Originally published as:

Morozova, D., Wandrey, M., Alawi, M., Zimmer, M., Vieth, A., Zettlitzer, M., Würdemann, H. (2010): Monitoring of the microbial community composition of the saline aquifers during CO2 storage by fluorescence in situ hybridisation. - International Journal of Greenhouse Gas Control, 4, 6, 981-989

DOI:10.1016/j.ijggc.2009.11.014

Daria Morozova[a,*], Maren Wandrey[a], Mashal Alawi[a], Martin Zimmer[b], Andrea Vieth[c], Michael Zettlitzer[d], Hilke Würdemann[a] and the CO_2SINK group

Monitoring of the microbial community composition in saline aquifers during CO_2 storage by fluorescence *in situ* hybridisation

[a] *Helmholtz Centre GeoForschungsZentrum Potsdam, Centre for CO_2 storage, Telegrafenberg, 14473 Potsdam, Germany*
[b] *Helmholtz Centre GeoForschungsZentrum Potsdam, Inorganic and Isotope Geochemistry Section, Telegrafenberg, 14473 Potsdam, Germany*
[c] *Helmholtz Centre GeoForschungsZentrum Potsdam, Organic Geochemistry Section, Telegrafenberg, 14473 Potsdam, Germany*
[d] *RWE Dea AG, Laboratory Wietze, Industriestraße 2, 29323 Wietze, Germany*

*Corresponding author. Tel. + 49 331 288 1579; Fax: +49 331 288 1529. Email: Daria.Morozova@gfz-potsdam.de

ABSTRACT

This study reveals the first analyses of the composition and activity of the microbial community of a saline CO_2 storage aquifer. Microbial monitoring during CO_2 injection has been reported. By using Fluorescence *in situ* Hybridisation (FISH), we have shown that the microbial community was strongly influenced by the CO_2 injection. Before CO_2 arrival, up to $6x10^6$ cells ml^{-1} were detected by DAPI-staining at a depth of 647 m below the surface. The microbial community was dominated by the domain *Bacteria* that represented approximately 60 to 90 % of the total cell number, with *Proteobacteria* and *Firmicutes* as the most abundant phyla comprising up to 47 % and 45 % of the entire population, respectively. Both the total cell counts as well as the counts of the specific physiological groups revealed quantitative and qualitative changes after CO_2 arrival. Our study revealed temporal shifts in the microbial community from chemoorganotrophic to chemolithotrophic populations, as evidenced by the outcompetition of sulphate-reducing bacteria by methanogenic archaea. In addition, an enhanced activity of the microbial population after five months CO_2 storage indicated that the bacterial community was able to adapt to the extreme conditions of the deep biosphere and to the extreme changes of these atypical conditions.

Keywords: CO_2 storage, FISH (fluorescence *in situ* hybridisation), microbial community

1. Introduction

Investigations of the possibilities to reduce CO_2 emissions are receiving a significant amount of attention as a consequence of the effects of global warming due to the accumulation of carbon dioxide gas in the atmosphere. One possibility to reduce the volume of CO_2 emitted into the atmosphere is the concept of CO_2 capture and storage in the deep underground. The EU funded CO_2SINK project is aimed as a pilot storage of CO_2 in a saline aquifer located near Ketzin, west of Berlin, Germany. The main aim of the project is to develop efficient monitoring procedures for assessing the processes that are triggered in the reservoir by long-term injection.

Until now, our knowledge about the potential influence that injection and long-term storage of CO_2 into saline aquifers have on the microbial community is extremely limited. Shifts in microbial community composition and activity, which should result from the introduction of the exogenous CO_2, in addition to possible effects on carbon and energy sources, are expected. Furthermore, bacterial populations and activity can be strongly influenced by changes in the pH value, pressure, temperature, salinity and other abiotic factors, which will be all influenced by CO_2 injection into the deep subsurface. Therefore, it is of great importance to characterise the microbial community of the deep subsurface before and with the injection of CO_2. Thorough studies of samples from deep boreholes, using a variety of molecular techniques, have shown an active biosphere composed of diverse groups of microorganisms (Parkes at al., 1994; Sahl et al., 2008; Whitman et al., 1998). In addition, numerous studies of microbial communities in the deep biosphere have revealed that the most important metabolic pathways in the deep subsurface are sulphate reduction, fermentation and possibly methanogenesis (Baker et al., 2003; Basso et al., 2009; Kotelnikova, 2001; Takai et al., 2001). A description of microbial communities that originated from varied deep subterrestrial settings has shown that those subsurface microbial communities could represent the greatest reservoir of living organisms on our planet (Whitman et al., 1998). Furthermore, analyses of the composition of microbial communities and its changes should contribute to an evaluation of the effectiveness and reliability of the long-term CO_2 storage technique. The interactions between microorganisms and the minerals of both the reservoir and the cap rock may cause major changes to the structure and chemical composition of the rock formations, which may influence the porosity and permeability within the reservoir. In addition, precipitation and corrosion may occur around the well affecting the casing and the casing cement. Moreover, the growth of microorganisms on the material surface (biofilms) can have a profound effect on material performance (Beech et al., 2005).

Microbiological sampling opportunities in the deep subsurface are extremely limited, and the differentiation of indigenous microorganisms from newly introduced microorganisms remains a challenge. An observation well provides an almost ideal opportunity for microbiological monitoring. In order to analyse deep biosphere diversity, subsurface water samples have been collected from deep environments *via* downhole sampling prior to and during CO_2 injection as well as after CO_2 arrival in the observation well. However, the process of collecting microbiologically representative water samples from these wells requires specific techniques in order to avoid contamination (Basso et al., 2005; Kallmeyer et al., 2006; Pedersen et al., 1997). Therefore, a fluorescent dye, Na-fluorescein (Uranine), was added to the drill mud to allow the detection of the amount of drill mud in the collected fluid samples (Wandrey et al., this issue).

Notably, cultivation-based methods usually fail to describe the composition of complex communities. Yet, molecular techniques may provide complementary insight into complex environments. Fluorescence *in situ* hybridisation (FISH) is one of many nucleic acid techniques that are useful for studying microorganisms in their natural environments. In recent years, the FISH method has become one of the most widely used tools in microbial ecology (Amann et al., 1990). FISH coupled with rRNA-targeted oligonucleotide probes allows direct visualisation, identification and localisation of bacterial cells from selected phylogenetic groups in environmental samples. This technique is based on the detection of rRNA and therefore is related to the activity state of the target cells.

In this study we concentrated on the results obtained from the observation well 1 (Ktzi 200), where CO_2 has arrived after two weeks of injection. We were able to study the effects of the five months CO_2 storage. The main objective was to characterise and visualise the microbial community of a deep saline aquifer and to assess the influence of CO_2 exposure on the composition of the microbial community by using the FISH technique without previous incubation.

2. Materials and methods

2.1 Study site

The storage site is located in the north eastern Germany near Ketzin, The target reservoir for CO_2 storage is the Stuttgart Formation of Triassic age (Förster et al., 2006). In the 600 m to 700 m depth it was 80 m thick with sand channels measuring up to 20 m. For the injection and monitoring of the CO_2 in a natural saline aquifer, three 700 to 850 m deep holes were drilled by mud rotary drilling in March and April 2007 (Fig. 1). As proposed for CO_2-storage (Borm and Förster, 2005; Würdemann et al., this issue), a variety of geophysical, geochemical and microbiological methods were subsequently applied in order to characterise the state of the reservoir formation. The Triassic Stuttgart formation consists of siltstones and sandstones interbedded by mudstones deposited in a fluvial environment (Juhlin et al., 2007). The temperature and pressure of the formation fluid were approximately 35 °C and 62 bar, and the salinity was roughly 235 mg l^{-1}.

