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Analysis of the microbial community from a saline aquifer prior to CO_2 injection in Ketzin using improved Fluorescence *in situ* Hybridisation method

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Abstract

This study reports a procedure combining a cell extraction method and Fluorescence *in situ* Hybridisation (FISH) for monitoring and quantification of bacteria in aquifer samples with high particulate loads from the CO_2 storage reservoir at Ketzin (Germany). FISH was applied to cells extracted by density centrifugation. This method was tested and evaluated on slurry from the injection well amended with *E.coli* cells and applied to fluid samples from the observation well. Cell extraction efficiency averaged out at 80-90%. Up to 10^6 cells ml^{-1} were detected at a depth of 675 m, with dominance of the domain *Bacteria*.

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Keywords: Fluorescence *in situ* Hybridisation; CO_2 storage; cell extraction; microbial community

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1. Introduction

Carbon dioxide capture and storage (CCS) is receiving a great attention as an option for the mitigation of the CO_2 emissions for stabilization of the greenhouse gas concentration. In order to investigate the possibility of underground CO_2 storage, the first onshore storage site in Europe was established as a research facility in Ketzin (Germany, west of Berlin). Since 2008 CO_2 is injected and stored in an approximately 650m-deep subsurface saline aquifer [1, 2].

The Earth subsurface is known to be a major habitat for a high number of different groups of active microorganisms [3-7]. Interactions between microorganisms, gas (either dissolved or in the supercritical state) and the mineral content of both the reservoir rock and the cap rock have to be characterized, since microorganisms are known to influence such process as mineral creation and dissolution [8, 9], permeability changes and filter clogging [10] and corrosion [11]. Therefore, analyses of microbial community composition and its changes should provide information about the effectiveness and reliability of long-term CO_2 storage technique.

Our previous study revealed that up to 10^6 cells ml^{-1} were detected in the first observation well (CO_2 Ktzi 200/2007), where CO_2 arrived after injection of 500 t [12, 13]. For the identification and enumeration of the microorganisms, the robust and widely applied method Fluorescence In Situ Hybridisation (FISH) was implemented. FISH coupled with rRNA-targeted oligonucleotide probes allows direct visualisation, identification and localisation of bacterial cells from selected phylogenetic groups. However, its application to the samples from the second observation well (CO_2 Ktzi 202/2007) where CO_2 arrived after injection of approximately 11.000 t was hampered. The presence of solids and particles in the reservoir fluids significantly interfered with the cell visualization using epi-fluorescent microscopy. Furthermore, bacteria attached to solid particles lead to underestimation of actual cell counts due to masking [14]. Since it is difficult to distinguish cells among particles and this strongly hinders the identification and enumeration of bacteria, an optimization of the FISH method was done with a newly developed method. A combination of chemical and physical treatment followed by density centrifugation through a cushion of Nycodenz, an universal density gradient medium, allowed to separate bacteria from the sediment matrix, and to avoid false fluorescent signals given by some organics and minerals. Recently, Nycodenz density centrifugation prior to FISH was successfully applied for the cell separation from soil particles, clays and marine sediments [15-18]. This study describes a FISH application for monitoring indigenous bacterial populations in aquifer samples prior to CO_2 injection by an improved cell extraction method developed based on the combination of chemical and physical treatments followed by density centrifugation.

2. Methodology

2.1. Study site

The pilot CO_2 storage site is located in the North German Basin near the city of Ketzin [1, 2]. The saline aquifer target reservoir for the CO_2 injection is the Stuttgart Formation. It has a thickness of approximately 75 m, at a depth of 630 to 700 m [19]. Three 750-800 m deep vertical wells, the CO2 Ktzi 200/2007, CO2 Ktzi 201/2007 and CO2 Ktzi 202/2007 boreholes (short names: Ktzi 200, Ktzi 201, and Ktzi 202), were drilled in 2007 [20]. The injection of CO_2 into the Ktzi 201 well was started on the end of June 2008 [21]. The temperature and pressure of the formation fluid prior to CO_2 injection were approximately 35 °C and 62 bar, and the salinity was roughly 235 g l^{-1}. This study focus on the results

obtained from the observation well Ktzi 202, where the application of a new developed method was necessary in order to obtain quantification results from FISH.

