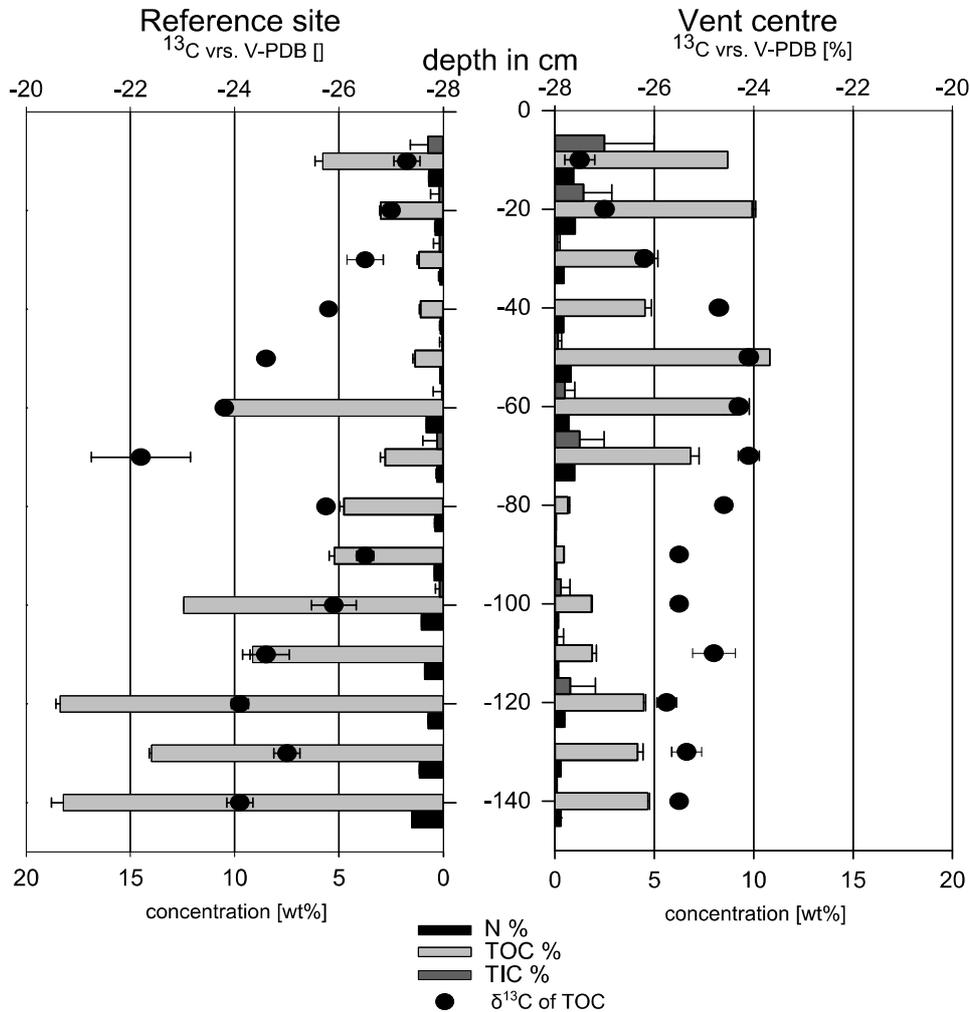


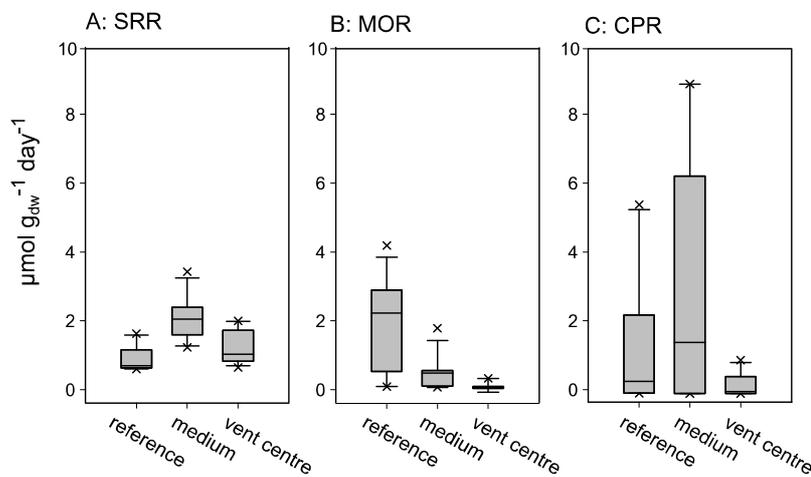
Figure 36: Development of total nitrogen (black bars), total organic carbon (TOC; light grey), and total inorganic carbon (TIC; dark grey) in relative concentrations, and of the $\delta^{13}\text{C}$ -values of soil TOC (black circles with standard error mean; SEM n=3) within the depth profile of 2007 [depth in cm on the vertical axis]. For better comparison of the spatial differences between the sites the horizontal axes for relative concentration [wt%] and $\delta^{13}\text{C}$ vs. V-PDB [‰] were mirrored to each other.

Microbial activities in the surface soil layers

The surface soils of 2007 showed generally low microbial mineralisation activity with potential CO_2 production rates (CPR) between $0.016\text{--}0.031 \mu\text{mol g}_{\text{dw}}^{-1}\text{d}^{-1}$, corresponding to rates found in other soil environments (Kirschbaum, 1995). The sulphate reduction rates (SRR) were highest under medium CO_2 concentration ($1.6 \pm 0.3 \mu\text{mol g}_{\text{dw}}^{-1}\text{d}^{-1}$) and

decreased at the reference site ($0.9 \pm 0.1 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$). In contrast, the methane oxidation rates (MOR) were highest at the reference site ($2.4 \pm 0.55 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$), and decreased with increasing CO_2 influence to $0.7 \pm 0.55 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$ (medium site) and further to $0.13 \pm 0.14 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$ in the vent centre. Below the Ah-horizon in the deep cores collected in 2007, high potential rates of sulphate reduction were detected for both reference site and vent centre albeit only low methanogenesis was detected in the depth profile (data not shown).

Figure 37: Statistical analysis of rate dependent and site specific heterogeneity of potential microbial activities measured in the surface soils of 2007 and 2008.



heterogeneity of potential microbial activities measured in the surface soils of 2007 and 2008. The sulphate reduction rate (SRR), methane oxidation rate (MOR), and CO_2 production rate (CPR) were measured in triplicate incubations from two composite soil samples for each year ($n=24$). The median (solid line) was calculated from the dataset while error bars represented the confidence interval (CI 95%) and given outlines were marked as thin X.

In 2008, the CPR in surface soil samples were highest at medium CO_2 concentrations with $5.8 \pm 2.9 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$. The SRR were again highest under medium concentrations with $2.5 \pm 0.5 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$ (vent centre: $1.3 \pm 0.6 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$), while at the reference site the rates were similar to the potential activity in 2007 ($0.69 \pm 0.14 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$). Likewise, the MOR were highest for the reference site again ($1.8 \pm 1.7 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$). At the medium site and vent centre, 0.4 ± 0.2 to $0.2 \pm 0.1 \mu\text{mol g}_{\text{dw}}^{-1} \text{d}^{-1}$ of methane were oxidised, respectively. In this study, no methane production was detected in anaerobic microcosms with soils samples from the top 10 cm, both from 2007 and 2008. In contrast, low rates of methanogenesis have been reported with samples from deeper soil horizons (Krüger, *et al.*, 2011).

The relatively heterogeneous distribution and temporal variation of the potential activities along the transect, with sampling depth and also between the two years, required a closer statistical investigation of potential influencing factors (Figure 36). In the first step, a linear regression model did not show a significant correlation between selected microbial activities and the increasing CO₂ concentrations (p: >0.2; data not shown). Subsequently, a more complex model (Two-way ANOVA, Table 11) was applied, which revealed an abundant interaction between the CO₂ concentrations and seasonal factors affecting the potential sulphate reduction rates. Due to the generally low SRR in September 2007, CO₂ showed no significant influence on SRR (using Holmes-Sidak t-test for pairwise comparisons), while the influence became significant during midsummer in 2008 (p: <0.01). In contrast to this, the methane oxidation rates were influenced by the increasing CO₂ concentrations (p: <0.001, Table S2) in both years.

Quantitative microbial community composition

The copy numbers for *Bacteria* were relatively similar at all sites, with $8.3 \pm 4 \times 10^9$, $1.6 \pm 0.8 \times 10^{10}$, and $1.4 \pm 0.5 \times 10^{10}$ 16S *rRNA* copies g⁻¹ for reference, medium site and vent centre, respectively. The archaeal 16S *rRNA* gene copy numbers were about two orders of magnitude lower than the bacterial with approx. 10⁸ gene copies g⁻¹ in the soil samples (Figure 37). The overall community size (total abundance of 16S *rRNA* gene copies) in the soil surface samples (only 2008) was not affected by the increasing CO₂ concentrations (p: >0.05).

