

# Tracing long-distance electron transfer and cable bacteria in freshwater sediments by agar pillar gradient columns

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**One sentence summary:** Novel agar pillar gradient columns selectively enrich for cable bacteria and differentiate electrogenic populations from surface and groundwater sediments.

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## Abstract

Cable bacteria (CB) perform electrogenic sulfur oxidation (e-SO<sub>x</sub>) by spatially separating redox half reactions over centimetre distances. For freshwater systems, the ecology of CB is not yet well understood, partly because they proved difficult to cultivate. This study introduces a new 'agar pillar' approach to selectively enrich and investigate CB populations. Within sediment columns, a central agar pillar is embedded, providing a sediment-free gradient system in equilibrium with the surrounding sediment. We incubated freshwater sediments from a streambed, a sulfidic lake and a hydrocarbon-polluted aquifer in such agar pillar columns. Microprofiling revealed typical patterns of e-SO<sub>x</sub>, such as the development of a suboxic zone and the establishment of electric potentials. The bacterial communities in the sediments and agar pillars were analysed over depth by PacBio near-full-length 16S rRNA gene amplicon sequencing, allowing for a precise phylogenetic placement of taxa detected. The selective niche of the agar pillar was preferentially colonized by CB related to *Candidatus Electronema* for surface water sediments, including several potentially novel species, but not for putative groundwater CB affiliated with *Desulfurivibrio* spp. The presence of CB was seemingly linked to co-enriched fermenters, hinting at a possible role of e-SO<sub>x</sub> populations as an electron sink for heterotrophic microbes. These findings add to our current understanding of the diversity and ecology of CB in freshwater systems, and to a discrimination of CB from surface and groundwater sediments. The agar pillar approach provides a new strategy that may facilitate the cultivation of redox gradient-dependent microorganisms, including previously unrecognized CB populations.

**Keywords:** freshwater cable bacteria, agar pillar columns, PacBio near-full-length 16S rRNA gene amplicon sequencing, sediment microprofiling, electrogenic sulphur oxidation (e-SO<sub>x</sub>)

## Introduction

The traditional view of diffusion-based redox stratification in sediments has been challenged by the discovery of filamentous cable bacteria (CB), which are able to transfer electrons between reactants over centimetre distances (Pfeffer et al. 2012). The proposed model of electrogenic sulfur oxidation (e-SO<sub>x</sub>) by CB has set a new paradigm for the biogeochemistry of sediments (Nielsen and Risgaard-Petersen 2015). Since their discovery, CB within the Desulfobulbaceae have been found to occur globally—not only in marine habitats, such as mangroves (Burdorf et al. 2016), salt marshes (Larsen et al. 2015) and the seagrass rhizosphere (Martin et al. 2019), but also in freshwater streambeds (Risgaard-Petersen et al. 2015), at roots of freshwater plants (Scholz et al. 2021) and in groundwater sediments (Müller et al. 2016). Moreover, the first stable enrichment culture of CB, *Candidatus* (*Ca.*) *Electronema* au-

reum strain GS, has recently been presented (Thorup et al. 2021). However, there is no pure culture of any CB to date, and their supposed strict dependence on opposing gradients of oxygen and sulfur complicates sustaining them in culture.

Long-distance electron transfer (LDET) has a direct effect on biogeochemical transformations and elemental cycling in sediments (Risgaard-Petersen et al. 2012). Via the ability to conduct electricity, CB shape the geochemistry of their habitat, removing potentially harmful sulfide from deeper sediments and forming an extended suboxic zone (Seitaj et al. 2015). Because of their unique conductive capacity, CB are thought to have a competitive advantage over other sulfur-oxidizing microbes in marine sediment (Meysman 2018). Due to their ability to uncouple redox half reactions in e-SO<sub>x</sub>, CB can capitalize on diffusion gradients especially in environments with high organic loading and limited

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electron acceptor availability, including polluted sediments (Meckenstock et al. 2015, Matturro et al. 2017). While a number of studies have demonstrated the presence of CB in freshwater systems to date (Risgaard-Petersen et al. 2015, Müller et al. 2016, Sandfeld et al. 2020, Dam et al. 2021, Liu et al. 2021, Scholz et al. 2021), the factors controlling their growth and ecology in freshwater systems remain poorly understood.

The present study introduces a new ‘agar pillar’ approach that allows for the targeted enrichment of CB in a (semi-) natural, but sediment-free experimental compartment. We tested this approach for a range of different freshwater sediments (from a streambed, a lake and an aquifer), hypothesizing that sediment origin and biogeochemistry will control the occurrence and/or diversity of CB detected, possibly even discerning CB populations derived from surface and groundwater. Our results expand the known diversity of species-level CB populations in freshwater sediment, and advance the available strategies for the investigation of their biogeochemistry and ecology in freshwater ecosystems.

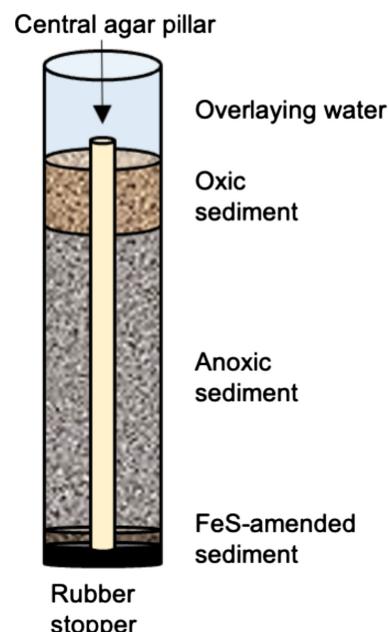
## Materials and methods

### Field sampling

A variety of terrestrial freshwater systems were selected for sampling, including an agriculturally impacted side stream of the Isar River, a pre-alpine meromictic lake, well known for its sulfidic hypolimnion (Oikonomou et al. 2014, 2015), and a previously investigated hydrocarbon-polluted aquifer sediment (Müller et al. 2016). Streambed sediment was taken in October 2018 by manual scooping from a side branch of the Isar River near Garching, Germany ( $48^{\circ}14'23.4''N$ ,  $11^{\circ}39'53.4''E$ ), running below trees in close proximity to agricultural fields. The water temperature at the time of sampling was  $13^{\circ}C$ . Surface sediment was sampled up to 15 cm depth, showing a blackish colour and a strong sulfidic smell. Sediment from the meromictic pre-alpine Lake Alatsee ( $47^{\circ}33'45.2''N$ ,  $10^{\circ}38'23.3''E$ ) (Oikonomou et al. 2014, 2015) was taken by scooping from around 1 m depth in October 2018 by wading in from the bank. The water temperature at the time of sampling was  $15^{\circ}C$ . Surface sediment was sampled up to 15 cm depth. This lake is known for its sulfidic monimolimnion (no seasonal mixing of lower water body) and redox transition zone, with seasonal blooms of purple sulfur bacteria. Contaminated aquifer sediment was sampled from 6–10 m depth below ground in November 2016 via drill coring at a previously investigated tar oil-contaminated aquifer in Flingern near Düsseldorf, Germany ( $51^{\circ}13'20.6''N$ ,  $6^{\circ}49'05.6''E$ ) (Wisotzky and Eckert 1997, Anneser et al. 2008, Pilloni et al. 2019). Sediment from the upper fringe of the hydrocarbon plume at 7 m depth was used for column incubations. All samples were stored anoxically in closed flasks in the dark at  $12^{\circ}C$  until laboratory microcosm set-up within 2 months after sampling.