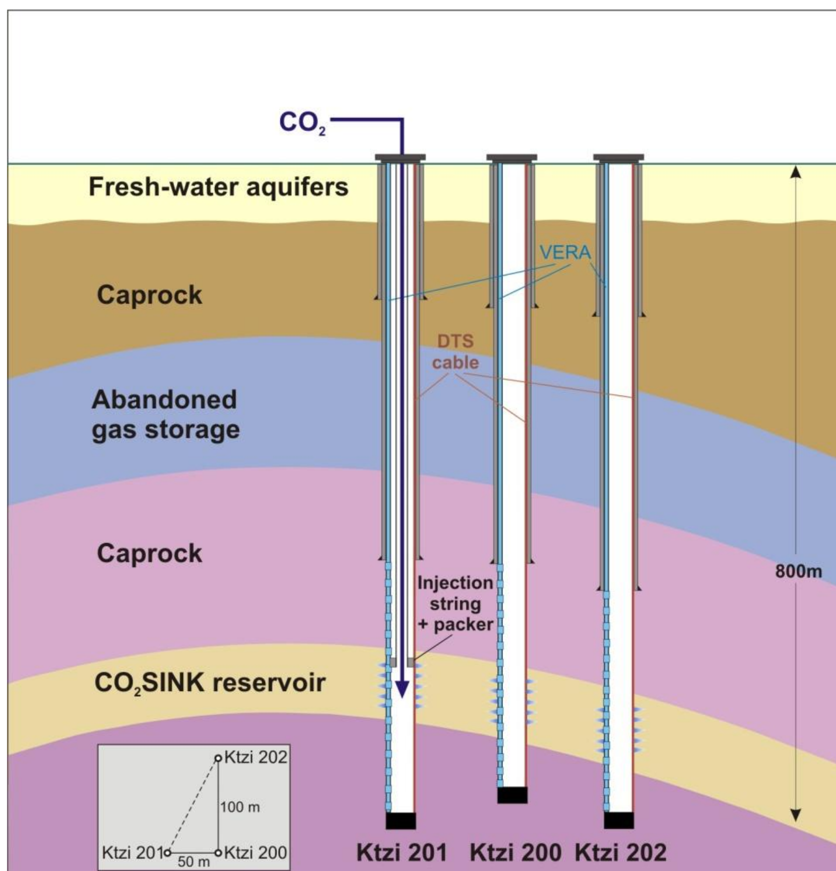

Fig. 1 Schematic concept of underground CO_2 storage in Ketzin: geology, drilling and monitoring

Prior to CO_2 injection, a number of hydraulic tests were performed on all wells. The so-called N_2 lift was performed in the injection well and the two observation wells two days prior to the CO_2 injection to “clean up” the wells from the rest of the drill mud (Zettlitzer et al., this issue). The injection of CO_2 into the Ktzi 201 well was started on the end of June 2008 (Schilling et al., 2008). The CO_2 arrival in the Ktzi 200 well was observed about two weeks later, after 500 t of CO_2 have been injected. The CO_2 arrival in the observation well 1 (Ktzi 200) was detected by the downhole measurements of the amount and nature of the dissolved gases in the fluid samples. Notably, detailed physical, geochemical and microbiological monitoring was performed in all three wells (Giese et al., 2009). However, this study concentrates on the results obtained from the observation well 1 (Ktzi 200), where it was possible to monitor the effects of the CO_2 exposure during the five months. Further detailed information on drilling, CO_2 injection and site description can be found at http://www.co2sink.de.

2.2 Sample collection

Downhole sampling was performed at a depth of 647 m (perforation depth) using DKB (double ball-lining) and PNL64 (flow-through) samplers. Each sampler was cleaned in the lab and washed with sterilised deionised water and ethanol shortly before sampling. The pH, conductivity, temperature and other parameters were measured directly during the sampling process. The fluids were transferred aseptically into sterilised 500 to 1000 ml glass vials (for FISH) and 100 to 250 ml polyethylene and glass vessels (for chemical analysis), cooled down to 4 °C and immediately transferred to the laboratory for microbiological and chemical analyses. Contamination control during sampling is described by Wandrey and co-authors (this issue). Gas sampling was performed using PNL64 sampler, which was controlled by a timer and collects fluid samples from a specific depth while maintaining *in situ* pressure conditions until sampling at the surface. At the surface samples were transferred into a 350 ml gas sampling pipe by connecting a hose to the sampler.

2.3 Chemical analysis

The total organic carbon content was determined using a TOC-analyser (Dimatec GmbH) according to DIN EN 1484-H3. The concentrations of cations and anions were measured after filtration of the sample (0.45 µm) using an ion chromatograph with an emission spectrometry (ICP-OES) according to DIN 38402-21. Quantification of the dissolved low molecular weight organic acids (e.g. acetate) has been done by ion chromatography with conductivity detection (ICS 3000, Dionex Corp.). For chromatographic separation of the anions the analytical column AS 11 HC (Dionex Corp.) was used. Standard deviation of sample and standard quantification was below 10 %. Electrical conductivity, pH and fluid temperature were measured during the sampling process using a portable pH/mV/Temperature meter (WTW). Gas composition was measured with mobile mass spectrometry (GSD 300 O1, Omnistar, Fa. Pfeiffer Vacuum GmbH). Detection limit is 0.001 mg l^{-1}.Tracer concentration (Na-fluorescein) was detected using a mobile light fluorometer (Hermes Messtechnik, Stuttgart, RS232).

2.4 Sample preparation for the cell counts

To obtain sufficient biomass for FISH, freshly collected fluid samples (500 to 1000 ml) were concentrated to a volume of approximately 0.5 ml by centrifugation (Heraeus Biofuge Pico, Sigma 6K15, 9600 g^{-1} for 1 h at 4 °C) and were then fixed as described previously by Pernthaler et al. (2001). Cell pellets (0.5 ml) were fixed with 1.5 ml freshly prepared in 4 % paraformaldehyde/phosphate-buffered saline (PBS) solution (pH 7.2 to 7.4) for 4 to 5 h at 4 °C. The fixed samples were then washed with PBS and stored in ethanol-PBS (1:1) at -20 °C. Before application to the slides, the fixed samples were diluted with 0.1 % sodium pyrophosphate in distilled water to obtain 100 to 200 cells (total) per microscopic field of view. The dilution was treated with mild sonication using an MS73 probe (Sonoplus HD70; Bandelin, Berlin, Germany) at a setting of 20 s to separate cells from solid particles. As a result of the comparatively much higher background fluorescence of solid particles observed after hybridisation on membrane filters (own observations), the dispersed samples were spotted on gelatine-coated Teflon-laminated slides (Zarda et al., 1997) with 10 wells. The slides were dried at 45 °C for 15 min and dehydrated in 50 %, 80 % and 96 % ethanol.

2.5 FISH and DAPI staining

All oligonucleotide probes used in this study were purchased from Thermo Scientific (Ulm, Germany). These probes were all labelled with the cyanine dye Cy3 and FLUOS. The probes for the domains *Bacteria* and *Archaea* and specific probes for the different phylogenetic groups of the domain *Bacteria* as well as sulphate-reducing bacteria were used. The probe names, details and references are summarised in Table 1. For *in situ* hybridisation, a 10 ml aliquot of hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl; pH 8.0, 0.02 % SDS), formamide in concentrations according to Table 1, and 30 ng ml^{-1} of the probe were dropped onto each well. The slides were transferred to an equilibrated 50 ml polypropylene top tube and incubated at 46 °C for 120 min. The slides were then washed at 48 °C for 10 min in a washing buffer (20 mM Tris-HCl; pH 8.0, 5 mM

EDTA, 0.01 % SDS w/v and 225 mM NaCl with a formamide concentration of 20 % in the hybridisation buffer). Afterwards, the slides were washed in ice-cold double-distilled water for a few seconds and quickly dried in an air stream. Subsequently, 10 ml of 4′6-Diaminodino-2-phenylindole (DAPI, 1 mg ml^{-1} working solution) was dropped onto each well and incubated in the dark at room temperature for 10 to 15 min. The slides were then washed in ice-cold double-distilled water and allowed to air-dry. Finally, the slides were embedded in Citiflour AF1 antifadent (Plano, Wetzlar, Germany) and covered with a coverslip.