The analysis of more specific gene markers revealed differences between the sites. The abundance of genetic markers for the *Crenarchaeota*, determined with primers for both 16S *rRNA* genes and the key gene for ammonia-oxidising *Archaea* (AOA with *amoA* gene), was highest at the vent centre, and significantly lower at the medium and reference sites (p-value for both genes <0.001). The copy numbers for ammonia-oxidising *Bacteria* (AOB) were compared with the numbers of AOA rather low at all sites and not correlated to CO₂ (p: >0.07).

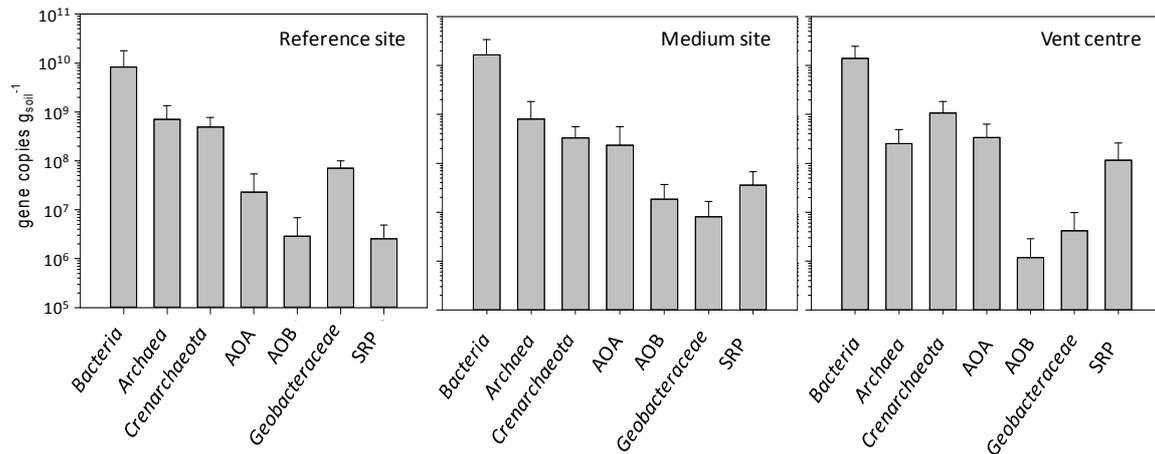


Figure 38: Site specific profiles of universal and specific 16S *rRNA* genes and functional genes analysed with quantitative PCR (as listed in Table S1). Bars represented the mean value for the surface soils of 2008 with error bars giving the standard deviation (SD; n=2 composite samples (5 to 6 measurement per sample in different dilutions)). AOA: Ammonium oxidising Archaea (*amoA* gene copies); AOB: Ammonium oxidising Bacteria (*amoA* gene copies); SRP: sulphate reducing prokaryotes (*dsrA* gene copies).

The functional gene *mcrA* for the methanogenic Archaea was detected at the reference site with $1.8 \pm 0.8 \times 10^7$ gene copies g^{-1} and slightly decreased at the medium site ($2.7 \pm 1.7 \times 10^6$ gene copies g^{-1}). At the vent centre the gene copies for *mcrA* were below the detection limit. Functional gene markers for sulphate reducing prokaryotes (*dsrA* gene), were highest in samples from the CO₂ affected sites (with up to $1.2 \pm 0.9 \times 10^7$ gene copies g^{-1}) compared to the reference site (p-value: <0.01) (Figure 3). The abundance of Fe(III)- and Mn(IV)-reducing *Geobacteraceae* was lowest at the vent centre ($4.2 \pm 3 \times 10^6$ gene copies g^{-1})

The quantitative community composition in samples from the deep cores collected in 2007 differed considerably from the surface samples (Table 8). The overall gene copy abundance showed only minor changes at the vent centre but a significant increase in the archaeal community size at the reference site with depth (p: <0.01). The analysis of specific gene markers revealed decreasing copy numbers for *Crenarchaeota* and AOA with depth at both sites. Nevertheless, both genes were, in contrast to the results for the surface samples, more abundant in the deeper sediments at the reference site than at the vent centre.

Table 8: Quantitative analysis of the microbial community composition in selected deep soil layers of 2007, determined using 16S *rRNA* genes and functional genes (for AOA, AOB, SRP, methanogenic *Archaea*) (\pm SD; n=2 samples; 5 to 6 measurements per sample).

gene copies g ⁻¹ _(wet soil)	reference site			vent centre		
	100 cm	130 cm	180 cm	50 cm	110 cm	130 cm
<i>Bacteria</i> (x10 ⁸)	12 \pm 1.1	3.9 \pm 0.3	2.7 \pm 0.3	1.2 \pm 0.2	2.2 \pm 0.2	1.4 \pm 0.2
<i>Archaea</i> (x10 ⁸)	4.0 \pm 0.1	15.2 \pm 0.5	13.5 \pm 0.8	0.2 \pm 0.03	1.6 \pm 0.1	0.2 \pm 0.01
<i>Crenarchaeota</i> (x10 ⁷)	14 \pm 1.9	5.6 \pm 0.9	5.4 \pm 0.08	2.6 \pm 0.5	2.6 \pm 0.2	3.4 \pm 0.2
AOA (x10 ⁶)	34 \pm 0.9	1.8 \pm 0.3	0.6 \pm 0.2	12.1 \pm 6	0.7 \pm 0.1	0.6 \pm 0.07
AOB (x10 ⁶)	0.1 \pm 0.08	0.5 \pm 0.1	-*	0.2 \pm 0.07	0.1 \pm 0.2	-*
SRP (x10 ⁶)	11 \pm 0.7	3.1 \pm 0.1	1.5 \pm 0.1	12 \pm 0.6	9.2 \pm 0.4	1.9 \pm 0.04
Methanogens (x10 ⁶)	3.5 \pm 1	3.8 \pm 0.6	4.3 \pm 0.6	0.02 \pm 0.01	0.2 \pm 0.02	0.16 \pm 0.04
<i>Geobacteraceae</i> (x10 ⁵)	129 \pm 14.1	12 \pm 0.1	8.2 \pm 0.8	0.8 \pm 0.2	0.2 \pm 0.01	3.8 \pm 1.1

AOA: Ammonium-oxidising Archaea

AOB: Ammonium-oxidising Bacteria

SRP: Sulfate-reducing prokaryotes

The *Geobacteraceae* 16S *rRNA* gene copy numbers and the *mcrA* gene copies for methanogenic Archaea were significantly increased by several orders of magnitude at the reference site (p : <0.0001), while the abundance of *SRP* was increased in the vent centre (p : <0.01).

Phylogenetic analysis of the microbial community

The phylogenetic analysis of the partial 16S *rRNA* gene sequences with DGGE revealed only minor differences between the two surface soil samples. The diversity of the bacterial was greater than that of the archaeal community.

The dominating part of the bacterial sequences was affiliated to the *Betaproteobacteria*, *Acidobacteria* and *Bacilli*, which have previously been associated to terrestrial soil communities. The isolation source of the closest relatives to the identified sequences was searched for indications about specific habitat conditions, e.g. acidic pH, anoxic environments, or increased CO₂ levels. The presence of such environmental parameters was similar for all sites, indicating no significant differentiation of the bacterial communities along the transect in the surface layers. With depth, (2007 deep cores; Table 12) both the vent centre and reference site showed a higher proportion of next relatives from

potentially anoxic and aquatic habitats, like earthworm gut, marine, and freshwater sediments. Further statistical testing of the distribution of selected phylotypes for site specific clustering showed that no significant differentiation along the gradients (CO_2 ; O_2 ; pH etc) occurred in the bacterial community. Therefore, no indicative bacterial phylum was specific or dominant at the CO_2 affected sites (Table 9 and Table 12).

Within the archaeal 16S *rRNA* DGGE profile only a few phylotypes were detected (Tab 2). However, in contrast to the bacterial community, the identified archaeal communities indicated a distinct shift in the community composition under elevated CO_2 . The community was significantly different ($p > 0.001$; pairwise comparison Bonferroni-corrected) between the vent centre and the reference or medium site, respectively. On the other hand, medium and reference site were not significantly distinct from each other ($p > 0.05$). The deep core (Table 13) samples clustered distinctively from the respective surface samples ($p < 0.001$) indicating a further depth-induced shift in the community, independent of the CO_2 .

Table 9: Identified sequences from DGGE bands of the surface soil samples (2008) for archaeal partial 16S *rRNA* genes. The sequences were identified using the RDP classifier and the seqmatch tool to search for next neighbours (% similarity giving the seqmatch similarity score; isolation sources as recorded in the GenBank entry). Sequences of deeper soil samples are provided in the supporting information.