### Column microcosms

Sediment samples were sieved individually through an analysis sieve (stainless steel, mesh aperture 2 mm; Haver & Boecker, Oelde, Germany) to remove larger plant debris or gravel. Sparging with  $N_2$  gas was used to reduce oxygen exposure during sieving. Glass cylinder columns of 2.8 cm diameter and 12 cm height were prepared by sealing them from the bottom with a rubber stopper of a syringe (Omnifix®, Luer Lock Solo, 50 mL; B. Braun, Melsungen, Germany). Approximately 150 mL of the homogenized sediment slurry was augmented with 2  $\mu\text{mol/g}$  (wet weight) of solid, anoxic FeS precipitate (prepared as described previously; Müller et al.



**Figure 1.** Scheme of the agar pillar gradient column set-up. The glass cylinder was sealed at the bottom with a rubber stopper. Sediment samples were filled into the column around a central agar pillar, spanning the entire length of the column. Bottom layer sediments were amended with FeS.

2016) until the slurry took a blackish colour. The bottom-sealed glass cylinders were filled up to 1 cm with the FeS-augmented sediment slurries as sulfidic bottom layer rich in electron donors. Then, a central agar pillar (Fig. 1) was cast before further sediment addition. For this, ethanol-rinsed cocktail straws (diameter 7 mm) were vertically placed into the glass cylinders, gently pressing them into the FeS-augmented bottom layer. A cardboard gauge with a central hole for the straw was used to maintain upright orientation, before further homogenized, non-FeS-augmented sediment slurry was filled on top of the bottom layer to surround the straw. Air bubbles were prevented while the slurry was filled up to  $\sim 2$  cm below the upper rim of the cylinder. 1.5% preboiled agar (microbiology grade; Carl Roth GmbH, Karlsruhe, Deutschland) cooled down to  $\sim 55^{\circ}C$  was then filled into the straws. After the agar had solidified, the straws were carefully pulled, with minimal mechanical disturbance of the slurry and the fresh-made agar pillar. The agar pillars were then cut to end  $\sim 2$  mm above the surrounding sediment surface, and columns were left to further settle and equilibrate. Columns were then submerged in a freshwater aquarium ( $\sim 20$  L) filled with nonsterile tap water and covered with a lid to minimize evaporation during incubation. The water was air-sparged during incubation by aquarium pumps (Tetra, Melle, Germany). For each sediment, triplicate columns were incubated at room temperature in the dark for 28 days. Microsensor profiling of diffusion gradients and signatures of LDET was done on days 3 and 28, both in agar and in surrounding sediment. Columns were sacrificed after 28 days.

For column sacrificing, the glass cylinders were fixed into a metal stand. Sediment subsections of 0–3, 3–6, 6–10, 10–15, 15–20, 20–25, 25–30, 30–40 and 40–50 mm column depth were then sampled. Sacrificing was done by fitting a syringe plunger (50 mL Omnifix) from below. The plunger was pressed against the syringe rubber stopper originally used to seal the glass cylinders. Thus, the entire sediment column, including agar pillars, was pushed

out of the glass cylinder without disturbing depth stratification. The sediment was pushed into a sterile, second glass cylinder of the same diameter with an attached millimetre scale. Each depth segment was then cut by using a thin sterilized metal plate and sliding it carefully between the two glass cylinders. The upper part of the sediment of a defined height was then carefully transferred to a sterile Petri dish for further handling. From each subsampled sediment section, 500 µL was transferred into 500 µL of 4% paraformaldehyde solution (1:1) for later fluorescence in situ hybridization (FISH). Further replicates of ~0.5 g were transferred into bead beating tubes for DNA extraction (storage at -80°C). Agar pillar segments were carefully rinsed of remaining sediment with sterile water. Approximately 500 µL of agar from inside the cross section was then squashed into 500 µL of 4% paraformaldehyde solution for FISH. For DNA extraction, 0.1–0.2 g agar was used per depth transect.

## Column geochemistry

Microprofiles of oxygen, sulfide and pH were measured with microsensors (~200–500 µm tip size for measurements in sediment and ~100 µm tip size for measurements in agar), purchased from Unisense (Aarhus, Denmark). In replicate columns, electric potential (EP) measurements were done at Aarhus University using self-prepared sensors (Damgaard et al. 2014). Calibration of microsensors was done according to the manufacturer's instructions, but raw signal was noted for EP measurements, respectively. Microsensor signals were recorded with the SensorTrace Pro software (Unisense, Aarhus, Denmark) and data were exported to Excel. Total sulfide concentrations  $\Sigma\text{H}_2\text{S} = [\text{H}_2\text{S}] + [\text{HS}^-] + [\text{S}^{2-}]$  were calculated using the simplified equation after Jeroschewski et al. (1996):  $[\text{H}_2\text{S}] = [\text{S}_{\text{tot}}^{2-}] / (1 + K_1 / [\text{H}_3\text{O}^+])$  with  $pK_1 = 6.9518$  after Millero et al. (1988). For EP, reverse profiles were taken (measuring from depth to the surface) to minimize noise. Mean values with standard deviation are shown.

## FISH

Attempts to perform microscopy with intact agar pillars and sediment failed due to their challenging physicochemical properties. All samples were fixed overnight in 2% paraformaldehyde at 4°C, and then carefully washed twice with 1× phosphate-buffered saline (PBS) buffer and stored at -20°C in a 1:1 solution with 96% ethanol. A dilution of 1:10 was used for analysis of sediment samples. A final volume of 20 µL was pipetted onto a polysine-coated slide (Polysine® Slides, Menzel-Gläser, Thermo Scientific, Dreieich, Germany). For all agar samples, a slice was squashed between an object slide and a cover slip. The slides were dried at 46°C for 30–60 min. Afterwards, they were dehydrated in consecutively increasing ethanol concentrations of 50%, 80% and 100% for 3 min each. Subsequent hybridization occurred for at least 2.5 h at 46°C and was followed by a 20 min stringent washing step according to Pernthaler et al. (2001). Filamentous Desulfobulbaceae were detected with the specific family-level oligonucleotide probe DSB706 (Loy et al. 2002) labelled with Atto 647N using 35% formamide in the hybridization buffer (Pernthaler et al. 2001). For detection of total bacteria, the oligonucleotide probe mix EUB I-III was used (Daims et al. 1999) coupled to Atto 488 with 35% formamide (FISH probes were 5'-labelled mono-probes, biomers.net GmbH, Ulm, Germany). Samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and embedded in an antifade mounting medium, a 1:5 mix of Vectashield® H-1000 (Vector Laboratories, Burlingame, CA/USA) and Citifluor (Electron Microscopy Sciences, Hatfield, PA/USA). Fluorescence microscopy was done

with an Axioskop 2 Plus (Zeiss, Jena, Germany) using specific filters (Zeiss filter sets 02 (DAPI), 10 (Atto 488), 50 (Atto 647N)). Imaging was done using a Leica DFC9000 camera and the Leica Application Suite X (Leica Microsystems, Wetzlar, Deutschland). Fluorescent images taken in different channels were overlaid in either LAS X or ImageJ software packages.