Table 1. rRNA-targeting oligonucleotide probes used for FISH

c	Target group	Sequence (5′–3′) of probe	Target site[a]	FA[b] (%)	Ref.
UNIV1390	All organisms	GACGGGCGGTGTGTACAA	16S rRNA (1390)	0	Zheng et al., 1996
EUB338	Domain *Bacteria*	GCTGCCTCCCGTAGGAGT	16S rRNA (338)	0-35	Amann et al., 1993
EUB338 II	Domain *Bacteria*	GCAGCCACCCGTAGGTGT	16S rRNA (338)	0-35	Daims et al., 1999
EUB338 III	Domain *Bacteria*	GCTGCCACCCGTAGGTGT	16S rRNA (338)	0-35	Daims et al., 1999
NON338	Complementary to EUB338	ACTCCTACGGGAGGCAGC	16S rRNA	0-35	Wallner et al., 1993
ALF968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	16S rRNA (968)	35	Neef, 1997
Bet42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	23S rRNA (1027)	35	Manz et al., 1992
Gam42a	*Gammaproteobacteria*	GCCTTCCCACATCGTT	23S rRNA (1027)	35	Manz et al., 1992
CF319a	Most *Flavobacteria*, some *Bacteroidetes* and *Sphingobacteria*	TGGTCCGTGTCTCAGTAC	16S rRNA (319)	35	Manz et al., 1996
CF319b	Same as CF319a	TGGTCCGTATCTCAGTAC	16S rRNA (319)	35	Manz et al., 1996
HGC69a	Gram-positive bacteria with high G+C content	TATAGTTACCACCGCCGT	23S rRNA (1901)	35	Roller et al., 1994
LGC354a	Gram-positive bacteria with low G+C content	TATAGTTACCACCGCCGT	16S rRNA (354)	25	Meier et al., 1999
LGC354b	Same as LGC354a	CGGAAGATTCCCTACTGC	16S rRNA (354)	35	Meier et al., 1999
LGC354c	Same as LGC354a	CCGAAGATTCCCTACTGC	16S rRNA (354)	35	Meier et al., 1999
Arc915	Archaea	GTGCTCCCCCGCCAATTCCT	16S rRNA (915)	n.d.	Stahl and Amann, 1991
SRB385	Most *Desulfovibrionales* and other Bacteria	CGGCGTCGCTGCGTCAGG	16S rRNA (385)	35	Amann et al., 1990
DEM1164r	*Desulfotomaculum* cluster I and other *Firmicutes*	CCTTCCTCCGTTTTGTCA	16S rRNA (1164)	10	Stubner and Meuser, 2000
DSS658	*Desulfobacteraceae* and other Bacteria	TCCACTTCCCTCTCCCAT	16S rRNA (658)	60	Manz et al., 1998

n.d. = not determined. [a]*E. coli* numbering. [b]Percentage (vol/vol) of formamide in the hybridisation buffer.

2.6 Microscopy and determination of cell counts

Microscopy experiments were performed using a Zeiss Axioskop 2 equipped with filters 02 (DAPI), 10 (FLUOS) and 20 (Cy3), a mercury-arc lamp and an AxioCam digital camera. The counting was done manually. For each hybridisation approach and sample, at least 800 DAPI stained cells were counted on 30 randomly chosen counting squares. Using FISH, only those cells with a sufficient number of ribosomes were detected (Amann et al., 1995). The number of these cells was calculated by counting probe-specific positive signals relative to DAPI counts. The counting results were always corrected by subtracting signals obtained with the probe NON338. For calculating the number of cells per ml of fluid (bacterial counts per volume, BCv), the mean count of bacteria per counting area (B), the microscope factor (area of sample spot/area of counting field, M), the dilution factor (D) and the volume of the fixed sample used for hybridisation (V) were determined and calculated as given in the equation 1:

$$BCv = B\ M\ D\ V^{-1} \quad (1)$$

3. Results

3.1 Fluid geochemistry

The fluid samples obtained from the observation well Ktzi 200 were analysed with regard to the concentrations of anions, cations, pH, conductivity, salinity, total organic carbon (TOC) and gas composition. Downhole samples were taken three and two months prior to CO_2 injection and during CO_2 injection, after two days, one, two and five months after arrival of the injected CO_2 in Ktzi 200. The CO_2 arrival was determined by the changes of the amount and nature of the dissolved gases in the fluid samples. The results are summarised in Table 2. Generally, the CO_2 arrival event resulted in changes in gas concentrations, of pH and iron content (Fig. 2, Tab. 2). Prior to CO_2 arrival, the pH values varied only slightly between 7.0 and 7.5 (neutral) and decreased to 5.3 (acidic) with CO_2 arrival. The concentrations of dissolved iron increased strongly from 0 - 12 mg l^{-1} before CO_2 arrival to around 200 mg l^{-1} after CO_2 arrival (Tab. 2). The concentrations of nitrate and lithium were constant with less than 5 mg l^{-1}. The TOC content ranged between 25.5 and 63.3 mg l^{-1} before injection and N_2-lift, during injection the fluid sample contained about 20 mg l^{-1} TOC and after arrival of the CO_2 the concentration showed high variation between 10.2 and 108.9 mg l^{-1}. Acetate concentrations were 36.3 and 49.3 mg l^{-1} three and two month before CO_2 injection, respectively. After CO_2 arrived in the observation well analyses yielded much lower values with less than 3.4 mg l^{-1} acetate (Fig. 2). Sulphate concentrations showed values of up to 4400 mg l^{-1}. In addition, the concentrations of sodium, calcium and magnesium varied only slightly. The tracer (Na-fluorescein) concentrations were constantly below 2 µg l^{-1} (Fig. 2). A detailed description of the contamination control by tracer during drilling in the CO_2 storage site at Ketzin is provided elsewhere (Wandrey et al., this issue).

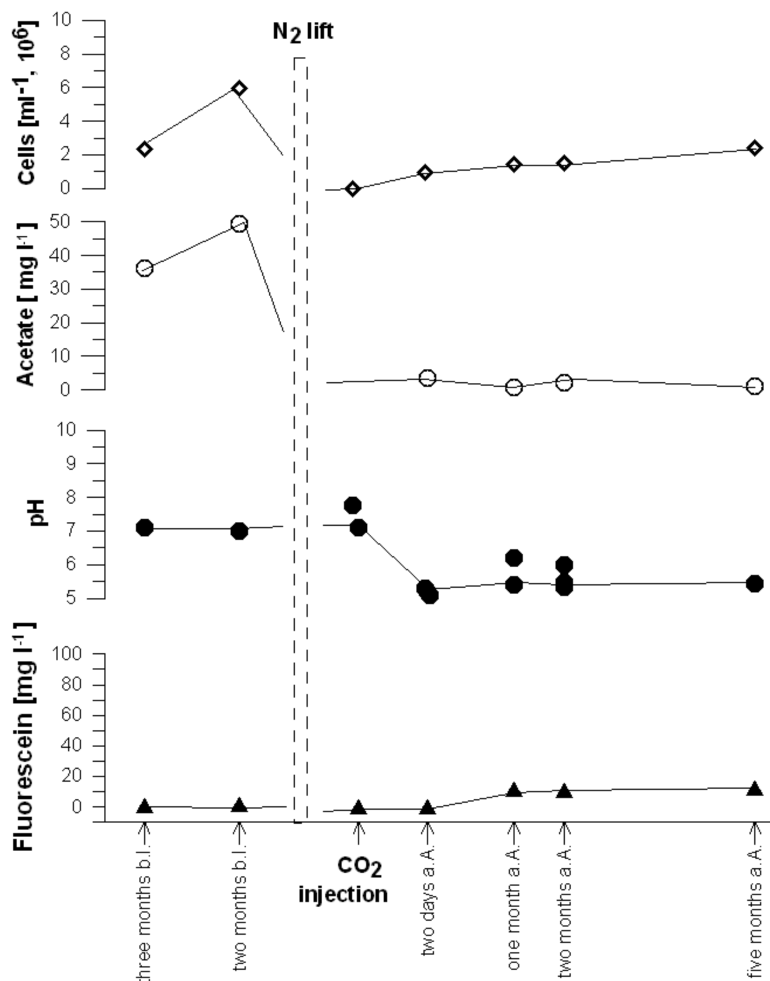

Fig. 2 Na-fluorescein, pH, acetate and total cell counts (DAPI) during CO_2 company monitoring. Means b.I. = before CO_2 Injection, a.A. = after CO_2 arrival