<i>Bacteria</i>	Acc. no.	assignment details	next relative (% similarity)	Acc. no.	isolation source	
Reference site (surface)	JF717698	<i>Betaproteobacteria</i>	<i>Ralstonia</i>	<i>Ralstonia</i> sp. OV225 (100)	AY216797	agricultural soil
	JF717699	<i>Acidobacteria</i>	<i>Edaphobacter</i>	uncult. Bacterium (89.9)	EF516251	grassland (pH: 4.9)
	JF717700	<i>Bacilli</i>	<i>Falsibacillus</i>	uncult. Bacterium (95.7)	DQ129455	urban aerosol
	JF717701	<i>Acidobacteria</i>	Gp1	uncult. <i>Acidobacteria</i> (98.8)	HM061990	agricultural soil (pH: 6.2)
	JF717702	<i>Acidobacteria</i>	Gp1	uncult. bacterium (96.2)	AY963480	forest soil (pH: 4.5)
	JF717703	<i>Clostridia</i>	<i>Desulfoviregula</i>	uncult. bacterium (98.5)	GU444065	agricultural soil
	JF717704	<i>Actinobacteria</i>	<i>Spirillospora</i>	uncult. <i>Actinobacteria</i> (99.2)	JF833791	potassium mine soil
	JF717705	<i>Gemmatimonadetes</i>	<i>Gemmatimonas</i>	uncult. <i>Gemmatimonadetes</i> (98.8)	AY395331	pasture soil
Medium site (surface)	JF717706	<i>Betaproteobacteria</i>	<i>Rivibacter</i>	uncult. bacterium(91.4)	DQ241393	mine drainage water
	JF717707	<i>Gammaproteobacteria</i>	<i>Luteibacter</i>	<i>Luteibacter rhizovicinus</i> (98.8)	JF778702	rhizosphere permafrost
	JF717708	<i>Gammaproteobacteria</i>	<i>Rugamonas</i>	<i>Undibacterium</i> sp. NBGD59 (89.5)	HQ003415	freshwater lake
	JF717709	<i>Acidobacteria</i>	Gp1	uncult. Acidobacteriaceae (98.2)	HQ598219	forest soil
	JF717710	<i>Actinobacteria</i>	<i>Thermoleophilum</i>	uncult. bacterium (88.7)	GU983322	metalliferous peat rhizosphere
JF717711	<i>Acidobacteria</i>	<i>Terriglobus</i>	uncult. <i>Acidobacteria</i> (86.5)	HQ598406	forest soil	
Vent centre (surface)	JF717665	<i>Betaproteobacteria</i>	<i>Ralstonia</i>	<i>Ralstonia</i> sp. OV225 (100)	AY216797	agricultural soil
	JF717666	<i>Actinobacteria</i>	<i>Arthrobacter</i>	<i>Arthrobacter globiformis</i> (99.2)	FJ011429	soil rhizosphere
	JF717667	<i>Betaproteobacteria</i>	<i>Nitrosospora</i>	uncult. bacterium (94.8)	GQ339172	iron(II)-rich freshwater seep
	JF717668	<i>Acidobacteria</i>	Gp3	uncult. Solibacter sp. (99.2)	FJ889211	agricultural soil (pH: 6.5)
	JF717669	<i>Acidobacteria</i>	Gp1	uncult. bacterium (100)	FJ466266	volcanic deposit
	JF717720	<i>Actinobacteria</i>	<i>Fodinicola</i>	<i>Streptomyces</i> sp. (96.3)	EF059965	mangrove soil
	JF717712	<i>Acidobacteria</i>	<i>Terriglobus</i>	uncult. <i>Acidobacteria</i> (86.5)	HQ598406	forest soil
	JF717713	<i>Acidobacteria</i>	Gp1	uncult. <i>Acidobacteria</i> (98.8)	HQ995662	grassland soil
	JF717714	<i>Acidobacteria</i>	Gp1	uncult. bacterium (99.2)	AY723969	forest soil (pH: 4.5)
	JF717715	<i>Actinobacteria</i>	<i>Kitasatospora</i>	<i>Streptomyces</i> sp. ACTY (100)	FM163174	Sphagnum peat (acidic)
	JF717716	<i>Acidobacteria</i>	Gp3	uncult. bacterium (96.5)	FR687555	red soil (Fe-rich clay)
	JF717717	<i>Gammaproteobacteria</i>	<i>Rudaaea</i>	uncult. <i>Xanthomonadaceae</i> (99.2)	FJ889342	agricultural soil (pH: 6.5)
	JF717718	<i>Bacilli</i>	<i>Falsibacillus</i>	<i>Bacillus</i> sp. IDA4715 (97.8)	EF515940	grassland soil

Archaea	Acc. no.	assignment details	next relative (% similarity)	Acc. no.	isolation source	
Reference site (surface)	JF717729	<i>Euryarchaeota</i>	<i>Methanomicrobia</i>	<i>Methanolobus vulcani</i> (T) (99.4 – 100)	U20155	marine sediments
	to			<i>Methanolobus taylorii</i> (T) (97.7-98)	U20154	estuarine sediments
	JF717733			<i>Methanolobus psychrophilus</i> (97)	EF202842	wetland (freshwater)
	JF717724	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (99.6)	AB541693	cattle manure compost
Medium site (surface)	JF717726	<i>Euryarchaeota</i>	<i>Methanomicrobia</i>	<i>Methanolobus vulcani</i> (T) (99.6)	U20155	marine sediments
	JF717728	<i>Euryarchaeota</i>	<i>Methanomicrobia</i>	<i>Methanolobus vulcani</i> (T) (97.5)	U20155	marine sediments
	JF717727	<i>Euryarchaeota</i>	<i>Methanomicrobia</i>	<i>Methanolobus vulcani</i> (T) (99.8)	U20155	marine sediments
	JF717723	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.4)	AJ535123	uranium mill tailings
Vent centre (surface)	JF717736	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (95.8)	FJ468479	quartz minerals
	JF717735	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	enrichment culture clone (95.3)	JF799601	plant root material
	JF717747	<i>Euryarchaeota</i>	<i>Methanomicrobia</i>	<i>Methanolobus vulcani</i> (T) (97.4)	U20155	marine sediments
	JF717748	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote(95.8)	FJ468483	feldspar minerals
	JF717749	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (98.8)	AB541693	cattle manure compost
	JF717734	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (98.3)	U62818	soil
	JF717725	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (98.5)	FJ468483	feldspar minerals
	JF717722	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.2)	AJ535123	uranium mill tailings
	JF717721	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.4)	FJ468484	feldspar minerals
	JF717750	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (95.9)	FJ468478	quartz minerals

*The pH of the isolation habitat was retrieved if available from the GenBank entry or the original publication

The genetic variance of the soil profiles was further analysed using a principal component analysis (Fast UniFrac), correlating the soil specific parameters, like depth, pH, etc. (see methods), responsible for the cluster formation. The first principal component contributed with about 85% to the variance, while the second principal component only accounted for 10% (Figure 40 in the supporting information) (cumulative pc1+ pc2= 95%).

The database searches for the archaeal sequences (Table 9) and the constructed maximum likelihood tree (Figure 38) showed that the majority of the vent centre associated sequences clustered into the group 1.1b of the *Thaumarchaeota*. Members of the group 1 *Thaumarchaeota* have previously been postulated as AOA for soil habitats (Pester *et al.*, 2011). Also the seqmatch hits (RDP) for isolated closest relatives showed that the majority of the archaeal sequences from the vent centre affiliated to *Nitrososphaera sp.* JG1 (JF748724), with sequence similarity varying from about 90 to 97%, *Nitrososphaera sp.* JG1 is a mesophilic AOA of the group 1.1b *Thaumarchaeota* which was cultivated from soil. In contrast to the vent centre the archaeal sequences of the reference and medium site were mainly affiliated to the methane-producing *Methanolobus* (Table 9 and Figure 38).