## DNA extraction and amplicon sequencing

DNA from sediment and agar subsamples was extracted according to the protocol described in Lueders et al. (2004). PacBio SMRT sequencing of bacterial near-full-length 16S rRNA gene amplicons was done as recently described for ~1 kb nitric oxide dismutase amplicons (Zhu et al. 2020) using universal bacterial 16S rRNA gene primers (Ba27f: AGRGTTYGATYMTGGCTCAG; Ba1492r: RGY-TACCTGTTACGACTT) (Lane 1991) tailed with PacBio universal sequence adapters for the first round of amplification. PCR was done in a total reaction volume of 25 µL, consisting of nuclease-free water, KAPA HiFi Buffer for GC-rich samples (Kapa Biosystems, Roche, Basel, Switzerland), 10 mM dNTP, 50 µM forward and 50 µM reverse primer, 1 U µL<sup>-1</sup> KAPA HiFi Hot Start DNA Polymerase (Kapa Biosystems) and ~1 ng of template DNA. The first round of amplification was done in 23 cycles of denaturation (30 s at 95°C), annealing (30 s at 57°C) and elongation (60 s at 72°C). Amplicons were purified with the MicroElute® DNA Clean Up Kit (Omega Bio-Tek Inc., Norcross, GA/USA) and quantified with a Fragment Analyzer (Agilent, Santa Clara, CA/USA) using a standard sensitivity kit (DNF-473; Agilent, Santa Clara, CA/USA). The second round of amplification was done with the PacBio barcoded universal F/R primers plate-96 (PacBio, Menlo Park, CA/USA) with ~0.5 ng template DNA from the first PCR. Amplification was done in 20 cycles using the same conditions as in the first round. Amplicons were then purified using PacBio's recommended magnetic bead protocol (AMPure PB beads; PacBio, Menlo Park, CA/USA). SMRTbell library preparation was done according to standard manufacturer protocols. 20 to 25 barcoded amplicon samples per SMRTcell were multiplexed by equimolar pooling. Quality was checked in between all steps using the Fragment Analyzer. Sequencing of the pooled amplicon mixes was done on a PacBio Sequel II System using version 2.1 chemistry and 2.1 polymerase.

## Sequence data analysis

Our PacBio near-full-length amplicon sequencing strategy, based on ~1.5 kb circular consensus sequences with a low error rate close to 0%, allows for increased taxonomic and phylogenetic detail as compared with other NGS approaches with much shorter read lengths. Fastq files were generated using the PacBio SMRTLink 6.0 portal. In a first step, pooled samples were demultiplexed, followed by generation of circular consensus sequences (CCS). Settings for CCS generation were as follows: predicted accuracy = 0.995, minimum number of passes = 3, minimal length 1300 bp, maximal length = 2000 bp. All other parameters were at default. Fastq files were submitted to the NCBI Sequence Read Archive (SRA) database under submission ID: SUB9561533 and BioProject ID PRJNA726381. Quality control and denoising of the sequencing reads was performed with mothur v.1.43.0 (Schloss et al. 2009), following previously described procedures (Schloss et al. 2016, Wagner et al. 2016). In a first step, Fasta files were generated with the fastq.info command using the pacbio = T option. Primer and adapter sequences, as well as sequences outside the expected size range (<1350 and >1650 bp) were removed using the trim.seqs command. Further, putative chimeras were

removed with the mothur-implemented Uchime algorithm (Edgar et al. 2011). Unique sequences were generated using unique.seqs command. The resulting denoised, high-quality sequences were uploaded to the SILVAngs server (<https://ngs.arb-silva.de>; all settings at default; Quast et al. 2013) for automated alignment against the SILVA SSU database (release 132) and taxonomic analysis.

## Phylogenetic tree construction

Desulfobulbaceae-affiliated near-full-length 16S rRNA sequences were aligned using the SINA online tool (Pruesse et al. 2007) and added to the SILVA Release 138 SSU Ref database (Quast et al. 2013) in ARB (Ludwig et al. 2004). The alignment was inspected manually, before a phylogenetic tree was calculated, using maximum likelihood algorithms and support by 1000 bootstraps (using a 50% sequence variability filter for Deltaproteobacteria). A selection of representative full-length Desulfobulbaceae 16S rRNA sequences from the SSU database was included as reference sequences.

## Results

### Column biogeochemistry

In streambed sediment columns,  $\sum\text{H}_2\text{S}$  concentrations strongly increased below 10 mm depth after 3 days, both in sediment and agar, rising up to  $\sum\text{H}_2\text{S}$  concentrations between  $\sim 17 \mu\text{M}$  and  $\sim 78 \mu\text{M}$  at 15 mm depth, respectively (Fig. 2). pH decreased gradually from  $\sim 8.5$  in the oxic zone to  $\sim 7.3$  at 10 mm depth. The pH values were comparable between sediment and the agar pillar. Oxygen was completely depleted below 4.5 mm sediment depth, while a minimum of  $47 \mu\text{M}$  was reached at 12 mm depth in the agar pillar, suggesting the upper  $\sim 10$  mm of the agar pillar remain oxic. The EP measured on day 3 remained below 3 mV over all depths both in sediment and agar. After 28 days of incubation, sulfide profiles in streambed columns were markedly shifted.  $\sum\text{H}_2\text{S}$  concentrations started to increase to maximally  $4 \mu\text{M}$  only below 20 mm in sediments, while  $\sim 1.4 \mu\text{M}$  were present below 8 mm in the agar pillar. pH dropped from  $\sim 8.7$  to  $\sim 6.7$  at the oxic-anoxic interface and further decreased over depth with a steeper gradient in sediment. The start of the suboxic zone was at 3 mm depth in the sediment and at 6 mm in the agar pillar. An electric field was clearly present after 28 days, indicated by increasing electric potentials (starting at the oxic-anoxic interface) to  $\sim 17$  and  $\sim 14$  mV with depth in sediment and agar, respectively.

After 3 days, microprofiling of Alatsee sediments revealed an oxygen penetration depth not deeper than 5 mm in sediments, while oxygen penetrated up to 10 mm into the agar pillar (Fig. 3). Sulfide concentrations were near zero from the surface to a depth of 10 mm in the agar pillar, with the sulfidic zone starting below, while they increased from 0 to  $\sim 40 \mu\text{M}$  between 3 and  $\sim 15$  mm depth in sediments, but decreased again below. The pH profiles in the agar pillar and sediment resembled each other, reaching minimum values of  $\sim 7.2$  at 8 mm depth. After 28 days, the sulfide gradient was shifted downwards by  $>10$  mm (increasing below  $\sim 17$  mm depth) in the sediment, with a marked suboxic zone detectable between 5 and 17 mm depth, while the suboxic zone started at 7 mm depth in the agar, extending at least 8 mm below. A minimal pH of  $\sim 6.5$  was reached below 10 mm (agar) and 15 mm (sediment), respectively, and was slightly more acidic than at the start of the experiment.

Microprofiling of aquifer sediments showed negligible  $\sum\text{H}_2\text{S}$  concentrations over depth after 3 days (Fig. 4). pH dropped from  $\sim 8.3$  to  $\sim 7.5$  over the first 10 mm in both sediment and agar. Oxygen concentrations decreased steadily over depth and anoxic con-

ditions were reached at 8 mm in sediment and at  $\sim 10$  mm in the agar pillar. An electric field was not present, as indicated by EP values below 2 mV over depth. After 28 days of incubation,  $\sum\text{H}_2\text{S}$  concentrations remained below  $3 \mu\text{M}$  over depth both in sediment and agar, suggesting increasing sulfide concentrations to be found only below 30 mm sediment depth. pH in the oxic zone was  $\sim 8.4$  (sediment and agar) and dropped below the oxic/anoxic interphase to  $\sim 7.6$  (sediment at 20 mm) or  $\sim 7.9$  (agar at 10 mm). An electric field was detectable in the sediment, as indicated by an EP increase of  $\sim 4$  mV over depth. However, no electric field was present in the agar pillar.