Table 2. Concentrations of selected anions, cations, conductivity, gases and TOC explored by fluid analyses obtained from observation well Ktz 200 by downhole sampling

	Fluid samples	Conductivity [µS cm⁻¹]	TOC [mg l⁻¹]	Sulphate [mg l⁻¹]	Ca [mg l⁻¹]	Fe [mg l⁻¹]	Mn [mg l⁻¹]	K [mg l⁻¹]	Gas composition				
									Lgas/ Lfluid	CH_4 [mg l⁻¹]	CO_2 [mg l⁻¹]	H_2 [mg l⁻¹]	N_2 [mg l⁻¹]
Before injection	3 months	225	63.3	4049	1992	12.2	899	620	0.017	0.17	0.08	0.14	17.9
	2 months	224	25.5	4397	2075	0	762	510	n.d	n.d	n.d	n.d	n.d
During CO_2 injection		224	7.4	4149	2005	0	766	348	n.d	n.d	n.d	n.d	n.d
After CO_2 arrival	2 days	223	12.8	3609	2116.5	139.7	811	336	15	2.46	26.19	2.6	1.89
	1 month	223	108.9	4346	2051	130.4	796	334	12.24	n.d*	n.d*	n.d*	n.d*
	2 months	223	10.2	4222	2070	279.4	810	332	6.8	0.97	12.13	0.54	0.67
	5 months	224	89.4	3805	2170	176.9	820	322	15.2	1.44	27.44	0.43	1.48

n.d = not determined (* = due to the technical problems)

3.2 Total and bacterial cell counts

Cell numbers detected in all fluid samples obtained from the observation well, are summarised in Table 3. DAPI staining revealed total cell numbers of 2 to 6x10^6 cells ml^{-1} while FISH using the UNIV probe for universal detection resulted in 2 – 4x10^6 ml^{-1} of metabolic active cells before CO_2 injection. A total of 2x10^6 ml^{-1} of bacterial cells were enumerated using the bacterial EUB338 probe. Interestingly, all of the cell numbers were significantly higher in the fluid samples before the N_2 lift and the CO_2 injection. Notably, cell concentrations in the fluid samples significantly decreased after N_2 lift and therefore any cells were detectable by fluorescence microscopy. However, total cell counts after the CO_2 arrival were approx. 10^5 cells ml^{-1}. During the following months, the total cell counts increased by one order of magnitude from 10^5 cells ml^{-1} to 10^6 cells ml^{-1}. After five months, almost all of the detected cells were active cells (FISH with UNIV probe) and were in the range of the samples taken two months prior to the injection (2x10^6 cells ml^{-1}).

Table 3. Total bacterial numbers in the fluid probes from observation well Ktzi 200 before, during and after CO_2 arrival analysed by DAPI staining and relative percentage of hybridised cells with specific probes

Fluid samples		Total cell counts[a] (cells ml⁻¹ [10⁶]) (mean ± SD)	Total (active) cell counts[b] (cells ml⁻¹[10⁶]) (mean ± SD)	% of all active cells (mean ± SD)[c]						Sum of Bacteria affiliated cells [%]
				Bacteria[d]	Proteobacteria[e]	High GC/ CFB[f] group	Low GC	SRB[g]	Arc	
Before injection	3 months	2.4±0.2	1.6±0.1	93.7±1.4	34.9±8.3	n.d.	44.8±25.4	11.2±5.8	n.d.	90.9
	2 months	5.9±0.4	4.8±0.4	89.3±7.7	7.7±3.3	n.d.	16.6±6.5	24.1±9.9	5.1±2.6	48.4
After CO_2 arrival	2 days	0.9±0.1	0.5±0.1	98.0±9.5	16.9±5.9	n.d.	25.7±18.7	2.3±1.2	16.9±7.9	45.8
	1 month	1.4±0.1	0.9±0.1	61.7±2.2	19.7±6.8	n.d.	19.5±6.3	n.d.	11.4±1.6	39.2
	2 months	1.5±0.1	1.3±0.1	80.0±3.8	46.9±9.2	n.d.	31.5±4.2	n.d.	0.5±0.0	78.4
	5 months	2.4±0.3	2.3±0.1	86.4±2.7	20.7±11.2	n.d.	33.8±16	18.9±13.8	n.d.	73.4

n.d. not determined, [a] obtained through DAPI staining, [b] obtained through hybridisation with UNIV, [c] percent detection compared to UNIV. Numbers have been corrected by subtracting NON38 counts, [d] probe mix of EUB I, II, III obtained through DAPI staining, [e] probe mix of ALF968, Bet42a, Gam42a, [f] probe mix of CFa and CFb, [g] probe mix of SRB385, DEM1164r, DSS658

The fraction of the cells that were detectable using the probe EUBmix, which identified members of the domain *Bacteria*, decreased after CO_2 arrival from 98 % to approximately 60 % for the next month (Tab. 3, Fig. 3). However, an increase in the fraction of the EUBmix labelled cells was observed two months after the CO_2 arrival. Between 16 and 45 % of the EUBmix labelled cells contributed to the bacteria with low G+C

content. The cell numbers calculated in the fluids varied between $0.1x10^6$ cells ml^{-1} and $0.8x10^6$ cells ml^{-1}. Our results indicated that members of the *alpha-, beta-, and gamma-Proteobacteria* were also very abundant in the fluid samples and contributed up to 46 % of all of the *Bacteria*-affiliated cells. In the fluid samples taken before and after the CO_2 arrival, the number of cells detected using the *Proteobacteria* probes (ALF968, Bet42a and Gam42a probes, Tab. 1) varied between $0.6x10^6$ cells ml^{-1} and $0.1x10^6$ cells ml^{-1}.