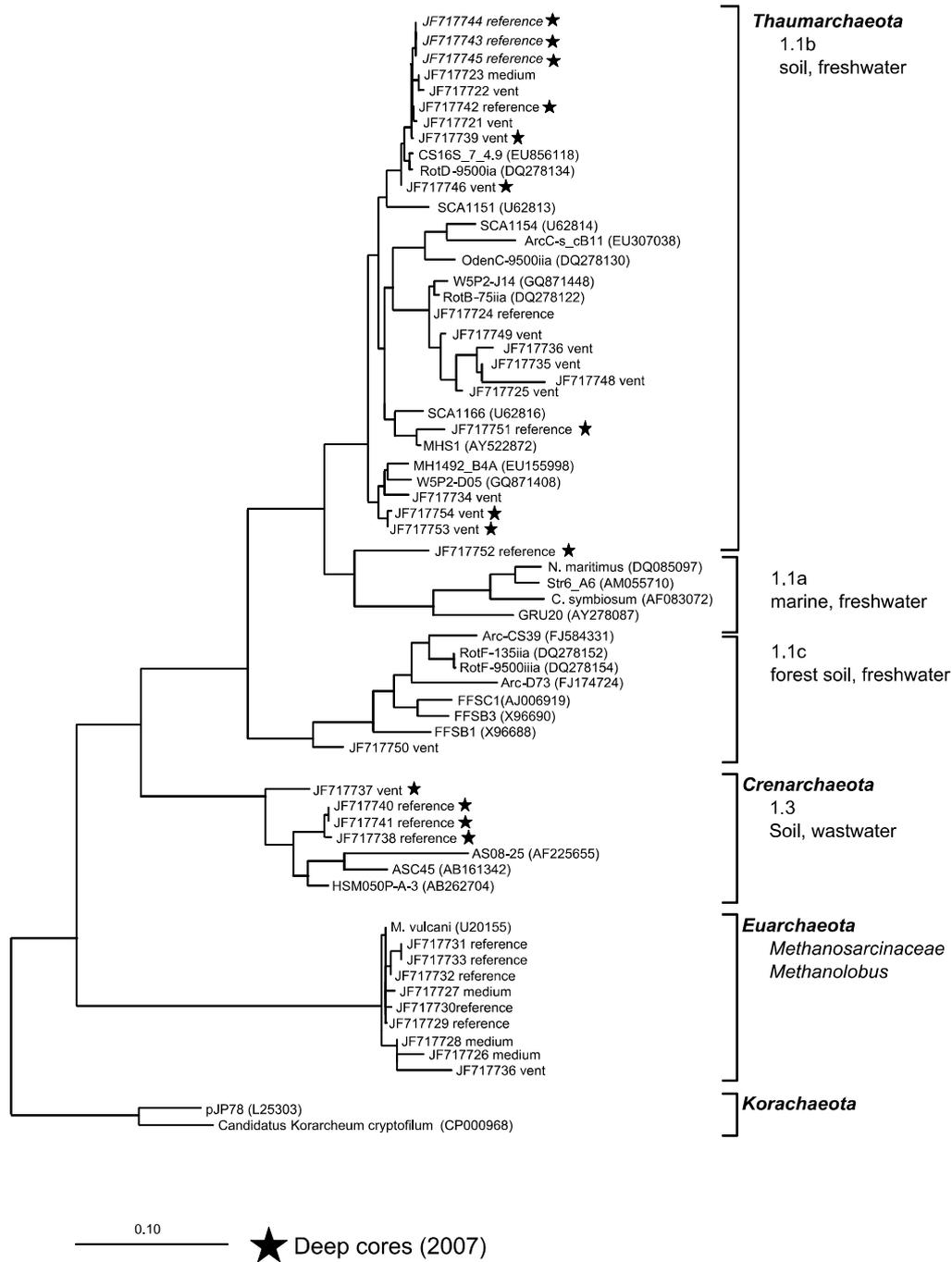


Figure 39: Phylogenetic analysis of archaeal 16S *rRNA* partial genes (~450 bp obtained with DGGE). The maximum likelihood tree (filter settings: pos-variation ssuref Arch) using the PHYML(DNA) method was constructed with the published reference sequences (name and accession numbers (Acc)) while adding the obtained sequences (GenBank acc. number with site) of this study without changing the overall tree topology. The clades were named according to previous publications (Brochier-Armanet *et al.*, 2008; Nicol *et al.*, 2008; Pester *et al.*, 2011) and separated between neutral (blue) and acidic (red) habitats. The *Korarchaeota* were used as outgroup and the scale bare represents 10% estimated sequence divergence. Sequences obtained from the deep core samples of 2007 are marked with [★].

Biomarker analyses of the bacterial and archaeal community

The concentrations of bacterial derived C14 to C18 fatty acids were highest at the vent centre depth (1608.4 $\mu\text{g g}^{-1}$ TOC; 110-120 cm) and decreased towards the surface to 959.3 $\mu\text{g g}^{-1}$ and 1057 $\mu\text{g g}^{-1}$ for vent centre and reference site, respectively. Also the relative distribution of fatty acids changed, as in the upper horizons a relative increase in terminally branched fatty acids was detected at both sites (supporting information with complete list in Table S2, e.g. i-15:0, ai-15:0, i-17:0 and ai-17:0). The deeper soil horizons (110-120 cm) contained higher concentrations of monounsaturated, straight chain fatty acids with even numbers of carbon atoms, e.g. 16 Δ 9, 18 Δ 9 and 18 Δ 10. In general, the bacterial biomarkers showed no characteristic changes that might have been used as marker molecules for CO₂ affected soils.

In contrast to the bacterial biomarkers, significant differences in the archaeal community were identified using the distribution of GDGT (Figure 39). The concentrations of especially the acyclic GDGTs were highest at the vent centre, showing an enrichment of the acyclic GDGT and crenarchaeol, with its typical cyclic moieties (GDGT #1 and #6 in Fig 39b), in 50-60 cm of the vent centre (#1/#6 = 1.7). At the reference site crenarchaeol was detected only in low concentrations (0.06 to 0.09 $\mu\text{g g}^{-1}$ TOC; 110-120 cm).

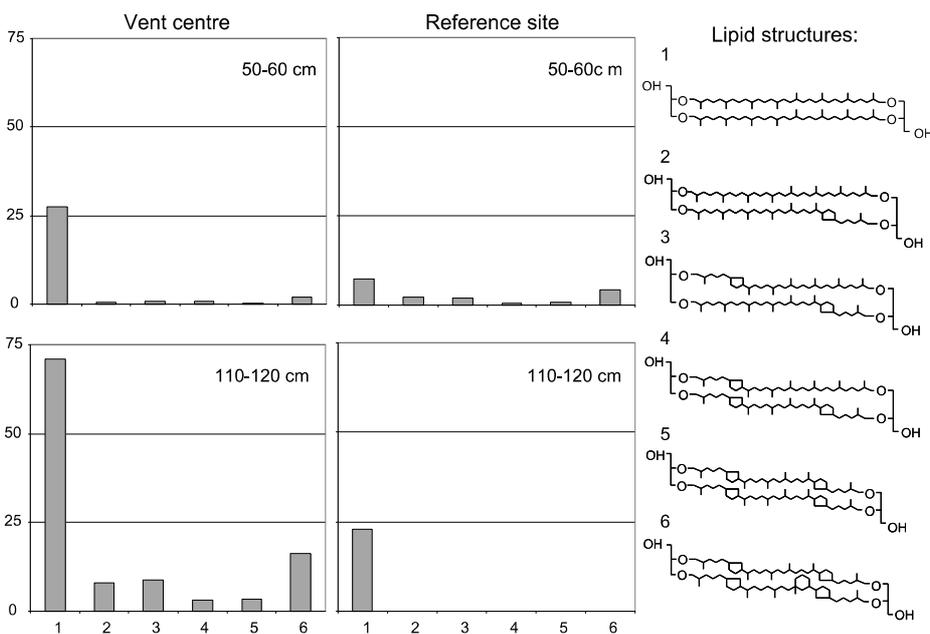


Figure 40: Differences in the distribution of archaeal GDGTs (a) with lipid structures (b) indicative for *Eurarchaeota* (structure 1 to 5; #1 typically Methanogens) and *Crenarchaeota/Thaumarchaeota* (crenarchaeol #6) in selected sample depths of the CO₂ vent centre and the reference site in September 2007.

4.4.4. Discussion

The Laacher See vent system was chosen as natural analogue to identify the long-term ecosystem effects of possible CO₂ leakages from deep storage sites, like oil and gas reservoirs, into surface soil environments.

Soil geochemical parameters under the influence of high CO₂ levels

The establishment of risk scenarios for CCS necessitates the investigation of potential environmental CO₂ effects to be expected in case of a potential leakage into surface ecosystems. In such a scenario, the most likely environmental consequences of increased CO₂ concentrations for soil ecosystems are reduced aeration and oxygen availability, reduced pH, mineralogical alterations and a reduction or change of the vegetation. For the Laacher See site, the geochemical results indeed indicated a reduced aeration and an increased acidification of the soil with depth due to elevated CO₂ concentrations and fluxes. Other geochemical alterations of the soil were less pronounced than in previous studies.

The soil organic carbon in surface soil samples from both reference and vent centre soils showed $\delta^{13}\text{C}$ values around -28‰ close to the values of C3 plants (Clark & Fritz, 1997). A relatively high number of dicotyledonous flowering plants using the C3 pathway was documented for the field site (Krüger *et al.*, 2011), although other studies reported monocotyledonous plants to be more tolerant towards CO₂ (West, *et al.*, 2009). In the deeper soil layers the $\delta^{13}\text{C}$ values showed a stronger enrichment in ¹³C at the reference site than at the vent centre. These results contradict previous findings at the Latera vent system, where the TOC was strongly affected by incorporation of ¹³C-enriched volcanic CO₂ (Oppermann, *et al.*, 2010). However, the concentrations of TOC were significantly lower at the Latera vent site. Therefore, at the Laacher See the relative enrichment of ¹³C in the TOC at the reference site might be caused by higher microbial degradation rates of organic material, while the degradation rates were lower or inhibited at the vent centre as consequence of elevated CO₂ concentrations.