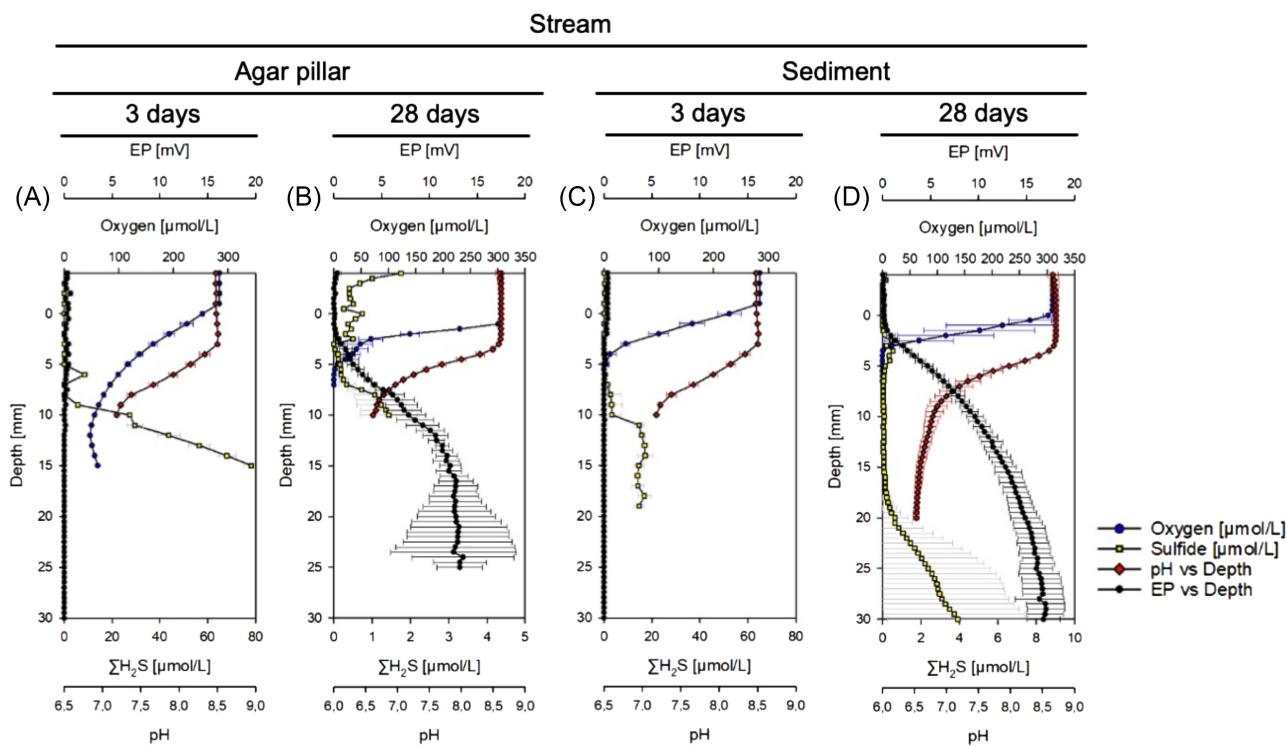
Comparing the three sites, a general observation was that oxygen penetrated deeper within the agar pillar than in the surrounding sediment, and that oxygen gradients were less steep in agar. The pH gradients in sediment and agar were mostly comparable. Geochemical profiles provided evidence for the establishment of a suboxic zone and the occurrence of LDET with time in all different column types, with interesting distinctions in patterns between the different locations. A characteristic pH peak as indicative for LDET by CB (Schauer et al. 2014) was observed at times in individual columns, but was largely hidden when averaged values were calculated for triplicate columns. Still, the marked shift of the sulfidic zone into deeper zones over time, along with the establishment of an electric field, was interpreted as a strong indication of e-SOx by CB.

### FISH and microscopy of filaments

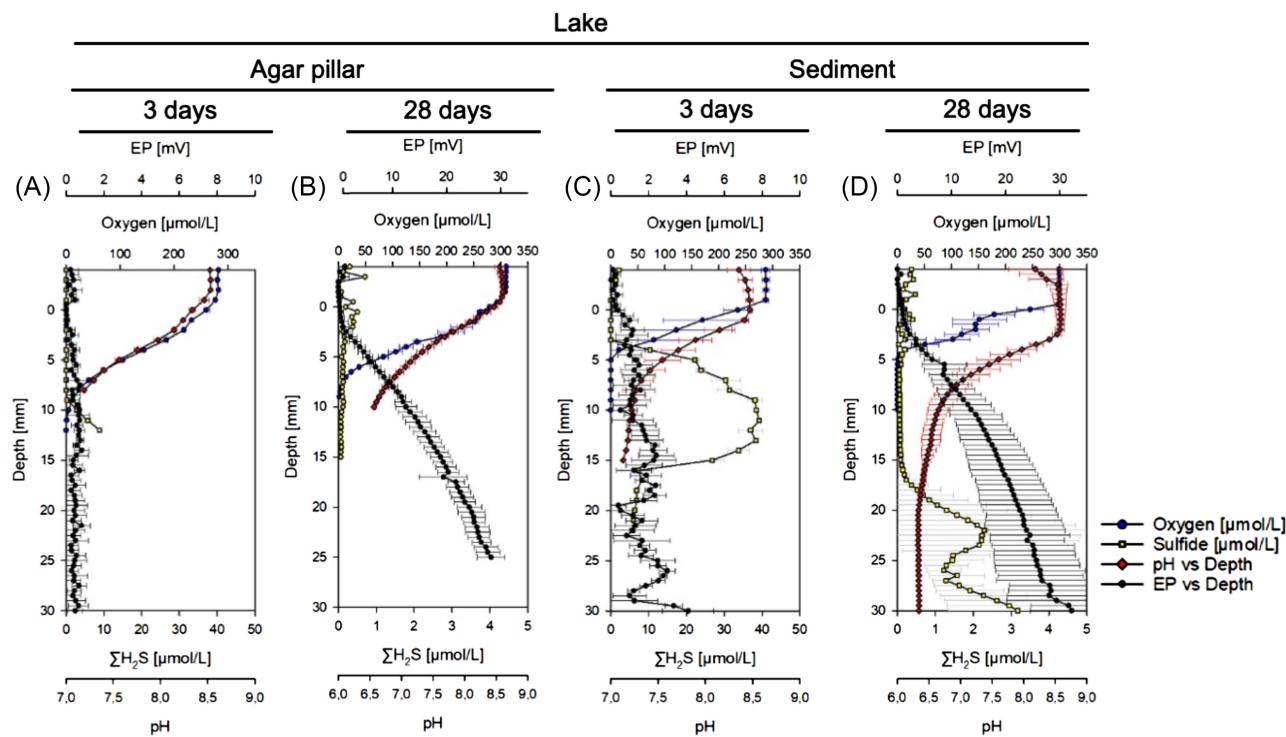
The presence of Desulfobulbaceae filaments in incubated sediment samples was determined by fluorescence in situ hybridization with probe Dsb706 (Loy et al. 2002) (Fig. 5). Indeed, bacterial filaments resembling typical freshwater CB and hybridizing with the Dsb706 probe were detected in column depths between 3 and 10 mm for all columns, suggesting the presence of CB within the Desulfobulbaceae family within all investigated sediments. For samples from agar pillars, filaments were also detected by FISH (shown for an Alatsee column in Fig. 5C).

### Near-full-length 16S rRNA gene amplicon sequencing

The bacterial community was investigated by PacBio SMRT sequencing of near-full-length 16S rRNA genes in sediment and agar pillars. Per sample, 1732–14 819 reads (after QC) with average read length  $>1450$  bp were obtained (for details see Table S1, Supporting Information). Communities clearly differed between sites, column depth and between sediment or agar. The initial bacterial community in the inoculum of the streambed sediment was characterized by a notable abundance of Gammaproteobacteria (22%), Alphaproteobacteria (9%), Deltaproteobacteria (6%), Firmicutes, Chloroflexi, Bacteroidetes, Actinobacteria, Acidobacteria and Planctomycetes (Fig. 6). After 28 days of column incubation, community profiles had clearly changed over depth. In the agar pillar, the uppermost oxic layer was dominated by Gammaproteobacteria (22%), amongst them members of the Rhodocyclaceae, Gallionellaceae and Burkholderiaceae. Below 3 mm (oxygen  $< 80 \mu\text{M}$ ), members of the Desulfobulbaceae appeared significantly enriched compared with the surrounding sediment (up to 8%, or  $\sim 10\times$  more;  $P = 0.006$ ), as well as the initial inoculum (up to 8%, or  $\sim 10\times$  more;  $P = 0.026$ ). Within these Desulfobulbaceae sequences, near-full-length amplicons also allowed for the unambiguous identification of sequences related to *Ca. Electronema* (up to 0.2% total abundance) (see below). *Ca. Electronema* were significantly enriched in the agar pillar compared



**Figure 2.** Geochemical depth profiles measured in column incubations of Garching streambed sediments: (A) agar pillar, 3 days; (B) agar pillar, 28 days; (C) sediment, 3 days; and (D) sediment, 28 days. Shown are means  $\pm$  standard deviations of triplicate profiles.