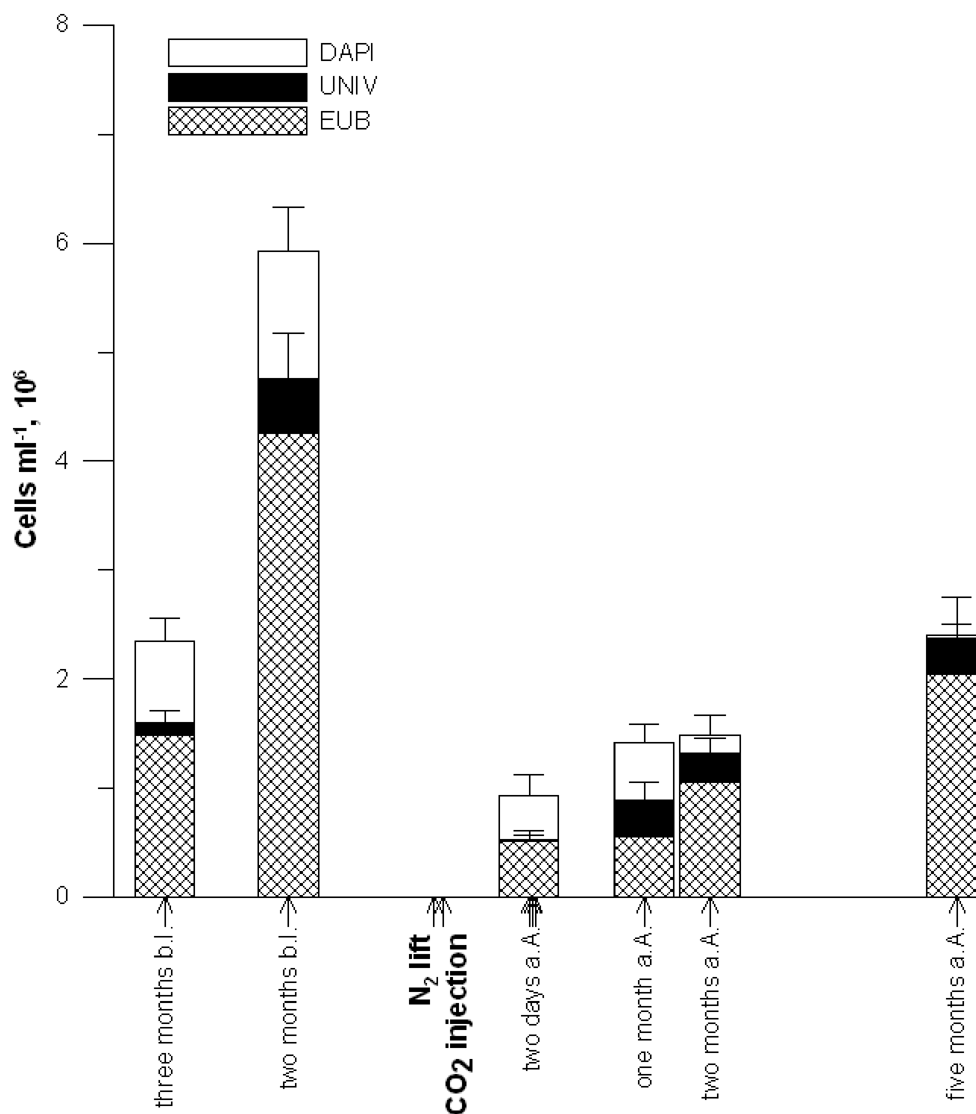

Fig. 3 Total cell numbers (DAPI), total number of active cells (FISH-UNIV probe), number of active bacterial cells (FISH-Eub probe) in the fluid probes from observation well Ktzi 200 before CO_2 injection and after CO_2 arrival. Means b.I. = before CO_2 Injection, a.A. = after CO_2 arrival

For the detection of sulphate-reducing bacteria, we used the SRB385 probe, which primarily targets the *Desulfovibrionales*, the DEM1164r probe, which targets the *Desulfotomaculum* cluster I and other *Firmicutes*, and the DSS658 probe, which targets the *Desulfobacteraceae* in addition to other *Bacteria*. Interestingly, sulphate-reducing bacteria could also be detected in the samples prior to CO_2 injection, where they made up approximately 24 % of the active cell counts (Figs. 4A and 5A). However, after CO_2 arrival, the SRB cell number decreased by one order of magnitude from $0.2x10^6$ cells ml^{-1} to $0.1x10^5$ cells ml^{-1}. The SRB-labelled cells became visible only after 5 months subsequent to CO_2 arrival (Fig. 5C); however, the cell numbers increased up to $0.5x10^6$ cells ml^{-1}, a value that was two times higher than that measured prior to the CO_2 injection. Notably, members of the *Bacteroidetes* phylum in addition to bacteria with a high G+C-content such as *Actinobacteria* were not detected.

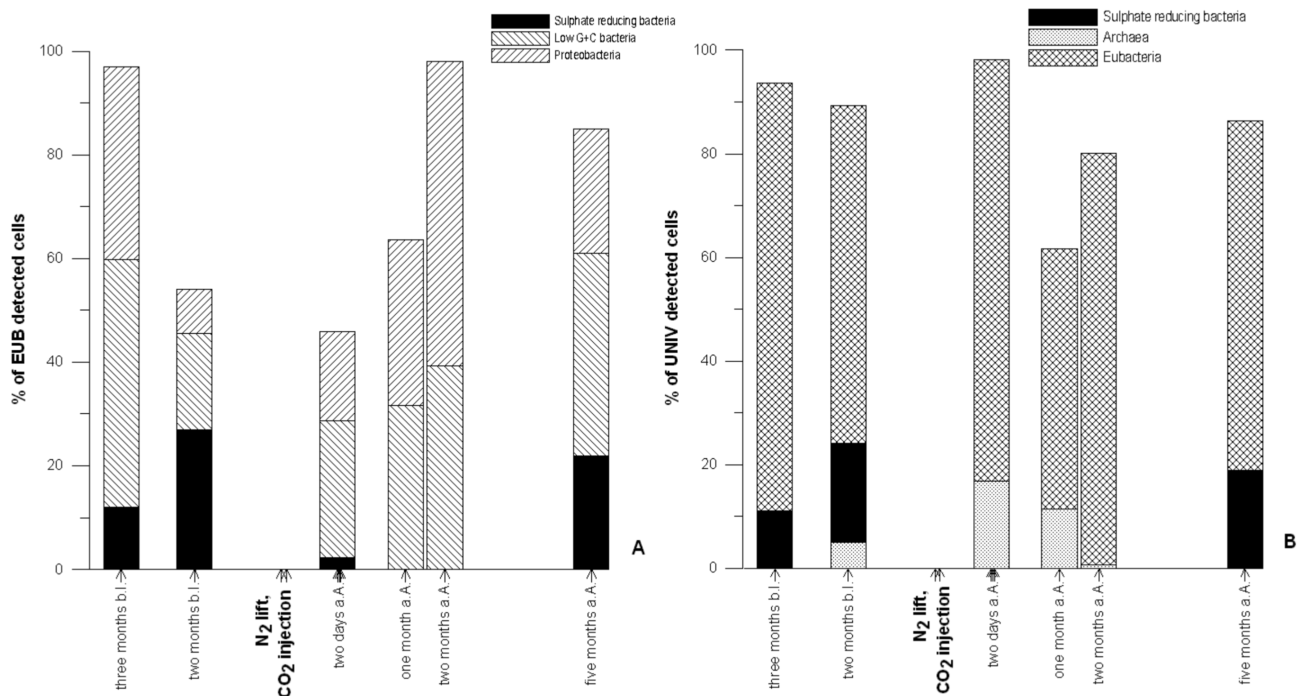

Fig. 4 A: Probe-specific counts relative to number of active bacterial cells (FISH-EUB-counts, percentages) in observation well. **B**: Numbers of active bacterial cells (FISH-Eub), sulphate-reducing bacteria (FISH-SRB) and Archaea (FISH- Arc) specific counts relative to all active cells (FISH-UNIV, percentages). Means b.I. = before CO_2 Injection, a.A. = after CO_2 arrival

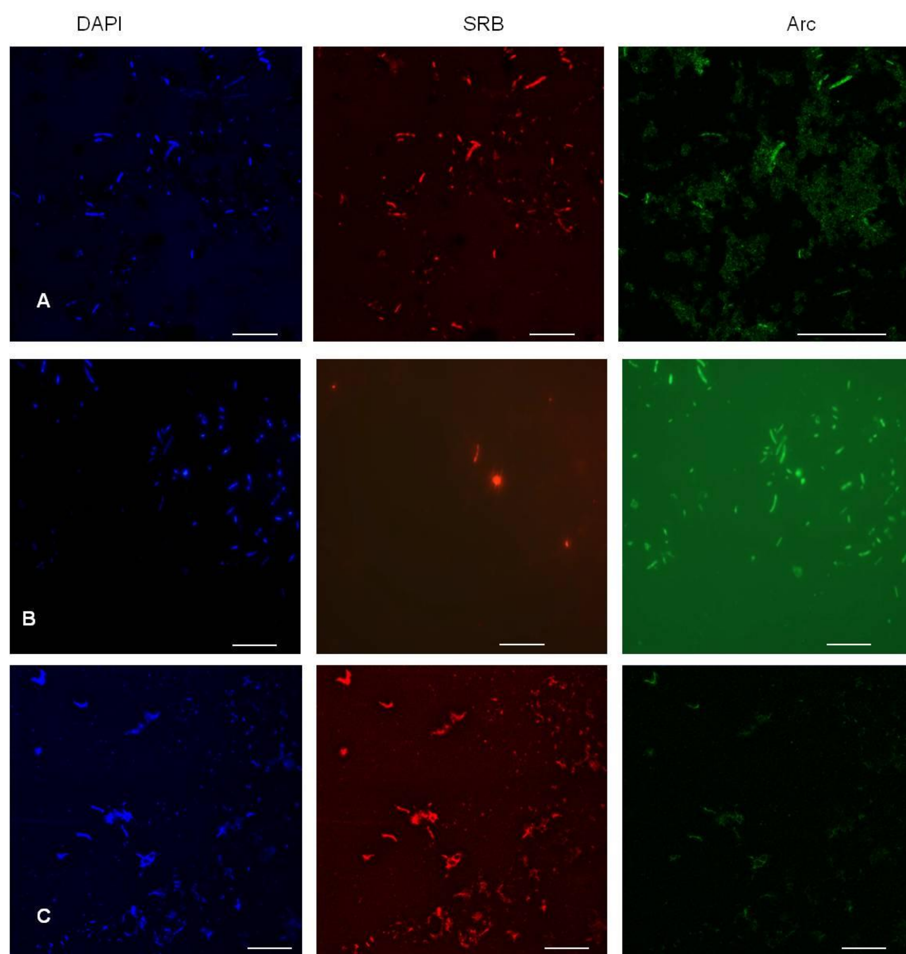

Fig. 5 Fluorescence microscope images showing total cell numbers (DAPI) and in situ hybridisation of bacteria in fluid samples from 647m depth. DAPI-stained cells (blue), and corresponding sulphate-reducing bacteria cells stained by the SRBmix_Cy3 (red) and methanogenic cells stained by Arc/MER_FLUOS (green) prior to N_2 lift and CO_2 injection (A), two days during CO_2 arrival (B), and five months after CO_2 arrival (C). Bars represent 5 µm.