The detailed geochemical profiles of the soil cores also showed substantial variations in TOC concentration (Figure 35), which might have been caused by the anthropogenic modifications of the lake's water level, i.e. a lowering in two steps of in total 15 m. Before the first lowering by about 10 m in the 12th century (the second by 3-5 m took place in the 19th century) the sampling sites should have been covered by a water column of up to 5 m height. After this human intervention, the sites were most likely located in the vicinity of the shoreline. The lowering of the water level increased the erosion rate from the newly surfaced land and organic material from dead water or land plants depending on the vicinity of the water table should have been transported to the lake. Within the soil depth profile the zones of high TOC concentrations were interpreted as reminiscence of this erosion process following water level lowering.

CO₂ induced formation of anaerobic microhabitats

The CO₂-induced alterations of geochemical parameters, changes in plant cover, gas flux rates or soil gas composition, etc., alone or in combination affect the soil ecosystem and consequently the indigenous microbial communities. The reduced aeration of the soil due to elevated CO₂ concentrations and fluxes in CO₂ vents, and the consequently anaerobic and more reduced conditions in the soil lead to decreasing microbial cell numbers with increasing CO₂ concentrations (Oppermann, *et al.*, 2010, Rousk, *et al.*, 2010). On the other hand, anaerobic microbial activities and communities, like sulphate-reduction or methanogenesis, have been described to be positively correlated with higher CO₂ levels (Beaubien *et al.*, 2008). However in contrast to previous studies, the quantification of 16S *rRNA* genes in the surface soils at the Laacher See site showed only minor differences in the overall distribution of archaeal and bacterial gene copy numbers between the sites.

The more specific gene markers, like the *Geobacteraceae* 16S *rRNA* gene, showed a significant decrease under high CO₂ concentrations in the surface soil samples and also in the analysed depth profiles down to 150 cm (p: 0.007). The mobilization of metal compounds in the soil matrix has been reported as one consequence of elevated CO₂ concentrations, as well as the reduced content of oxidised metals (Fe₂O₃) (Stephens &

Hering, 2004, Beaubien, *et al.*, 2008). Since the *Geobacter* group is well documented as metal reducers in e.g. anaerobic iron-rich aquifers (Holmes, *et al.*, 2007 /and reference therein), their reduced abundance might be indicative for the leaching effects of CO₂ in the vent centre soils.

The presence of high CO₂ concentrations and the consequently reduced aeration were correlated with the differences of microbial activities in surface soil layers. Especially the high activities observed in the soils samples of in 2008; e.g. for sulphate reduction, showed significant differences between the reference and the CO₂-affected sites. Accordingly, high abundances of *dsrA* genes (sulphate reducing prokaryotes) were detected with about 1 to 2 orders of magnitude higher gene copies at the vent centre ($p < 0.001$). Since volcanic CO₂ vents often emit gas with considerable amounts of H₂ and H₂S (Beaubien *et al.*, 2008), a general consequence for a CCS leakage scenario is seen in the development and persistence of anaerobic microhabitats around the localised CO₂ emitting source. The increased abundance and activity of sulphate reducing prokaryotes, i.e. H₂S production, represents a potential hazard for the surrounding environment (Roberts, *et al.*, 2011). This has to be further investigated for CCS leakage scenarios, especially the timescale from the onset of the leakage towards an altered microbial population adapted to the anaerobic conditions.

CO₂ induced alteration of the microbial community

The microbial community composition was investigated in more detail using DGGE to identify further indications for CO₂ induced changes of the soil environment. For the bacterial community no other environmental factor besides the sampling depth was identified, which influenced the community composition. For sequences with low similarity scores (% similarity) the hits in the GeneBank dataset, the environmental affiliation could not be reliably linked. The factor “depth” revealed sequences with next relatives in potentially anaerobic environments, e.g. from sediments of freshwater or marine habitats, which was seen as further indicator for the limnic origin of the Laacher See site.

In contrast, the isolated archaeal sequences shared surprisingly well the environmental conditions with their next cultivated relatives identified from the databases, regarding the limnic (sedimentary) and volcanic origin of the soil environment. Two habitat conditions were repeatedly affiliated with the vent centre community: uranium contaminated soil, and Si-oxide minerals (quartz and feldspar). Both fit well to the Laacher See site, since the soils and sediments in and around Maar lakes of the Eifel and Röhn have been described to be rich in uranium (Scharpenseel, *et al.*, 1975), while the remnants of weathered volcanic soils are rich in silica-minerals (Wada, 1985), and CO₂ can enhance these weathering processes (Stephens & Hering, 2004).

Furthermore, the archaeal community composition showed differences along the transect with increasing CO₂ concentrations, i.e. a predominance of *Crenarchaeota* / *Thaumarchaeota* associated sequences in the vent centre. Especially sequences belonging to the group 1.1b were abundant at the vent centre. This group was reported to be highly abundant in acidic soils (Jurgens *et al.*, 1997; Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2010). Thus, in contrast to the *Bacteria*, the phylogenetic affiliation of the archaeal community showed a significant correlation with the CO₂ concentration and the dependent secondary environmental effects (vegetation, soil aeration, and pH). Although the bulk soil pH measurements did not indicate a CO₂-induced lower pH in the surface soils (Krüger *et al.*, 2011), the microenvironment in and around soil particles has been shown to vary considerably from the macro-environmental conditions in such parameters, like pH or redox state (Foster, 1988). Reasons for their development might be small-scale diffusional barriers or local differences in substrate / organic matter availability and composition, and thus in microbial turnover rates.

Interestingly, the abundance of *Crenarchaeota* / *Thaumarchaeota* increased with depth in layers that were presumably largely anoxic. The next cultivated relative, *Nitrososphaera* sp. JG1 (Kim, *et al.*, 2012 (online first)), was identified as a mesophilic ammonium oxidising Archaeon. This cultivated organisms shared between 90-97% sequence identities with the DGGE bands along the CO₂ gradient and also in the depth profile. This

increasing phylogenetic diversity of the archaeal sequences might reflect a broader physiological capability of the community, important for the soil biosphere.

The dominating archaeal GDGT was a typical membrane compound of methanogenic *Archaea* (De Rosa & Gambacorta, 1988 /reference therein). However, the community profile and group abundance suggests for the vent centre that at least a fraction of these GDGTs has been produced by *Thaumarchaeota* (DeLong, *et al.*, 1998), especially since the methanogens only represent a smaller fraction of the CO₂ associated community. Corresponding to this, crenarchaeol was the dominant lipid biomarker found in the vent centre, thus confirming the abundance of *Thaumarchaeota* as already indicated by qPCR and DGGE analyses.

In conclusion, while the bacterial diversity did not show significant changes, the elevated CO₂ concentrations at the vent centre induced an environmental differentiation favouring *Thaumarchaeota* (group 1.1b), which might thus be considered as “indicator species”. Another question currently under debate is about the definition of “thresholds” for CO₂ related environmental consequences. Decreasing root respiration, changes in plant species composition, and altered microbial activities were already reported at concentrations of 5-20 % CO₂ in the upper layers of the soil column (Macek *et al.*, 2005; Pfanz *et al.*, 2007; Pierce & Sjögersten, 2009). The Laacher See vent site showed changes already at the medium site (qPCR and potential activities) with around 20 % CO₂ in the gas phase. Consequently, a potential environmentally relevant threshold for CO₂ for soil environments might indeed be defined at 10-20 %.

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4.4.5. Supporting Information

Soil gas concentration and flux measurements

The 60 m long traverse across the terrestrial vent was intensively investigated during both field campaigns in 2007 and 2008, respectively. In short (for a closer description refer to (Krüger *et al.*, 2011)): Soil gas concentration (for CO₂, O₂, CH₄, and N₂) and flux measurement equipment used combined steel probes and handheld infrared gas sensors (a Geotechnical Instrument plus Li-Cor and Dräger instruments) pulling out soil gas for concentration recording, and custom-made accumulation chambers for CO₂ flux quantifications. The presented data was concentrated on the three selected sites used for the microbiological investigations.

Composition of the medium for activity measurements

For the artificial mineral medium a basal solution was prepared (Widdel & Bak, 1992) containing (all in g per litre of distilled water): NaCl, 5.0; MgCl₂ · 6 H₂O, 0.4; KCl, 0.5. After autoclaving, the media was completed within the anaerobic chamber with separately sterilised stock solutions (final concentration given in g per litre (mM)): NaHCO₃, 2.52 (30); NH₄Cl, 0.25 (4.7); KH₂PO₄, 0.2 (1.5); trace element solution; vitamins; selenite and tungstate solution (Widdel *et al.*, 1983; Tschech & Pfennig, 1984; Widdel & Bak, 1992). The medium was reduced with Na₂S in 0.05 mM final concentration. The final pH of the media was 7.3.