2.2. Fluid and slurry sampling

Fluid samples from the Ktzi 202 were collected using downhole sampling (Erdöl-Erdgas Workover GmbH) at a depth of 675 m using double ball-lining (Doppelkugelbüchse, DKB) sampler. Slurry samples from Ktzi 201 were collected using DKB sampler from at a 727 m depth (bottom of the well). Both samplers were flushed with sterilised deionised water and ethanol immediately before sampling. The pH, conductivity, temperature and other parameters were measured directly after the sampling process. The fluids and slurry were transferred aseptically into sterilised 100 to 1000 ml glass vials, refrigerated to 4 °C and transferred immediately to the laboratory for microbiological and chemical analyses. Quantitative solid analyses were carried out by filtration of 100 ml fluid through 1.2-µm Millipore membrane filtrations. The filters were incubated for 24 h at 60 °C in the oven and weighted before and after incubation to assess the dry solid content. The total organic carbon content was determined using a TOC-analyser (Dimatec GmbH) according to DIN EN 1484-H3. In order to detect possible contamination in the fluid samples, 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 [22]. Tracer concentration (Na-fluorescein) was detected using a mobile light fluorometer (Hermes Messtechnik, Stuttgart, RS232).

2.3. Sample fixation and preparation

To obtain sufficient biomass for FISH analysis, freshly collected fluid samples from Ktzi 202 (500 to 1000 ml) were concentrated to a volume of approximately 0.5 ml by centrifugation (at 1000 *g* for 1 h at 4 °C, Heraeus Biofuge Pico, Sigma 6K15) and were then fixed with paraformaldehyde (1% end concentration) over night at 4 °C [23]. About 1 g of slurry from Ktzi 201, 727 m was transferred into a sterile 10 ml tube and fixed overnight according to Pernthaler et al. [23]. Cell numbers in the slurry were in a range of detection limits of FISH analyses and were about 10^3 cells ml^{-1}. A laboratory culture of *E. coli* JM 109 was grown overnight in LB media and preserved as described above. Fixed slurry was mixed with fixed cells of *E. coli* (10^8 cells) in order to determine the cell recovery after the cell extraction method. Slurry with *E. coli* and pellet were stored at -20 °C until further analysis.

2.4. Cell extraction prior to FISH

Cells were separated from a sediment matrix in few steps.

Chemical treatment – Aliquots of 200-400 µl from the slurry with *E. coli* cells or pellet remaining from fluid centrifugation were diluted in a ratio 1:2 with a detachment solution containing a detergent mix. The detergent mix was made as follows: 580 µl H_2O_{dest} and 400 µl (500 mM) EDTA, 1000 ml (100 mM) sodium pyrophosphate decahydrate, and 20 µl Tween 20. Detachment solution was composed of 250 µl detergent mix, 250 µl methanol, 150 µl NaCl (5 M) and 1350 µl H_2O_{dest}.

Physical treatment – After the addition of the detachment solution, the samples were shaken on an orbital shaker for 1 h to detach particle-associated microorganisms.

Cell separation by density centrifugation – After the physicochemical treatment, 1 vol of pre-heated Nycodenz solution (Nycomed, Oslo, Norway; density 1.3 g ml^{-1}) was added below 1 vol of sample using a syringe with an appropriate needle and centrifuged in a swing-out rotor (10000 *g* for 30 minutes at 4 °C). After centrifugation, layers of supernatant, bacterial cells, Nycodenz and a pellet were clearly visible.

The layers of supernatant and bacterial cells were harvested together, and were transferred to another Eppendorf tube for subsequent washing.