The quantification of the domain Archaea in the fluid samples taken before the CO_2 arrival and N_2 lift, resulted in number of cells detected using the Arc915 probes of about $2x10^5$ cells ml^{-1}. The most of the Archaea were found in the fluid samples after the CO_2 arrival and made up between 11 and 16 % of the active cells that were detected (Tab. 3, Fig. 5B). Our data indicated that the archaeal cells decreased in number during the next months and were not observed after five months (Fig. 5C). All of the samples where a high number of cells were detected by the probe Arc915 were hybridised with the probe MER1, which is specific for the 16S rRNA of methanogenic archaea. Therefore, the cell counts obtained by the Arc915 would be characterised as the cell count of methanogens.

4. Discussion

In this study, we have characterised for the first time the influence of CO_2 injection on the microbial community composition in a saline aquifer. Herein, we have reported the identification, quantification and visualisation of microbial communities using the FISH technique and absolute cell counts made by DAPI investigated in different samples taken at a depth of 647 m in the observation well Ktzi 200 of the CO_2 storage site in Ketzin, Germany.

Although saline aquifers could be characterised as an extreme habitat for microorganisms due to reduced conditions, high pressure and salinity, a high number of microorganisms were found in all the fluid samples. A total of 2 to $6x10^6$ cells ml^{-1} were determined in samples from a depth of 647 m (Tab. 3) whereas increased numbers due to contamination of the sampled fluids by residual drill mud can be excluded detecting only low tracer concentrations in the fluids. The observed cell numbers are at least one order of magnitude higher than the values identified by previous analyses of anaerobic aquifers and enumerated by acridine orange direct microscopic counts (Stevens et al., 1993; Pedersen et al., 1997). The enumeration of the detectable cells using the probe EUBmix revealed the dominance of the domain *Bacteria* in the saline aquifer. Based on both the relative and absolute abundance, we showed that the bacterial population was dominated by bacteria with low G+C content (predominantly *Firmicutes*) and by *Proteobacteria*. Similar observations were already reported in other deep microbial ecosystems (Fry et al., 1997; Sahl et al., 2008). For example, Basso and colleagues (2009) showed that the microbial community of a deep subsurface gas storage aquifer was primarily composed of *Firmicutes* and δ-*Proteobacteria*. Other studies reported that the microbial community in groundwater collected in Japan at a depth of 900 m was composed of 40 % *Firmicutes* and 27 % δ- *Proteobacteria* (Shimizu et al., 2007), in the same range as the deep aquifer in a Miocene formation (Shimizu et al., 2006).

In addition to the possible contribution by *Proteobacteria* and *Firmicutes*, we used more specific probes that targeted phylogenetic groups of the SRB within the δ-subclass of *Proteobacteria*. Most of the known members of this group naturally occur in groundwater and can produce hydrogen sulphide from sulphate that is prevailing here. Previous microbiological and molecular analyses of deep saline groundwater revealed a sulphate-reducing microbial population belonging to *Firmicutes* (Lin et al., 2006). Several other studies have discovered evidence of *Desulfotomaculum* spp. and other sulphate-reducing bacteria in the deep subsurface (Baker et al., 2003; Detmers et al., 2004; Fredrickson et al., 1997; Havemann et al., 1999; Olson et al., 1981; Onstott et al., 1998).

Notably, drastic reduction of the numbers of bacteria was seen after clean up the well (Fig. 2), which was also the case for the injection well Ktzi 201 after N_2 lift (Morozova et al., 2009; Zettlitzer et al., this issue). Also TOC and acetate concentrations decreased after N_2 lift (Fig. 2, Table 2).

The CO_2 arrival in the observation well was detected by the downhole measurements of the amount and nature of the dissolved gases in the fluid samples by GMS (Gas Membrane Sensor) and the increase of the well head pressure (Giese et al., 2009). The CO_2 arrival resulted in a significant decrease in pH and the number of bacteria (Fig. 2). A rapid changes of the environmental parameter, among others the pH decrease, is known to have deleterious effect on the cell function and biochemistry, affecting bacterial activity and composition (Booth et al., 2002; Wall et al., 2007). The bacterial population recovered in the following months after CO_2 arrival as indicated by the total cell counts and FISH using the UNIV probe (Fig. 3). Although the FISH signals cannot be directly translated into the cell activity status, it is accepted that there is a positive relationship between bacterial metabolic rates and the capacity to detect active cells (Bouvier and del Giorgio,

2003). The quantification of the cell numbers using the UNIV probe revealed significant differences in the active cell counts when compared to the total cell counts (TCC). Thus, the proportion of the active cells in the microbial community was drastically decreased from approximately ¾ to ½ during the first days after CO_2 arrival (Fig. 3). However, five months after CO_2 arrival, almost all of the counted cells were active (Fig. 3, Tab. 3). This strengthened the assumption that the microorganisms are capable to adapt to the extreme shifts of environmental condition in the deep biosphere and to their active involvement in global biogeochemical cycling.

The clean-up process for the wells following by CO_2 arrival and pH decrease contributed to the shift in the microbial community from chemoorganotrophic to chemolithotrophic populations. This correlates well with the observed increase in the methanogenic archaea community for the samples where no or only an insignificant number of SRB was detected (Fig. 4B, Fig. 5B). Further evidence for the existence of a methanogenic community was provided by gas analyses of the downhole samples, which detected the presence of methane, CO_2 and hydrogen in the samples, where methanogenic archaea were detected (Tab. 2). Methanogenic archaea are highly resistant microorganisms that may use only hydrogen and CO_2 as energy and carbon sources and do not require other organic substrates. Interestingly, certain strains of methanogenic archaea have been shown to grow at low pH (Maestrojuan and Boone, 1991). Furthermore, resent studies revealed shifts in methanogenic community to the H_2-dependent methanogenesis at low pH (Kotsyurbenko et al., 2007). CO_2 injection into the saline aquifer, followed by well head pressure increase, CO_2 inflow and fluid outflow within the observation well, will had resulted in fluid circulations and turbulences (Würdemann et al., this issue). These circulations could be a possible explanation for the fluctuations seen for the chemical signatures like TOC and sulphate in the fluid (Tab. 2). It has been generally accepted that carbon availability and environmental conditions are controlling microbial growth (Aldén et al., 2001; Brockman et al., 1992; Fredrickson and Balkwill, 2006; Fry et al., 1997; Jørgensen and Boetius, 2007; Whitman et al., 1998). Analyses of the organic acids in the fluids revealed that acetate seems to be the major constituent of the dissolved organic carbon (DOC). Before CO_2-injection, high concentrations of acetate correlated to fluid samples with the highest bacteria numbers (Fig. 2). Since the arrival of the CO_2 in the observation well, acetate concentrations dropped. As acetate is an important substrate for microorganisms such as sulphate-reducing bacteria and methanogenic archaea changes in the concentration are of special interest. Above mentioned groups of microorganisms are important terminal oxidisers in the anaerobic mineralisation of organic matter and can be observed as ecological equivalents, mineralising organic matter to CO_2 or to CO_2 and CH_4 in high-sulphate and low-sulphate environments, respectively (Takai et al., 2001). To summarise, our results suggest that in the presence of sulphate and acetate, SRB outcompeted the methanogens due to a reduction in sulphate, which is a major catabolic process due to the higher affinity of sulphate reducers for hydrogen and acetate and a higher energy yield of sulphate reduction (King, 1984; Schönheit et al., 1982). Under these conditions, methanogenesis occurs very slowly, and the cell activity is too low to be detected using FISH, as is the case for the samples obtained after five months of the CO_2 arrival (Figs. 4B and 5C).