Quantitative PCR (qPCR)

Defined concentrations for each target gene were prepared from pure cultures to provide specific standards for quantitative PCR (qPCR) as described previously ((Engelen *et al.*, 2008). A complete list of the tested quantitative PCR assays is given in Table 10. The qPCR master mix contained either SYBRGreen I (Invitrogen) or a combination of primer and a specific FAM/Tamra labelled probe were used (TaqMan®, Applied Biosystems). Melting curves were measured after the amplification with SYBRGreen I-specific fluorescence. Each DNA extract was measured in triplicate and in two to three different

dilutions to check for PCR inhibition. For *Crenarchaeota* and *Bacteria* the detection limit was 10^2 to 10^3 cells, while the other assays detected <50 cells (please refer to publication sources for detailed description). The results were calculated as gene copies g^{-1} of wet soil and for both composite samples (n=10 to 12 measurements) and averaged for each CO_2 concentration (\pm SD of 2 composite samples).

Table 10: Overview about quantitative PCR (qPCR) assays with target gene, primer name and sequence, amplification temperature, and publication source

qPCR assay	Target gene	Primer	Sequence (5'-3')	amplification temperature	publication
<i>Bacteria</i>	16S rRNA	Bac331F Bac797R Bac	TCCTACGGGAGGCAGCAGT GACTACCAGGGTATCTAATCCTGTT CGTATTACCGCGGCTGCTGGCAC	60°C	Nadkarni <i>et al.</i> 2002
<i>Archaea</i>	16S rRNA	Arch341F Arch806R Arch516	GYGCASCAGKCGMGAAW GGACTACVS GGGTATCTAAT TGYCAGCCGCGCGGTAHACCVGC	59°C	Takai <i>et al.</i> 2000
<i>Crenarchaeota</i>	16S rRNA	Cren771F Cren957R	ACGGTGAGGGATGAAAGCT CGGCGTTGACTCCAATTG	56°C	Ochsenreiter <i>et al.</i> 2003
AOA ¹	amoA+	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	53°C	Francis <i>et al.</i> 2005
AOB ²	amoA+	AmoA-1F AmoA-2R	GGGGTTTCTACTGGTGGT CCCCTCTGCAAAGCCTTCTTC	57°C	Nicolaisen <i>et al.</i> 2004
SRP ³	dsrA*	DSR-1F+ DSR-4R	ACS CAC TGG AAG CAC GGC GG GTG GMR CCG TGC AKR TTG G	60°C	Schippers <i>et al.</i> 2006
Methanogens ⁴	mcrA#	ME 1F ME 3R	GCM ATG CAR ATH GGW ATG TC TGT GTG AAS CCK ACD CCA CC	54°C	Wilms <i>et al.</i> 2007
<i>Geobacteraceae</i>	16S rRNA	GEO494F GEO825R	AGG AAG CAC CGG CTA ACT CC TAC CCG CRA CAC CTA GT	60°C	Holms <i>et al.</i> 2002

¹Ammonia-oxidising archaea; target gene *ammonia monooxygenase subunit A (archaeal typ)

²Ammonia-oxidising bacteria ; target gene *ammonia monooxygenase subunit A (bacterial typ)

³Sulphate-reducing prokaryotes; target gene *diss. sulphide reductase subunit A

⁴methanogenic archaea; target gene #methyl Coenzyme M reductase α -subunit

The primer specificity for the group specific qPCR assays was checked using the “probe check” tool (under: <http://www.microbial-ecology.de/probebase/>). As confirmed with this tool and by the literature, the *Crenarchaeota* assay (Ochsenreiter *et al.*, 2003) included the recently established phylum *Thaumarchaeota* with different reclassified group 1 *Crenarchaeota* (Brochier-Armanet *et al.*, 2008). Nevertheless, to avoid misinterpretation of the publication source the results and method were continuously referred as *Crenarchaeota*.

Denaturing gradient gel electrophoresis (DGGE) and sequence analysis

The protocols for PCR and following DGGE were described by Wilms and colleagues (2006) and references therein (del Panno *et al.*, 2005). In detail: a 626bp-fragment of the

bacterial 16S *rRNA* gene was amplified using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTGAGTTT) with 32-38 amplification cycles. For the amplification of the archaeal 16S RNA gene a nested PCR approach was used. In the first step the primers S-D-Arch-0025-a-S-17 (CTGGTTGATCCTGCCAG) and S*-Univ-151 7-a-A-21 (ACGGCTACCTT GTTACGACTT) were applied to obtain the full-length archaeal 16S *rRNA* gene fragment (about 35 amplification cycles). The final 550 bp long fragment was amplified with S-D13 Arch-GC-0344-a-S-20 (often also Arch344f; ACGGGGCGCAGCAGGCGC GA) and 915R (GTGCTCCCCGCCAATTCCT) with only a reduced number of cycles (20-25) to prevent an overamplification of the 16S *rRNA* gene. The negative control of the first PCR was used as template in the nested PCR to check for cross contamination and unspecific amplification.

DGGE was conducted using an INGENYphorU-2 system (Ingeny, Goes, Netherlands) with about 300 ng DNA from the PCR Product loaded onto polyacrylamide gels (6%, wt/vol) in 1x TAE. The gradient ranged from 50 to 70% for *Bacteria* and 30-80% for *Archaea* with 100% denaturant corresponding to 7 M urea and 40% formamide. The DNA was eluted from the DGGE bands in 50 µl PCR water (Fluka; Ampuwa®) for 48 h at 4°C. After centrifugation, 1-5 µl of the supernatant was taken as a template for re-amplification of the eluted DNA using the same reaction mix and PCR-program as for initial amplification.

Extraction, isolation and analysis of Biomarkers

Further protocol modifications developed from Oppermann *et al.* (2010): Glycerol dibiphytanyl glycerol tetraether (GDGT), contained in the alcohol fraction, were analysed and quantified using a Prostar Dynamax HPLC coupled to a 1200L triple-quad mass spectrometer (Varian, USA). Separation of compounds was achieved with a Nucleodur 100-3 CN column (2 x 150 mm, Macherey-Nagel, Germany, mobile phase: n-hexane and propanol, flow rate: 0.2 mL min⁻¹). Compounds were ionised applying atmospheric pressure chemical ionisation positive ion mode, Corona current: 5 µA, vaporising gas:

400°C (17 psi), drying gas: N₂, 200°C (12 psi), nebulising pressure: 58 psi, capillary voltage: 90 V). Masses with m/z 950 to 1400 were scanned. GDGTs were tentatively identified using LC- retention times and published mass spectra. The GTGT and GDGTs were quantified using the relative intensity of their [M+H]⁺ and [M+H]⁺ +1 ion compared to a standard of known concentration. A complete list of analysed lipid structures is given in Table 13.

Supplemented Tables & Figures

Table 11: Statistical analysis of variations for the activity profiles using Two-way ANOVA with post-hoc analysis¹ to identify influencing factors within the years (Season: mid-summer and autumn) and CO₂ concentrations of the transect stations.

Two-Way-ANOVA with 2x3 factors						Post-hoc analysis
Significance level p: <0.01 (1%)						Holm-Sidak method
SRR:						
Source of Variation	DF	SS	MS	F	P	CO ₂ within mid-summer ¹ :
Season	1	0.79	0.79	4.65	0.039	p: <0.01 significant
CO ₂ concentration	2	9.65	4.83	28.32	< 0.001	CO ₂ within autumn ¹ :
Interaction	2	2.14	1.07	6.28	0.005	p: >0.02 not significant
Residual	30	5.11	0.17			Season within CO ₂ :
Total	35	17.69	0.51			I: Reference p: >0.02
						II: Medium p: 0.001
						III: Vent p: >0.02
MOR						
Source of Variation	DF	SS	MS	F	P	
Season	1	1.10	1.10	1.72	0.20	
CO ₂ concentration	2	24.31	12.16	18.94	< 0.001	
Interaction	2	0.85	0.43	0.67	0.52	
Residual	30	19.25	0.64			No post-hoc analysis:
Total	35	45.52	1.30			Main factor identified without significant interaction!
CPR:						
Source of Variation	DF	SS	MS	F	P	CO ₂ within mid-summer ¹ :
Season	1	75.94	75.94	32.17	< 0.001	I vrs II p: 0.001
CO ₂ concentration	2	40.12	20.06	8.5	0.001	II vrs III p: >0.01
Interaction	2	40.35	20.18	8.55	0.001	III vrs I p: >0.02
Residual	29	68.45	2.36			CO ₂ within autumn ¹ :
Total	34	229.79	6.76			p: >0.9 not significant
						Season within CO ₂ :
						I: Reference p: >0.5
						II: Medium p: <0.001
						III: Vent p: <0.01

¹Post-hoc analysis using a t-test for the pairwise comparison of different factors to identify the interaction between seasonal and CO₂ effects (using Holm-Sidak method). The results were summarised if consistent for all pairwise comparisons within the group assignment.