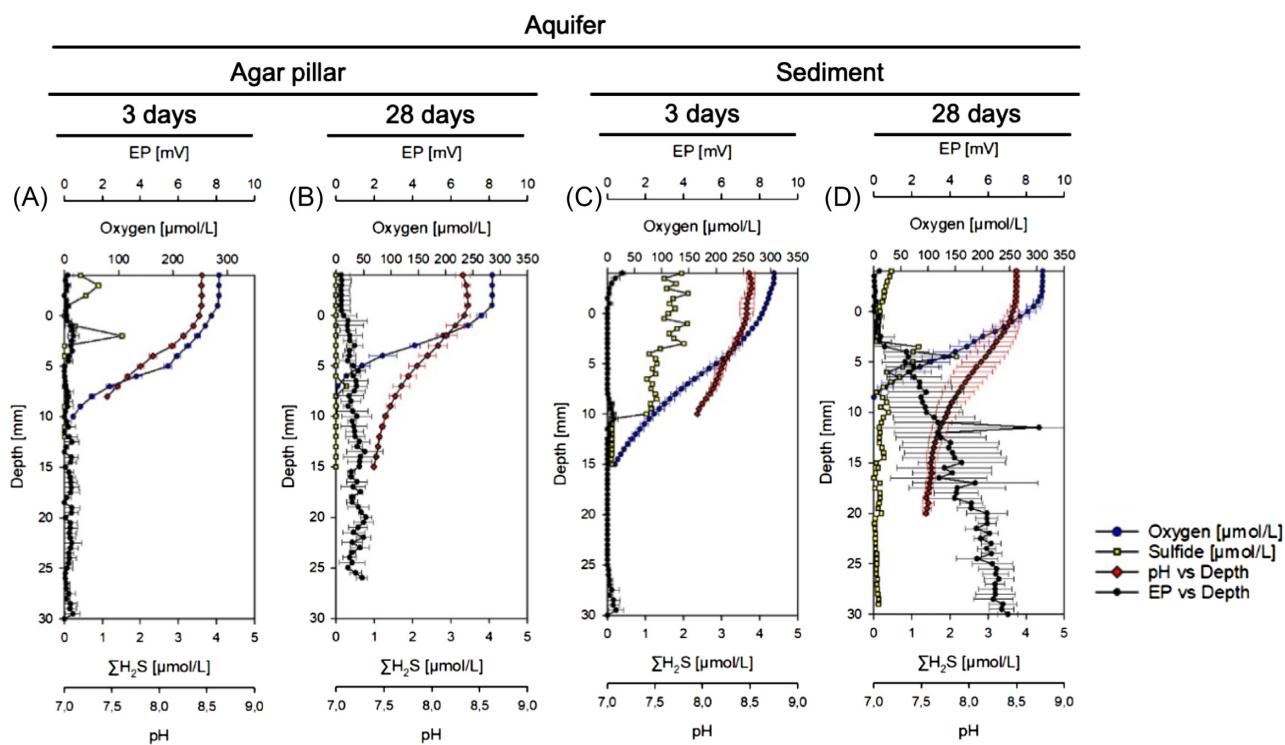


**Figure 3.** Geochemical depth profiles measured in column incubations of Lake Alatsee sediments. See legend of Fig. 2 for further details.

with the surrounding sediment (below 3 mm depth,  $\sim 10 \times$  enrichment,  $P = 0.041$ ).

The anoxic agar was dominated by members of the Bacteroidetes (up to 35%), Firmicutes (5–21%), but also Deltaproteobacteria (<10%) and Chloroflexi (<7%), whereas the uppermost

oxic sediment layer (0–3 mm) was mainly colonized by Alphaproteobacteria ( $\sim 30\%$ ), members of the Rhizobiales amongst them, Gammaproteobacteria (28%) and Chloroflexi (13%). The fraction of Alphaproteobacteria decreased over depth (to a minimum of 8%) while Bacteroidetes increased (up to 12%). Compared with the



**Figure 4.** Geochemical depth profiles measured in column incubations of Flingern aquifer sediments. See legend of Fig. 2 for further details.

agar, Firmicutes, Bacteroidetes and Spirochaetes were less abundant, while Alphaproteobacteria and Chloroflexi were more abundant in sediments. Distinctions in distribution over depth were not apparent.

In Lake Alatsee sediments (Fig. 7) the upper agar pillar was dominated by Alphaproteobacteria (mainly Beijerinckiaceae up to 23%) and Gammaproteobacteria (up to 54%). Below 30 mm, the community was more diverse with Gammaproteobacteria (mainly Aeromonadaceae, up to 20%), Firmicutes (up to 8%) and also Desulfobulbaceae (up to 2%) detected. Unfortunately, some agar pillar samples from Alatsee were degraded and overgrown with a black fungus after 28 days, so that DNA extraction failed and no amplicon data could be generated between 3 and 30 mm depth. Bacterial communities in sediments were rather similar over depth. They were dominated by Gammaproteobacteria (up to 36%) with mainly Burkholderiaceae and Rhodocyclaceae, while members of Alphaproteobacteria (up to 9%), Deltaproteobacteria (up to 12%, ~1% Desulfobulbaceae, including reads affiliated to *Ca. Electronema*, see Fig. 9), Chloroflexi (up to 5%), Bacteroidetes (up to 5%), Acidobacteria (up to 5%) were also present. The number of unclassified taxa was higher than for other examined sites (up to 30%).

The intrinsic microbiota of the investigated aquifer sediments was mainly characterized by Gammaproteobacteria (28%), Deltaproteobacteria (15%) and Epsilonproteobacteria (6%) (Fig. 8), comparable also to previous work at the site (Pilloni et al. 2019). After 28 days of incubation, the agar pillar was highly dominated by Gammaproteobacteria, with Burkholderiaceae and Rhodocyclaceae prominent (up to 63%) in the oxic zone (down to 10 mm) while Aeromonadaceae markedly increased in abundance over depth in deeper anoxic layers (up to 66% at 20–25 mm). A notable population of Desulfobacteraceae (15%) and Clostridiaceae (19%) was detectable in the agar pillar right below the oxic/anoxic interface (10 mm), but members of the Desulfobulbaceae were