The identification of the SRB, which are known to be involved in corrosion (Crombie et al., 1980; Hao et al., 1996; Lee et al., 1995; Pankhania, 1988), could be of great importance for the technical progress of the long-term CO_2 storage technique. Thus, our recent investigations showed that members of this group were able to rapidly and massively change the permeability of the injectivity in the near well bore area (Morozova et al., 2009; Würdemann et al., in prep.; Zettlitzer et al., this issue).

It should be noted that the microbial composition of the fluid samples in the observation well is remarkably heterogeneous. The abundance and diversity of the microorganisms may differ between the fluid- and sediment-associated populations within the same formation, as has been shown by Hazen and co-authors (Hazen et al., 1991). However, as previous studies have demonstrated, fluids from the well can provide representative samples for analyses of selected microbiological properties from the deep subsurface (Stevens and McKinley, 1995). Thus, our data presenting the chemical and microbiological composition of the formation fluids should provide an explicit correlation to the future analyses of the rock core samples.

6. Conclusions

Since microorganisms represent very effective geochemical catalysts, the thorough investigation of their distribution and physiology could be of great importance for the process of CO_2 storage. The reactions between the microorganisms and the minerals of both the reservoir rock and the cap rock may cause major changes in the structure and chemical composition of the rock formations, corrosion at the casing and the casing cement around the well. In order to draw broader conclusions about the microbial community in the deep biosphere, more intensive sampling and methodologies are necessary. The limiting factors of our work, such as taking undisturbed samples directly in the subsurface saline aquifer, the high costs of the downhole sampling and time-consuming analyses have to be taken into consideration. Further studies on the activity, quantity and physiology of these microbial communities using PCR-SSCP, DGGE, molecular cloning and real-time PCR are planned. This study can thus provide only an early insight into the community structure and its changes due to the CO_2 injection.

Acknowledgements

We would like to thank all partners in the CO_2SINK project and CO_2 storage centre for their continued support and contributions. Special thanks go to EEW (Erdöl-Erdgas Workover GmbH) and Andrea Seibt (BWG) for their most valuable advice and operational support during the downhole sampling. The CO_2SINK project receives its funding from the European Commission, the Federal Ministry of Economics and Technology, and the Federal Ministry of Education and Research. Dirk Wagner and the whole Geomicrobiology-Team at Alfred Wegener Institute are acknowledged for the friendly atmosphere and support during the FISH analysis in their lab. Rona Miethling-Graff, Ann-Kathrin Scherf and Jürgen Päpke are acknowledged for critical reading of the manuscript. Most important, this project would not have been possible without the never ending efforts and enthusiasm of Frank Schilling, the former leader of the CO_2SINK project.

References

Aldén, L., Demoling, F., Bååth, E., 2001. Rapid method of determining factors limiting bacterial growth in soil. Appl. Environ. Microbiol. 67, 1830-1838.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919-1925.

Amann, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. FEMS Microbiol. Rev., 59, 143-169.

Baker, B.J., Moser, D.P., MacGregor, B.J., Fishbain, S., Wagner, M., Fry, N.K., Jackson, B. et al., 2003. Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of south Africa and deep basalt aquifers of Washington state. Environ. Microbiol. 5, 267-277.

Basso, O., Lascourreges, J.F., Le Borgne, F., Le Goff, C., Margot, M., 2009. Characterization by culture and molecular analysis of the microbial diversity of a deep subsurface gas storage aquifer. Res. Microbiol., in press.

Basso, O., Lascourreges, J.F., Jarry, M., Margot, M., 2005. The effect of cleaning and disinfecting the sampling well on the microbial communities of deep subsurface water samples. Environ. Micobiol. 7, 13-21.

Beech, I.B., Sunner, J.A., Hiraoka, K., 2005. Microbe-surface interactions in biofouling and biocorrosion processes. Int. Microbiol. 8, 157-168.

Bouvier, T., del Giorgio, P.A., 2003. Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridisation (FISH): A quantitative review of published reports. FEMS Microbiol. Ecol. 44, 3-15.

Booth, I.R., Cash, P., O'Byrne, C.O., 2002. Sensing and adapting to acid stress. Antonie van Leeuwenhoek 81, 33–42.

Borm, G., Förster, A., 2005. Tiefe salzwasserführende Aquifere – eine Möglichkeit zur geologischen Speicherung von CO_2. Zeitschrift für Energiewirtschaft, Recht, Technik und Umwelt 8, 15-20.

Brockman, F.J., Kieft, T.L., Fredrickson, J.K., Bjornstad, B.N., Li, S.W, Spangenburg, W., Long, P.E., 1992. Microbiology of Vadose Zone Paleosols in South-Central Washington State. Microbiol. Ecol. 23, 279-301.

Crombie, D.J., Moody, G.J., Thomas, J.D.R., 1980. Corrosion of iron by sulfate-reducing bacteria. Chem. Ind. 21, 500-505.

Detmers, J., Strauss, H., Schulte, U., Bergmann, A., Knittel, K., Kuever, J., 2004. FISH shows that *Desulfotomaculum* spp. are the dominating sulfate-reducing bacteria in a pristine aquifer. Microb. Ecol. 47, 236-242.

Diams, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient fort the detection of all Bacteria; Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434-444.

Förster, A., Norden, B., Zinck-Jørgensen, K., Frykman, P., Kulenkampff, J., Spangenberg, E., Erzinger, J., Zimmer, M., Kopp, J., Borm, G., Juhlin, C.-G., Cosma, C., Hurter, S., 2006. Baseline characterization of the CO_2SINK geological storage site at Ketzin, Germany. Environmental Geosciences 133, 145–161.

Fredrickson, J.K., Balkwill, D.L., 2006. Geomicrobial processes and biodiversity in the deep terrestrial subsurface. Geomicrobiol. J. 23: 345-356.

Friedrickson, J.K., McKinley, J.P., Bjornstad, B.N., Long, P.E., Ringelberg, D.B., White, D.C., et al., 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a Late Cretaceous shale-sandstone sequence, north-western New Mexico. Geomicrobiol. J. 14, 183-202.

Fry, N.K., Friedrickson, J.K., Fishbain, S., Wagner, M., Stahl, D., 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl. Environ. Microbiol. 6, 1498-1504.

Giese, R., Henninges, J., Lüth, S., Morozova, D., Schmidt-Hattenberger, C., Würdemann, H., Zimmer, M., Cosma, C., Juhlin, C., CO2SINK Group, 2009. Monitoring at the CO_2SINK Site: A Concept Integrating Geophysics, Geochemistry and Microbiology. Energy Procedia 1, 2251-2259.

Hao, O.J., Chen, J.M., Huang, L., Buglass, R.L., 1996. Sulfate-reducing bacteria. Critical Reviews in Environ. Sci. & Technol. 26, 155-187.

Havemann, S.A., Pedersen, K., Ruotsalainen, R., 1999. Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers in Finland. Geomicrobiol. J. 6, 277-294.

Hazen, T.C., Jimenez, L., Lopez de Victoria, G., Fliermans, C.B., 1991. Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microb. Ecol. 22, 293-304.

Jørgensen, B.B., Boetius, A., 2007. Fast and famine - microbial life in the deep-sea bed. Nature 5, 770-781.

Juhlin, C., Kim Zinck-Jørgensen R. G., Cosma, C., Kazemeini, H., Juhojuntti, N., Lüth S., Norden, B., Förster, A., 2007. 3D baseline seismics at Ketzin, Germany: The CO_2SINK project. Geophysics 72, B121–B132.

Kallmeyer, J., Mangelsdorf, K., Cragg, B., Horsfield, B., 2006. Techniques for contamination assessment during drilling for terrestrial subsurface sediments. Geomicrobiol. J. 23, 227-239.

King, G.M., 1984. Utilization of hydrogen, acetate, and "non-competitive" substrates by methanogenic bacteria in marine sediments. Geomicrobiol. J. 3, 275-306.