Table 12: Complete list of identified sequences of the deep core samples for bacterial and archaeal partial 16S *rRNA* genes. The partial sequences were identified using the RDP classifier and seqmatch to search for relatives (isolation sources as recorded in the GenBank entry).

<i>Bacteria</i>	Acc. no.	assignment details	next relative (% similarity)	Acc. no.	isolation source	
reference site	JF717680	<i>Acidobacteria</i>	<i>Edaphobacter</i>	uncult. Acidobacteria (91.6)	HM061757	non-agricultural soil (surface)
(deep core)	JF717681	<i>Betaproteobacteria</i>	<i>Methyloversatilis</i>	uncult. bacterium (93.2)	DQ297977	hydrocarbon contaminated soil
	JF717682	<i>Bacilli</i>	<i>Bacillus</i>	uncult. bacterium DA001 (99.6)	X99967	grassland soil
	JF717683	<i>Actinobacteria</i>	<i>Asanoa</i>	<i>Actinoplanes</i> sp. (98.6)	AJ488563	soil
	JF717684	<i>Gammaproteobacteria</i>	<i>Rugamonas</i>	uncult. bacterium (91)	AY345556	maar freshwater lake
	JF717685	<i>Thermomicrobia</i>	<i>Sphaerobacter</i>	uncult. bacterium (98.4)	EU135322	grassland (undisturbed prairie)
	JF717686	<i>Clostridia</i>	<i>Tepidanaerobacter</i>	uncult. bacterium (98.2)	HQ330625	freshwater sediments
	JF717687	<i>Dehalococcoidetes</i>	<i>Dehalogenimonas</i>	uncult. Chloroflexi (96.4)	FJ902028	biomat in acidic sediments
	JF717688	<i>Bacilli</i>	<i>Saccharococcus</i>	<i>Bacillus</i> sp. HZ-B (93.8)	HQ652870	marine sediments
	JF717689	<i>Deltaproteobacteria</i>	<i>Desulfobacca</i>	delta proteobacterium (88.2)	GU339469	groundwater (gas reservoir)
	JF717690	<i>Acidobacteria</i>	Gp13	uncult. bacterium (96.9)	HM243859	freshwater sediments
	JF717691	<i>Actinobacteria</i>	<i>Paraeggerthella</i>	uncult. bacterium (97.3)	HQ330625	freshwater sediments
	JF717692	<i>Dehalococcoidetes</i>	<i>Dehalogenimonas</i>	uncult. bacterium (97.1)	HM186867	water saturated soil (Hanford Site)
	JF717693	<i>Bacilli</i>	<i>Bacillus</i>	<i>Bacillus drentensis</i> (100)	AJ542505	agricultural grassland
	JF717694	<i>Ignavibacteria</i>	<i>Ignavibacterium</i>	uncult. bacterium (93.4)	FJ716463	anoxic freshwater
	JF717695	<i>Betaproteobacteria</i>	<i>Azospira</i>	enrichment culture clone CL21 (99.8)	GU134935	acidic fen
	JF717696	<i>Acidobacteria</i>	Gp13	uncult. bacterium (96.9)	HM243859	freshwater sediments
	JF717697	<i>Acidobacteria</i>	Gp13	uncult. bacterium (97.2)	HM243859	freshwater sediments
vent centre	JF717671	<i>Bacilli</i>	<i>Paucisalibacillus</i>	uncult. Bacillaceae (88.3)	FJ543002	earthworm gut
(deep core)	JF717670	<i>Bacilli</i>	<i>Bacillus</i>	<i>Bacillus</i> sp. HZ-B (100)	HQ652870	marine sediments
	JF717672	<i>Actinobacteria</i>	<i>Actinoallomurus</i>	uncult. actinobacterium (99)	JF833791	potassium mine soil
	JF717673	<i>Acidobacteria</i>	Gp1	uncult. Acidobacteria (99.8)	HM061759	pristine soil (pH: 6.2)
	JF717674	<i>Sphingobacteria</i>	<i>Ferruginibacter</i>	uncult. Bacteroidetes (99.6)	FR749757	soil (Antarctic Peninsula)
	JF717675	<i>Ignavibacteria</i>	<i>Ignavibacterium</i>	uncultured bacterium (98.8)	FN870283	acidic freshwater sediments
	JF717676	<i>Bacilli</i>	<i>Bacillus</i>	<i>Bacillus</i> sp. HZ-B (99.6)	HQ652870	marine sediments
	JF717677	<i>Actinobacteria</i>	<i>Arthrobacter</i>	<i>Arthrobacter globiformis</i> (98.6)	AF408952	agricultural soil (Southern Apennine)
	JF717678	<i>Acidobacteria</i>	Gp1	uncult. bacterium (99)	GQ339162	iron(II)-rich freshwater seep
	JF717679	<i>Actinobacteria</i>	<i>Actinoallomurus</i>	uncult. actinobacterium (99.4)	JF833791	potassium mine soil
	JF717719	<i>Betaproteobacteria</i>	<i>Ralstonia</i>	<i>Ralstonia</i> sp. OV225 (99.8)	AY216797	agricultural soil

<i>Archaea</i>	Acc. no.	assignment details	next relative (% similarity)	Acc. no.	isolation source	
Reference site	JF717751	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99)	AY522901	agricultural soil
(deep core)	JF717752	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (92.8)	AJ535123	uranium mill tailings
	JF717745	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.6)	AJ535123	uranium mill tailings
	JF717744	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.6)	AJ535123	uranium mill tailings
	JF717743	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.6)	AJ535123	uranium mill tailings
	JF717742	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (100)	AJ535123	uranium mill tailings
	JF717741	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (96.4)	AB262704	peat soil
	JF717740	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (96.7)	GU135491	freshwater sediments
	JF717738	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (95.9)	AB262704	peat soil
Vent centre	JF717746	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (100)	AJ535123	uranium mill tailings
(deep core)	JF717739	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.8)	FJ468484	feldspar minerals
	JF717753	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. crenarchaeote (99.6)	FJ468482	feldspar minerals
	JF717754	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (99.2)	U62818	soil
	JF717737	<i>Crenarchaeota</i>	<i>Thermoprotei</i>	uncult. archaeon (99.2)	HM187508	water saturated soil ("Hanford Site")

Table 13: Concentrations of bacterial fatty acids (C₁₄ to C₁₈) from two sampling depths from the CO₂-vent and the reference site (with relative abundance in brackets). Fatty acids were abbreviated; giving the numbers of carbon atoms, and the position unsaturated bounds. Position of methyl branches were abbreviated with *i*-, *ai*- and *m*- for iso-, anteiso-, and mid chain methyl branched, respectively. Site and/or depth specific biomarkers showing a relative increase in comparison to the other samples were indicated in bold script.

Site:	Vent centre $\mu\text{g g}^{-1}$ TOC (rel. %)		Reference site $\mu\text{g g}^{-1}$ TOC (rel. %)	
Depth:	50-60 cm	110-120 cm	50-60 cm	110-120 cm
<i>i</i> -14:0	21.7 (2.3)	28.8 (1.8)	24.7 (1.9)	27.0 (2.6)
14:0	60.2 (6.3)	77.2 (4.8)	84.0 (6.5)	77.8 (7.4)
<i>i</i> -15:0	71.7 (7.5)	74.8 (4.6)	89.7 (7.0)	28.7 (2.7)
<i>ai</i> -15:0	49.8 (5.2)	69.9 (4.3)	80.1 (6.2)	31.2 (3.0)
15:0	23.7 (2.5)	22.7 (1.4)	21.9 (1.7)	17.0 (1.6)
<i>i</i> -16:0	24.7 (2.6)	64.6 (4.0)	43.4 (3.4)	18.0 (1.7)
16:1	7.7 (0.8)	n.d.	13.1 (1.0)	n.d.
16:1	28.7 (3.0)	8.1 (0.5)	34.2 (2.7)	3.6 (0.3)
16:1 Δ 9	7.4 (0.8)	76.2 (4.7)	33.5 (2.6)	51.0 (4.8)
16:1	8.1 (0.8)	34.2 (2.1)	2.9 (0.2)	11.2 (1.1)
16:0	302.7 (31.6)	461.8 (28.7)	346.8 (27.0)	343.3 (32.5)
<i>m</i> -17:1	11.2 (1.2)	13.0 (0.8)	10.3 (0.8)	n.d.
<i>m</i> -17:0	25.7 (2.7)	46.6 (2.9)	36.3 (2.8)	7.3 (0.7)
<i>m</i> -17:0	10.6 (1.1)	9.7 (0.6)	n.d.	n.d.
<i>i</i> -17:0	35.2 (3.7)	62.4 (3.9)	53.7 (4.2)	26.4 (2.5)
<i>ai</i> -17:0	29.6 (3.1)	41.6 (2.6)	52.2 (4.1)	13.2 (1.3)
cycl17:0	10.7 (1.1)	32.0 (2.0)	9.8 (0.8)	n.d.
17:1	4.7 (0.5)	n.d.	n.d.	n.d.
17:0	20.3 (2.1)	n.d.	25.5 (2.0)	n.d.
<i>m</i> -18:0	19.5 (2.0)	19.9 (1.2)	24.5 (1.9)	n.d.
<i>i</i> -18:0	32.4 (3.4)	19.2 (1.2)	28.2 (2.2)	28.3 (2.7)
18:1	22.7 (2.4)	n.d.	52.1 (4.1)	n.d.
18d9	18.2 (1.9)	117.9 (7.3)	31.0 (2.4)	52.3 (5.0)
18:1 Δ 10	5.2 (0.5)	104.9 (6.5)	14.4 (1.1)	174.8 (16.5)
18:1 Δ 11	5.3 (0.6)	45.4 (2.8)	19.9 (1.6)	30.2 (2.9)
18:0	101.7 (10.6)	177.8 (11.1)	152.4 (11.9)	115.8 (11.0)
total	959.3	1608.4	1284.4	1057.0