Washing – Aliquots of800-1000 µl from the supernatant and bacterial cells layers were washed with 1xPBS and centrifuged (16000 *g*, 20 minutes at 4 °C). This step was repeated several times in order to remove any remaining traces of Nycodenz.

Due to high background fluorescence of solid particles observed after hybridisation on membrane filters (own observations), the dispersed samples were spotted on gelatine-coated Teflon-laminated slides [24] with 10 wells. The slides were dried at 46 °C for 15 min and dehydrated in 50 %, 80 % and 96 % ethanol.

2.5. Fluorescence in situ Hybridisation (FISH)

FISH coupled with rRNA-targeted oligonucleotide probes was applied for direct visualisation, identification and localisation of bacterial cells from selected phylogenetic groups in environmental samples. The probes for the domains *Bacteria* and *Archaea, Firmicutes* (bacteria with low G+C content) and specific probes for the sulphate reducing bacteria, labelled with the cyanine dye Cy3 and FLUOS (Thermo Scientific) were used. For the detection of sulphate reducing bacteria, the SRB385 probe, which primarily targets the *Desulfovibrionales*, the DEM1164r probe, which targets the *Desulfotomaculum* cluster I and other *Firmicutes*, and the DSV687 probe targeting *Desulfovibrionales* and many *Desulfuromonales* were used. Details of the procedures and probe description are given in Morozova et al. [12]. Total cell counts were determined using 4′6-Diaminodino-2-phenylindole (DAPI, 1 mg ml^{-1} working solution). Microscopy experiments were performed using a Zeiss Axio Imager M2 equipped with filters 49 (DAPI), 38 (FLUOS) and 49 (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. 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. Method assessment and first results

3.1 Cell extraction and separation by density gradient centrifugation

The cell extraction and separation by Nycodenz purification method was applied to the slurry from Ktzi 201, 727 m, amended with *E. coli* cells, and to the aquifer samples from observation well Ktzi 202. Geochemical analyses revealed high dry total dissolved solid (TDS) content of 7.9 g l^{-1} with 17.8 mg l^{-1} of total organic carbon (TOC) in the slurry from Ktzi 201, 727 m. For the aquifer samples from Ktzi 202, dry TDS content was 2.4 g l^{-1} with 134.7 mg l^{-1} TOC.

Physical and chemical treatments revealed to be successful for improving the separation. Four distinct layers were formed during centrifugation with Nycodenz. The fraction on top of the Nycodenz layer contained most of the detached cells and was clearly visible. Cell counts from the other layers were very low and not relevant statistically. Furthermore, the improved separation method succeeded in lowering the content of organics and minerals in the slurry and fluid samples (Fig. 1). Experiments with *E. coli*

cells mixed with the slurry from the bottom of the injection well Ktzi 201 were performed in order to calculate the method efficiency by determining the recovery of *E. coli* using DAPI staining (total number of extracted cells as percentage of cells prior to mixing with slurry, determined by DAPI staining). The results revealed a high cell recovery after Nycodenz treatment between 80 and 90 %. These values were in the range or even higher than previously reported for the soil samples [16, 18, 25]. The reason might be that in comparative protocols the samples treated with Nycodenz were immersed directly in paraformaldehyde without using an extraction buffer first. Furthermore, treatment with dispersants EDTA and Tween 20 [17] before the centrifugation with Nycodenz also increased the yield of the free cells. For comparison, samples extracted (previously) using the non-improved extraction yielded in low cell numbers and most fluorescence was caused by minerals and organic compounds present in the samples. In contrast, the application of the improved extraction protocol resulted in clearly visible cells and a lack of sediment particles. Therefore, the new developed method allowed the quantification of the cells with more accuracy since the detection limit was at least two order of magnitude lower than in untreated samples [18].