Kotelnikova, S., 2002. Microbial production and oxidation of methane in deep subsurface. Earth-Science Reviews 58, 367-395.

Kotsyurbenko, O. R., Friedrich, M. W., Simankova, M. V. , Nozhevnikova, A. N. , Golyshen, P. N., Timmis, K. N., Conrad, R., 2007. Shift from acetoclastic to H_2-dependent methanogenesis in a West Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacterium* strain. Appl. Environ. Microbiol. 73, 2344-2348.

Lee, W., Lewandowski, Z., Nielsen, P.H., Hamilton, W.A., 1995. Role of sulphate-reducing bacteria in corrosion of mild steel: A review. Biofouling 8, 165-194.

Lin, L.-H., Wang, P.-L., Rumble, D., Lippmann-Pipke, J., Boice, E., Pratt, L.M., Lollar, B.S., Brodie, E.L., Hazen, T.C., Andersen, G.L., DeSantis, T.Z., Moser, D.P., Kershaw, D., Onstott, T.C., 2006. Long-Term sustainability of a high-energy, low-diversity crustal biome. Science 314, 479 – 482.

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H., 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. Syst. Appl. Microbiol. 15, 593-600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer K.H., 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. Microbiology 142, 1097-1106.

Manz, W., Eisenbrecher, M., Neu, T.R., Szewzyk, U., 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. 25, 43-61.

Maestrojuan, G., Boone, D., 1991. Characterization of *Methanosarcina bakeri* MS^T and 227, *Methanosarcina mazei* $S\text{-}6^T$, and *Methanosarcina vacuolata* $Z\text{-}761^T$. Int. J. Bacteriol. 41, 267-274.

Meier, H., Amann, R., Ludwig, W., Schleifer, K.-H., 1999. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22, 186-196.

Morozova, D., Wandrey, M., Vieth, A., Würdemann, H., 2009. Microbial community characterisation of the CO_2 reservoir in saline aquifer in Ketzin, Germany. Biospektrum: Proceedings of the VAAM annual conference 2009 (8. – 11. March 2009, Bochum, Germany), p. 214.

Neef, A., 1997. Anwendung der *in situ* Einzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozinösen. Ph.D. Thesis, Technische Universität München.

Olson, G.J., Dockins, W.S., McFeters, G.A., Iverson, W.P., 1981. Sulfate-reducing and methanogenic bacteria from deep aquifers in Montana. Geomicrobiol. J. 2, 327-340.

Onstott, T.C., Phelps, T.J., Ringelberg, D., White, D.C., Boone, D.R., et al., 1998. Observations pertaining to the origin and ecology of microorganisms recovered from deep subsurface of Taylorsville Basin, Virginia. Geomicrobiol. J. 15, 353-385.

Pankhania, I.P., 1988. Hydrogen metabolism in sulfate-reducing bacteria and its role in anaerobic corrosion. Biofouling 1, 27-29.

Parkes, R.J., Cragg, B.A., Bale, S.J., Getliff, J.M., Goodman, K., Rochelle, P.A., Fry, J.C., Weightman, A.J., Harvey, S.M., 1994. Deep bacterial biosphere in Pacific Ocean sediments. Nature 371, 410-413.

Pedersen, K., Hallbeck, L., Arlinger, J., Erlandson, A.C., Jahromi, N., 1997. Investigation of the potential for microbial contamination of deep granitic aquifers during drilling using 16S rRNA gene sequencing and culturing method. J. Microbiol. Methods 30, 179-192.

Prevedel, B., Wohlgemuth, L., Legarth, B., Henninges, J., Schütt, H., Schmidt-Hattenberger, C., Norden, B., Förster, A., Hurter, S., 2009. The CO2SINK boreholes for geological CO2 -storage testing. Energy Procedia 1, 2087-2094.

Roller, C., Wagner, M., Amann, R., Ludwig, W., Schleifer, K.H., 1994. *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA- targeted oligonucleotides. Microbiology 140, 2849-2858.

Sahl, J.W., Schmidt, R., Swanner, E.D., Mandernack, K.W., Templeton, A.S., Kieft, T.L., Smith, R.L., Sanford, W.E., Callighan, R.L., Mitton, J.B., Spear, J.R., 2008. Subsurface microbial diversity in deep-granitic-fracture water in Colorado. Appl. Environ. Microbiol. 74, 143-152.

Schilling, F., Borm, G., Giese, R., Schmidt-Hattenberger, C., Wohlgemuth, L., Würdemann, H., Zimmer, M., CO_2SINK Group, 2008. CO_2 injection at Ketzin. In: Kukla, P., Littke, R. (Eds.). Schriftenreihe der Deutschen Gesellschaft für Geowissenschaften, Heft 60, International Conference and 106th Annual Meeting of the Deutsche Gesellschaft für Geowissenschaften e.V. (DGG) and 98th Annual Meeting of the Geologische Vereinigung e.V. (GV), p. 126.

Schönheit, P., Kristjansson, J.K., Thauer, R.K., 1982. Kinetic mechanism for the ability of sulphate-reducers to outcompete methanogens for acetate. Arch. Microbiol. 132, 285-288.

Shimizu, S., Akiyama, M., Ishijima, Y., Hama, K., Kunimaru, T., Naganuma, T., 2006. Molecular characterization of microbial communities in fault-bordered aquifers in the Miocene formation of Northern-most Japan. Geobiology 4, 203-213.

Shimizu, S., AkiyamaNaganuma, T., Fujioka, M., Nako, M., Ishijima, Y., 2007. Molecular characterization of microbial communities in deep coal seam groundwater of northern Japan. Geobiology 5, 423-433.

Stahl, D. A., Amann., D., 1991. Development and application of nucleic acid probes. 205-248. In Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., Chichester, England.

Stevens, T.O., McKinley, J.P., 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. Science 270, 450-454.

Stevens, T.O., McKinley, J.P., Friedrickson, J.K., 1993. Bacteria associated with deep, alkaline, anaerobic groundwaters in southeast Washington. Microb. Ecol. 25, 35-50.

Stubner S., Meuser, K., 2000. Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol. Ecol. 34, 73-80.

Takai, K., Moser, D.P., DeFlaun, M., Onstott, T.C., Fredrickson, J.K., 2001. Archaeal diversity in waters from deep South African gold mines. Appl. Environ. Microbiol. 67, 5750-5760.

Wall., J:D., Bill Yen., H.C., Drurry, E.C., 2007. Evalutaion of stress response in sulphate-reducing bacteria through genome analysis. In: Barton, L.L., Hamilton, W.A. (Eds.) Sulphate-reducing bacteria. Environmental and engineered systems. Cambridge Unniversity press, Cambridge, 141-167.

Wallner, G., Amann, R., Beisker, W., 1993. Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136-143.

Wandrey, M., Morozova, D., Zettlitzer, M., Würdemann, H., subm. Contamination control of rock core and brine samples of the legs Ktzi 200-2007, Ktzi 201-2007, and Ktzi 202-2007 at the CO_2SINK location Ketzin/Germany using Fluorescein as a tracer. This issue.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: The unseen majority. Proc. Natl. Acad. Sci. USA 95, 6578-6583.

Würdemann, H., Morozova, D., Wandrey, M., Vieth, A. Zettlitzer, M. and the CO_2SINK Group. Temporary loss of injectivity due to microbial processes creating iron sulphide in Ketzin. In prep.

Würdemann, H., Schilling, F.R., Moeller, F., Borm, G., Kühn, M., and CO_2SINKGroup, subm. The field-laboratory for CO_2 storage “CO_2SINK” at Ketzin (Germany): an overview on the first 10 months of operation. This issue.

Zarda, B., Hahn, D., Chatzinotas, A., Schönhuber, W., Neef, A., Amann, R., Zeyer, J., 1997. Analysis of bacterial community structure in bulk soil by *in situ* hybridisation. Arch. Microbiol. 168, 185-192.

Zettlitzer, M., Moeller, F., Würdemann, H., Lokay, P., and the CO2SINK group, subm. Re-Establishment of proper injectivity of the CO_2-injection well Ketzin-201. This issue.

Zheng, D., Alm, E.W., Stahl, D.A., Raskin L., 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62, 4504-4513.