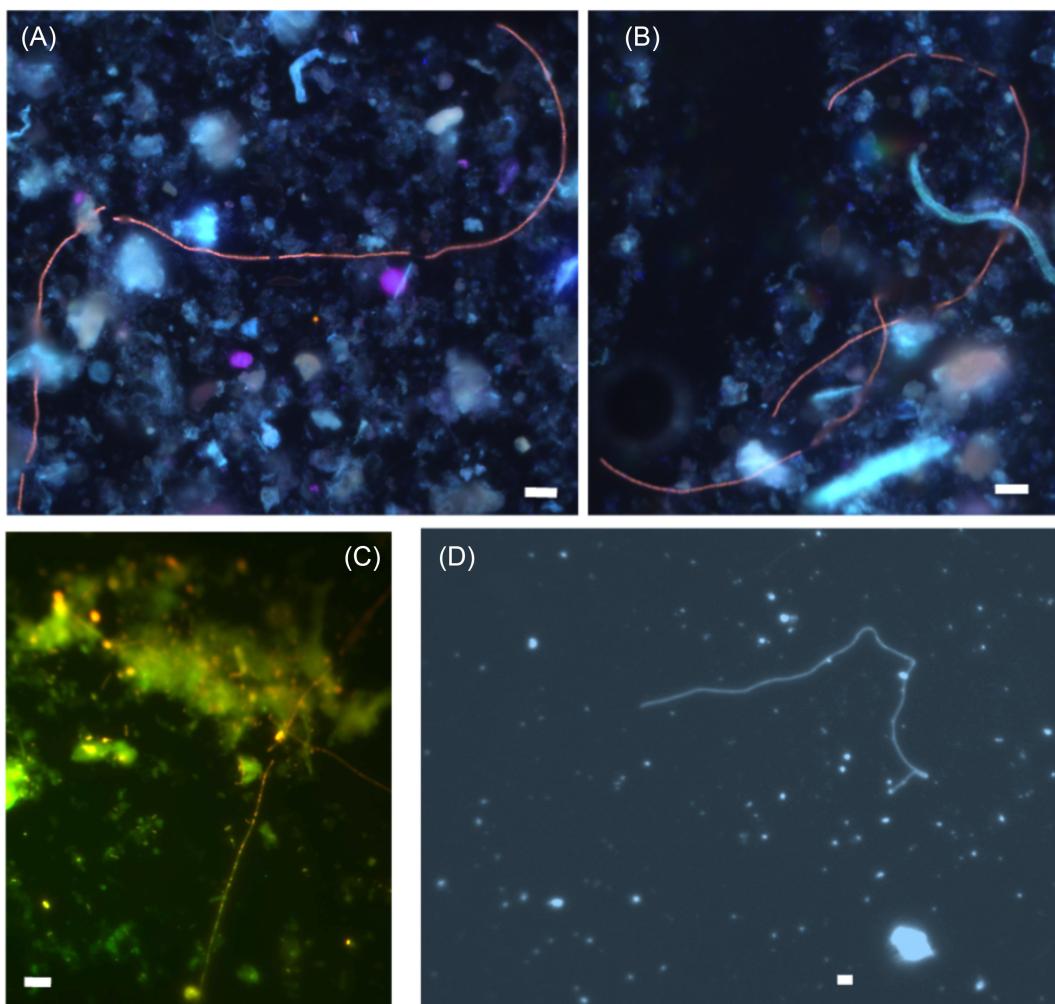
not abundant. Upper sediment depths were again dominated by Gammaproteobacteria; however, Sulfuricellaceae were especially enriched (up to 34%) down to 6 mm. Over depth, sequences related to Thiovulaceae and Desulfobulbaceae became constantly more abundant (up to 19% and 11%, respectively). As opposed to the agar, Firmicutes were not abundant in deeper sediment samples (<1%).

### Phylogenetic analysis of putative CB sequences

The generated near-full-length amplicons allowed for a robust phylogenetic placement of the obtained CB 16S rRNA gene sequences. For streambed and Lake Alatsee samples, three putative species-level clusters of freshwater CB sequences branched close to *Ca. Electronema palustris* (Fig. 9, groups 1, 2 and 3), while two putative species-level clusters branched closer to *Ca. Electronema nielsenii* and *Ca. Electronema aureum* (group 4 and 5). Sequences within these clusters showed maximum sequence similarities of ~94–95% to either *Ca. Electronema palustris* or *Ca. Electronema nielsenii*, all but for group 5, which was <~99% similar to *Ca. Electronema nielsenii* (Table S2, Supporting Information). For aquifer sediment, one cluster of groundwater CB sequences branched with previously described groundwater CB, as sister group to *Desulfurivibrio* spp. (Müller et al. 2016), while a second cluster found in sediment and agar was even more deeply branching (Fig. 9).

### Discussion

Here, we introduce a novel enrichment strategy for CB, in the form of a central agar pillar embedded within laboratory sediment gradient columns. Complemented by microsensor measurements and near-full-length 16S rRNA gene amplicon sequencing, we demonstrate the presence of LDET and diverse, potentially



**Figure 5.** Fluorescent microscopy of filamentous CB detected in sediment columns. **(A)** Micrograph of filaments from Garching streambed sediments. Filaments are visualized in an overlay of two images taken with filters for specific fluorescence of the DSB706 FISH probe (red) and DAPI counterstaining (blue). **(B)** Micrograph of filaments from Lake Alatsee sediments. Other details are as in panel (A). **(C)** Micrograph of filaments from Lake Alatsee agar pillar (6–10 mm depth). Filaments are visualized in an overlay of two images taken with filters for specific fluorescence of the DSB706 (red) and EUB I–III (green) FISH probes. **(D)** Micrograph of filaments from Flingern aquifer sediments. Filaments are visualized in images taken with filters for the DAPI fluorescence (blue).

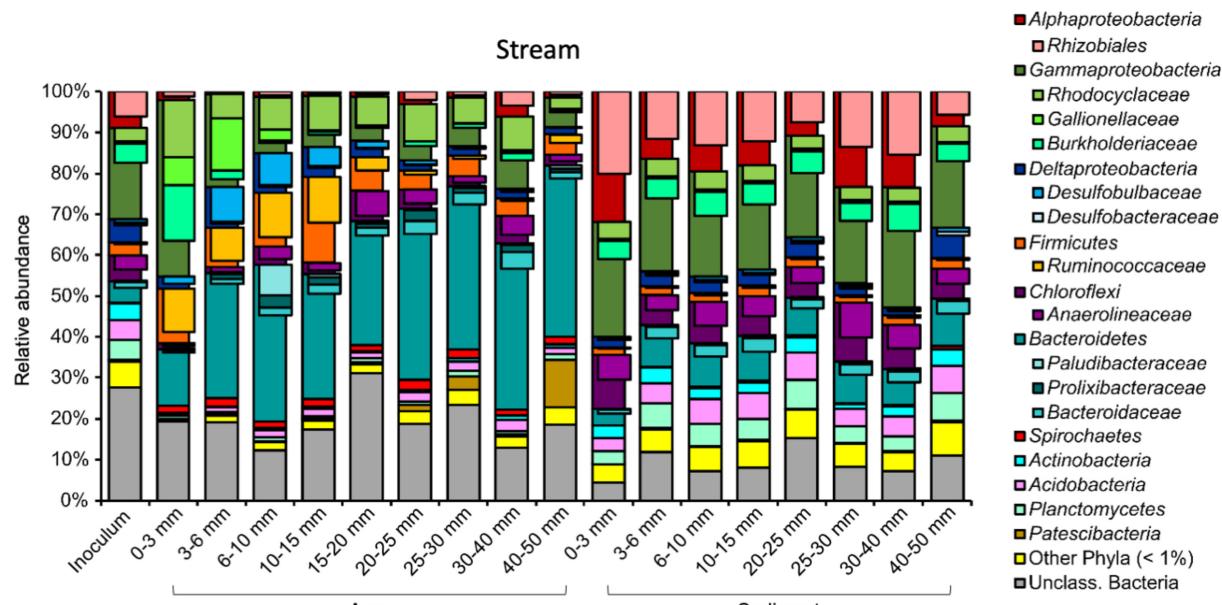
novel species-level populations of CB in the investigated freshwater sediments.