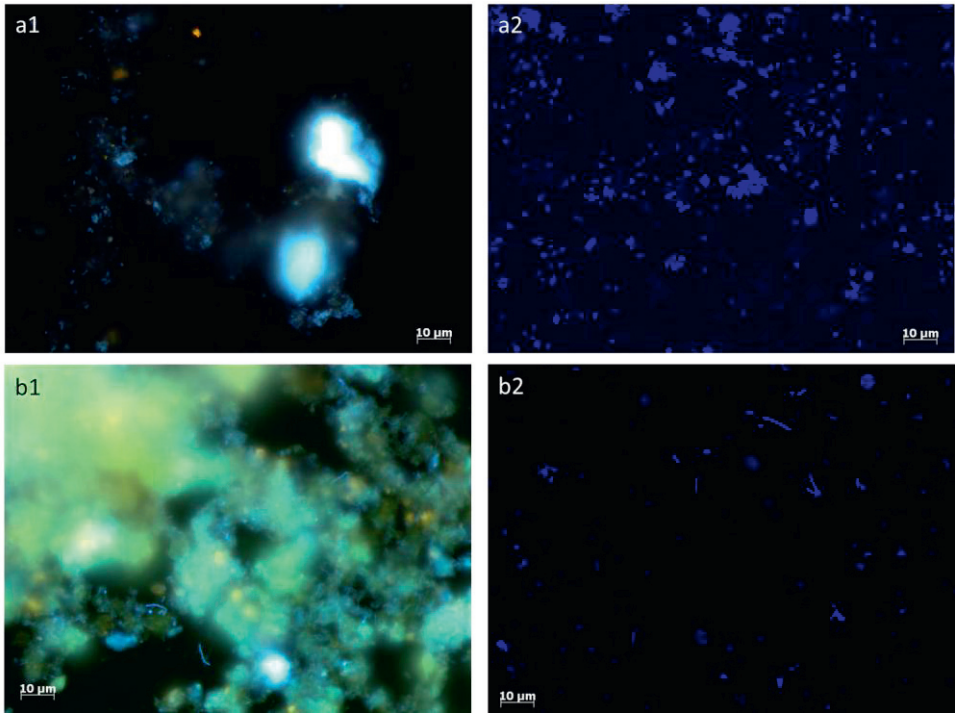

Fig. 1. (a) fluorescence microscope images showing DAPI-stained cells (blue) and *in situ* hybridisation of bacteria in slurry from Ktzi 201 mixed with *E. coli* cells before (1) and after (2) cell extraction followed by Nycodenz treatment; (b) fluorescence microscope images showing DAPI-stained cells (blue) and *in situ* hybridisation of bacteria in fluid samples from Ktzi 202 before (1) and after (2) cell extraction followed by Nycodenz treatment

3.2 FISH application to the extracted microorganisms

FISH was performed with specific probes for *Archaea*, *Eubacteria* (most *Bacteria*), *Firmicutes* and different groups of sulphate reducing bacteria. DAPI staining was applied for total cell count determination. The quality of the extraction was evaluated in terms of signal intensity and occurrence of unspecific or background fluorescence.

Nycodenz-treated samples were characterized by a lack of background fluorescence and a clear signal was observed during FISH analysis (Fig. 1b). Up to 10^6 cells ml^{-1} were enumerated in fluid samples from the second observation well Ktzi 202 using DAPI staining. These values were similar to those previously reported for the first observation well Ktzi 200 [12] and about one order of magnitude lower than in the injection well Ktzi 201 [1, 10]. Due to the differences in technical procedures during well completion for the injection and observation wells [1, 10] some differences in fluid composition were observed. Carbon availability and environmental conditions are the most important factors controlling microbial growth [3, 26]. Comparable geochemical analyses revealed highest TOC content of almost 400 mg l^{-1} in the injection well [10], which corresponds to the highest cell numbers enumerated in the fluids [27]. Fluids from the first observation well were characterized by relatively low TOC content (up to 63.3 mg l^{-1}) and comparably lower cell numbers prior to CO_2 injection [12]. Notably, due to the low particles loads in the fluids from Ktzi 201 and Ktzi 200, the samples from those two wells were not treated with Nycodenz prior to FISH analyses and therefore cell numbers could not be directly compared; however, similar trend could be observed.