UniFrac PCoA analysis of archaeal sequence distribution

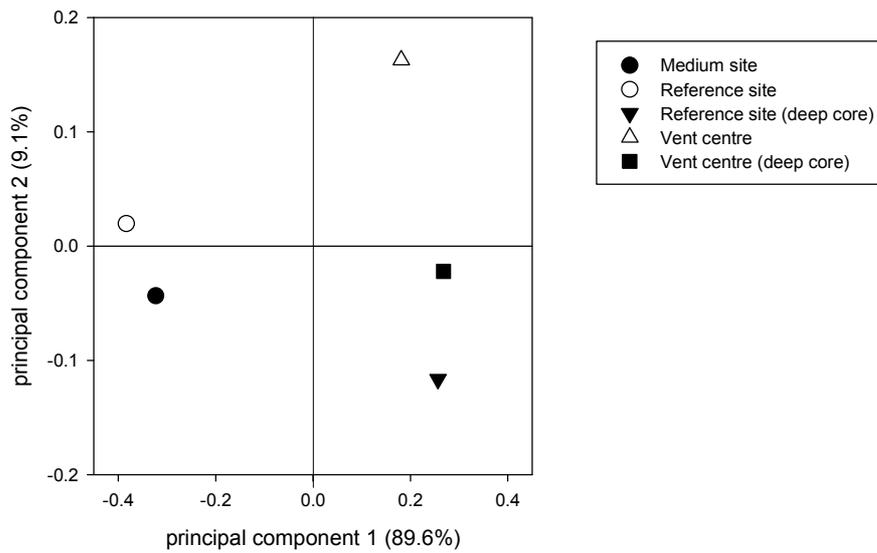


Figure 41: Principal component analysis demonstrating the clustering within the archaeal community (16S *rRNA* partial sequences) using the FastUniFrac tool with the tree generated in ARB (Fig 4; maximum likelihood tree PHYLIP(DNA) program). The Principal components 1 and 2 explained cumulative 94% variation within the cluster.

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List of publications

Journal publications (peer review)

Janin Frerichs, B.I. Oppermann, I. Möller, M. Herrmann, S. Gwosdz, and M. Krüger (2012):

“Microbial community changes at a terrestrial volcanic CO₂ vent induced by soil acidification and anaerobic microhabitats within the soil column” *FEMS Microbial Ecology* (online first December 2012)

Krüger, M., D. Jones, **J. Frerichs**, B.I. Oppermann, J. West, P. Coombs, K. Green, T. Barlow, R. Lister, R. Shaw, M. Strutt, and I. Möller (2010):

“Effects of elevated CO₂ concentrations on the vegetation and microbial populations at a terrestrial CO₂ vent at Laacher See, Germany.” *International Journal of Greenhouse Gas Control* **5**:1093-1098.

Krüger, M., J. West, **J. Frerichs**, B.I. Oppermann, M.-C. Dictor, C. Jouliand, D. Jones, P. Coombs, K. Green, J. Pearce, F. May, and I. Möller (2009):

“Ecosystem effects of elevated CO₂ concentrations on microbial populations at a terrestrial CO₂ vent at Laacher See, Germany.” *Energy Procedia* **1**:1933-1939.

Oppermann, B.I., W. Michaelis, M. Blumenberg, **J. Frerichs**, H. M. Schulz, A. Schippers, S. E. Beaubien, and M. Krüger (2010):

“Soil microbial community changes as a result of long-term exposure to a natural CO₂ vent.” *Geochimica et Cosmochimica Acta* **74**:2697-2716.

Manuscripts in preparation:

Janin Frerichs, C. Gniese, J. Rakoscy, M. Schlömann, N. Hoth, and M. Krüger

“Pronounced microbial population dynamics in a Northern German gas reservoir connected to production-related technical measures”

Janin Frerichs, C. Gniese, NA, and M. Krüger

„ 4.2. Viability and adaptation potential of indigenous microorganisms from natural gas field fluids in high pressure incubations with supercritical CO₂”

Attendance at conferences (since 2008)

- *Vereinigung für Angewandte und Allgemeine Mikrobiologie - VAAM annual meeting*
 - 2008 - *Frankfurt*: Tracing the migration of bacteria from the water column into deep sediments layers (poster)
 - 2009 - *Bochum*: Adaptation of terrestrial microbial communities to elevated CO₂ concentrations (poster)
 - 2010 *Hannover*: Active microbial communities in gas reservoirs in the North German Plain and the effects of high CO₂ concentrations (poster)
 - 2011 – *Karlsruhe*: High CO₂ concentrations negatively effect methanogenesis and sulfate reduction in gas fields of the North German Plain (poster)
 - 2012 – *Tübingen*: The influence of supercritical CO₂ on sulphate reducing and methanogenic enrichment cultures from hydrocarbon reservoir formations in Germany (poster)

- *Deutsche Geologische Gesellschaft; GEODresden 2009, Dresden; Germany*
 - Active microbial community in gas reservoir (Schneeren) in the North German Plain and the effects of high CO₂ concentrations (oral presentation)
- *GEODarmstadt 2010, Darmstadt, Germany*
 - High CO₂ concentrations negatively effect methanogenesis and sulfate reduction in gas fields of the North German Plain (poster)

- *European Geological Union - EGU annual meeting*
 - 2010, Vienna; Austria
 - Active microbial community in gas reservoirs in the North German Plain and the effects of high CO₂ concentrations (oral presentation)

- *International Society of Microbial Ecology; ISME-13 2010, Seattle; US*
 - High CO₂ concentrations negatively affect methanogenesis and sulfate reduction in gas fields of the North German Plain (oral presentation)

- *IEAGHG Natural Releases of CO₂ workshop 2010, Maria-Laach; Germany*
 - The effects of high CO₂ concentrations on microbial community structure and activity at natural CO₂ seeps and depleted natural gas reservoirs (oral presentation)

- *Goldschmidt-Conference 2011, Prague; Czech Republic*
 - High CO₂ concentrations negatively affect methanogenesis and sulphate reduction in gas fields of the North German Plain (poster)

- *International Symposia on Subsurface Microbiology ISSM 2011, Garmisch-Partenkirchen, Germany*
 - The influence of supercritical CO₂ on sulphate reducing and methanogenic enrichment cultures from hydrocarbon reservoir formations in Germany (poster)

- *2nd Microenergy Workshop 2012 Aarhus, Denmark*
 - The influence of supercritical CO₂ on sulphate reducing and methanogenic enrichment cultures from hydrocarbon reservoir formations in Germany (poster)

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Persönliche Erklärung zur Dissertation

Gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried-Wilhelm-Leibniz-Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

„The influence of high CO₂ concentrations on microorganisms in different Ecosystems“

selbstständig verfasst habe und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Curriculum Vitae

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- March 2013: Institute for Molecular Biosciences of the Goethe University Frankfurt a. M.
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- April 2007- Mai 2008: Diploma thesis ICBM of the Carl von Ossietzky University Oldenburg
- October 2002- April 2008: Academic studies in biology at the Carl von Ossietzky University in Oldenburg (Diploma)
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- January – June 2005: Academic exchange with the ERASMUS-Program of European Union at the University of Bergen, Norway
- June 2002: Abitur at the Fachgymnasium BBS III in Oldenburg (Stadt Oldenburg), Germany