### Biogeochemistry and LDET in freshwater sediments

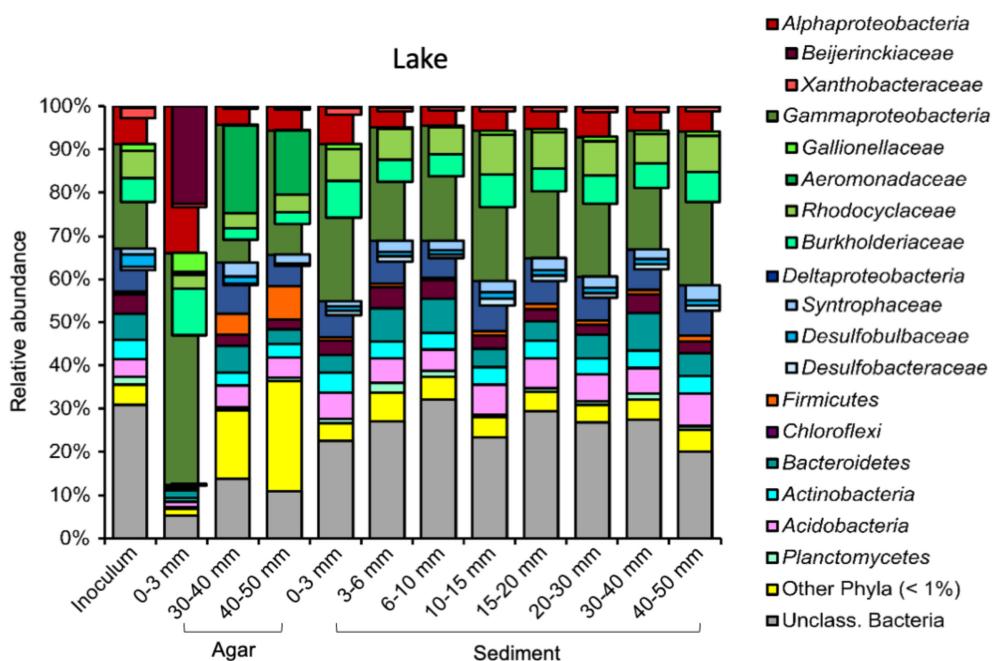
The results of our study demonstrate the potential for electrogenic processes and the presence of CB for a range of different freshwater habitats. The development of CB filaments within homogenized sediments in our study is comparable to time frames previously reported from marine sediment (Schauer et al. 2014). Most distinctively, we observed biogeochemical patterns of LDET in the investigated streambed and lake sediments. Both sediments showed characteristic counter-gradients of oxygen and sulfide separated by a pronounced suboxic zone after incubation, along with a marked rise in EP over depth (Figs 2 and 3). The EP increase of 10–15 mV in streambed columns (sediment and agar) and of up to 30 mV in Alatsee columns (sediment and agar) after 28 days of incubation was in a similar range as previously reported for creek sediments with active freshwater CB (Risgaard-Petersen et al. 2015).

CB can be quite abundant in organic-rich marine sediments, where they spatially separate redox half reactions by acting as living electron-conducting cables between the sulfidic and oxic zones (Nielsen et al. 2010, Pfeffer et al. 2012). Freshwater sediments are generally characterized by not only lower salt but also much lower sulfide concentrations. The prompt detectability of LDET fuelled by sulfide oxidation in the sediments (albeit FeS-amended) investigated here could indicate a continuous and rapid recycling of different sulfur species at low concentrations *in situ*. Such ‘cryptic sulfur cycling’ (Holmkvist et al. 2011) has been described for marine habitats and may play an important, possibly underestimated role also in freshwater habitats (Pester et al. 2012), especially in the presence of CB (Sandfeld et al. 2020).

In the investigated streambed sediments, an electrogenic microbial community clearly developed over incubation, even more pronounced within the agar pillar. While the relative abundance of Desulfobulbaceae in sediment after incubation was comparable to their initial abundance (0.7–0.9%), a notable enrichment of Desulfobulbaceae (up to 8% of total 16S reads) was detected right below the oxic surface (at 3–6 mm depth) in the agar pillar. Within these Desulfobulbaceae sequences, a small fraction of sequences



**Figure 6.** Relative sequence abundance for bacterial taxa obtained by amplicon sequencing of samples from Garching streambed sediment column after 28 days of incubation. All phyla with average relative sequence abundance >1% in at least one of the libraries are shown. Selected abundant subphylum taxa are highlighted.

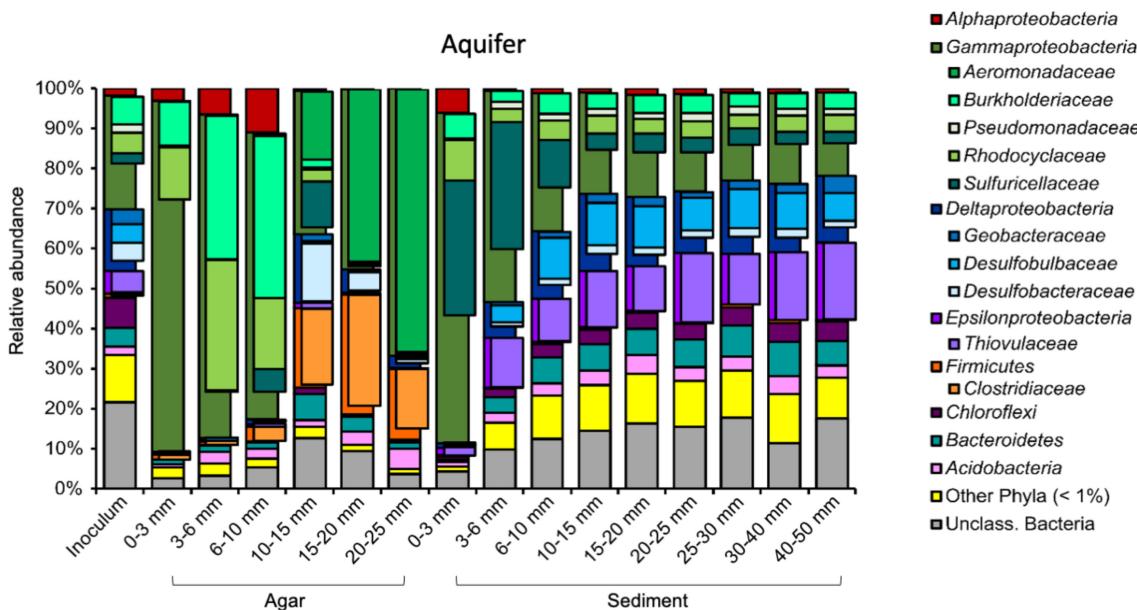


**Figure 7.** Relative sequence abundance for bacterial taxa obtained by amplicon sequencing of samples from Lake Alatsee sediment column after 28 days of incubation. See legend of Fig. 6 for further details.

affiliated with *Ca. Electronema* (~0.2% total abundance) was also significantly enriched (~10-fold) within the agar pillar, compared with the surrounding sediment. For the investigated streambed sediments, the agar pillar was thus proven as a selective niche for CB enrichment, possibly serving as a viable strategy for the further cultivation and isolation of CB from these and other sediment inocula.

Lake Alatsee is a stratified lake with a sulfide-rich monimolimnion and dense seasonal populations of phototrophic sulfur-oxidizing bacteria (Oikonomou et al. 2015). Our gradient col-

umn data indicate that chemolithoautotrophic sulfur oxidation via e-SOx can also occur in Lake Alatsee sediments. Consistent with its sulfidic nature (Fritz et al. 2012), we observed a high initial abundance of Desulfobulbaceae (~2.7%) in fresh Alatsee sediments. After column incubation, Desulfobulbaceae showed an average abundance of ~1.2% in sediments without any notable pattern of depth distribution. Unfortunately, several important depth sections of the agar pillar (3–30 mm) were lost during sampling due to disintegration of the agar. Still, based on the increase in EP, which was highest amongst all samples investigated, we presume



**Figure 8.** Relative sequence abundance for bacterial taxa obtained by amplicon sequencing of samples from Flingern aquifer sediment column after 28 days of incubation. See legend of Fig. 6 for further details.

that e-SOX and the responsible CB populations must have been highly active in the Alatsee agar pillars.

In contrast, incubations of contaminated aquifer sediments showed a very distinct microbial community pattern. The EP was lower and apparently only found within sediments (up to 6 mV), but not within the agar pillar. This observation coincided with the absence of any Desulfobulbaceae from the agar pillar, while they were most abundant (~10% in average) below a depth of 6 mm in these sediments compared with the surface sediment samples. This further adds to the need for a more careful differentiation between groundwater and surface freshwater CB, as discussed later.