FISH analyses revealed a high diversity of the microbial community (Tab. 1). The eubacterial abundance expressed as a percentage of total DAPI-stained cells was 85.1 %. Between 21.6 and 54.8 % of the DAPI labelled cells contributed to the bacteria with low G+C content, which indicates that bacterial population was dominated by *Firmicutes*. Similar observations were already reported for the first observation well [12] as well as for the other deep microbial ecosystems [7, 28] like deep subsurface gas storage aquifer [29] or in the 900 m deep coal seam groundwater [30]. The quantification of the domain *Archaea* in the fluid samples resulted in up to 28.2 % of the detected cells, higher than these enumerated in the fluids from the other wells in Ketzin [12, 27].

High abundance of sulphate reducing bacteria (SRB) with up to 60.1% for the *Desulfovibrionales* and *Desulfomonales* groups were quantified. Detection of sulphate reducing bacteria with the SRB385 probe, which primarily targets the *Desulfovibrionales*, and the DEM1164r probe, which targets the *Desulfotomaculum* cluster I and other *Firmicutes*, resulted in 33.3 % and 24.6 % SRB, respectively. High numbers of the sulphate reducing bacteria were also reported for the first observation well, where up to 24 % SRB were detected in the fluid samples prior to CO_2 injection and N_2 lift [12] as well as for the injection well, where up to 32 % SRB were enumerated prior to CO_2 injection and N_2 lift [10, 27]. High TOC content in the fluids with acetate – an important substrate for microorganisms – as a major component (between 5 and 25 mg l^{-1}) favour the presence of sulphate reducers under these conditions. 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* [31]. Several other studies have discovered evidence of *Desulfotomaculum* spp. and other sulphate reducing bacteria in the deep subsurface [26, 32, 33].

Table 1. Total bacterial numbers in the fluid probes from observation well Ktzi 202 prior to CO_2 injection analysed by DAPI staining and relative percentage of hybridised cells with specific probes

Ktzi 202 675m	DNA staining/rRNA-targeting oligonucleotide probe	Cell numbers (cells ml^{-1} [10^6]) (mean ± SD)	% of total cell number
Total cell numbers	DAPI	5.1 ± 1.7	
Eubacteria	EUB338 mix	4.3 ± 1.4	85.1
Gram-positive bacteria with low GC-content (*Firmicutes*)	LGC354a	2.8 ± 1.8	54.8
	LGC354b	1.1 ± 0.9	21.6
	LGC354c	1.2 ± 1.1	24.1
Sulphate reducing bacteria	SRB385	1.7 ± 1.2	33.3
	DEM1164r	1.2 ± 3.3	24.6
	DSV687	3.1 ± 1.9	60.1
Archaea	Arc915	1.4 ± 1.2	28.2

The identification of the sulphate reducing bacteria, which are known to be involved in corrosion [34, 35], 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 change rapidly and massively the permeability of the injectivity in the near well bore area [1, 10, 27]. Further detailed analyses of the saline aquifer probes from the second observation well using Nycodenz-based FISH analysis as well as PCR-based methods like fingerprinting and real time PCR are planned. The results of those analyses should be compared with the results gained from the first observation well [12, 36] in order to draw broader conclusions about the microbial community in the deep aquifers and its changes during CO_2 storage.

4. Conclusions

Cell extraction prior to FISH visualization and quantification was improved significantly by applying Nycodenz-based density centrifugation in combination with physico-chemical extraction for samples characterized by high particulate loads. In this case, established for deep aquifer samples, this method - with few modifications- could be also used for the separation of bacterial populations inhabiting rock cores. Moreover, alternative PCR-based identification methods can be applied to Nycodenz-extracted cells. Thus, the Nycodenz-based cell extraction described here represents a valuable and promising tool for the *in situ* analysis of bacterial communities in saline aquifers proposed for the CO_2 storage.

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