### Selective niche of the agar pillar

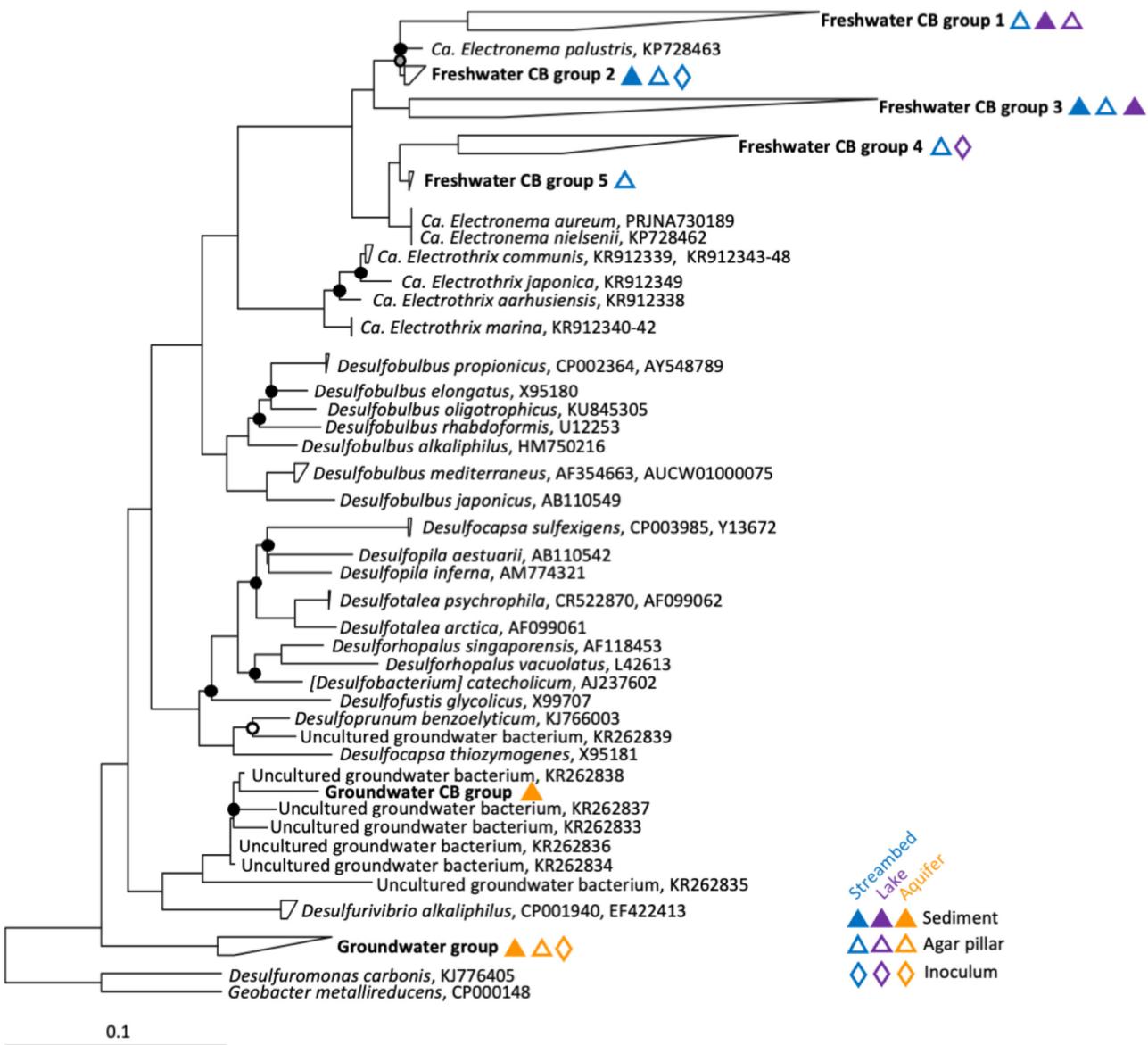
The findings of our study demonstrate that the 'agar pillar' approach is a viable strategy to enrich for CB from the investigated freshwater sediments. Microbial colonization of the initially sterile agar pillar must have occurred laterally from the homogenized sediments. We assumed the gel-like agar to promote the growth of CB filaments and their movement into and within the agar medium. The previously reported gliding motility of CB filaments (Bjerg et al. 2016) might be more viable in the agar pillar compared with more particle-rich sediments. Over incubation time, the agar pillars equilibrated with the surrounding pore water chemistry, as supported by mostly consistent microprofiles of H<sub>2</sub>S, pH and EP after column set-up (Figs 2–4). However, oxygen penetrated several millimetres deeper within the agar. Growing within the agar pillar could thus offer CB filaments the advantage of populating a more extensive zone with reduced oxygen availability over depth (5–8 mm in agar vs 3–4 mm in lake and streambed sediment). Cathodic cells of CB filaments are proposed to use oxygen as electron sink alone, rather than conserving energy via aerobic respiration (Kjeldsen et al. 2019). Consistent with the previous findings that cathodic cells within CB filaments are positioned at <14% air saturation (Scilipoti et al. 2021), we only observed CB several millimetres below the oxic surface (>3 mm depth). In contrast to surface freshwater sediments, CB from groundwater sediments were not

enriched in the selective niche of the agar pillar. Again, this substantiates the need to discern surface water and groundwater CB, likely exhibiting distinct physiologies and possibly also motility (Müller et al. 2019).

### Phylogenetic placement of terrestrial CB

Within the two genus-level branches of CB separating the marine *Ca. Electrothrix* and the freshwater *Ca. Electronema* genera (Trojan et al. 2016), sequences from Lake Alatsee and streambed sediments clearly clustered within the freshwater CB branch (Fig. 9). However, 16S rRNA gene sequence identity to the proposed species *Ca. Electronema nielsenii* and *Electronema palustris* was below the 98.7% sequence identity threshold proposed for species-level differentiation (Stackebrandt and Ebers 2006) for freshwater CB groups 1, 3 and 5 (Fig. 9; Table S2, Supporting Information), and thus could represent new species-level taxonomic units of freshwater CB within the *Ca. Electronema* genus.

For the investigated aquifer sediments, the presence of filamentous Desulfobulbaceae was previously established. Here, the generated 16S rRNA sequences revealed two distinct clusters, one as a sister group to *Desulfurivibrio alkaliphilus* affiliated with previous putative groundwater CB (Müller et al. 2016) and one even more basal within the Desulfobulbaceae. The similarity of the generated near-full-length 16S rRNA sequences of the groundwater CB group (Fig. 9) to that of other CB was <88%. Moreover, while groundwater CB were present in our sediment columns, they were not enriched in the agar pillar. This further supports the notion that the ecophysiology of groundwater CB may be distinct from that of CB found in surface water sediments. Groundwater CB have been suggested to gain energy via sulfur disproportionation and can also grow independently of redox gradients (Müller et al. 2019). Thus, the selective niche of the agar pillar may not be equally suited for the enrichment of such subsurface CB populations. Still, as an EP increased and filamentous Desulfobulbaceae were apparent in aquifer sediments in our columns over time, this work substantiates that also groundwater CB might actually be capable of LDET.



**Figure 9.** Phylogenetic placement of full-length 16S rRNA gene reads of presumed species-level CB groups reported in this study (in bold) within the Desulfobulbaceae. The accession numbers of selected sequence entries are given. The tree is rooted with outgroup sequences of *Geobacter metallireducens* and *Desulfuromonas carbonis*. Branching points without symbol indicate bootstrap values >99. Black circles: bootstrap support >90; grey circles: bootstrap support >70; and open circles: bootstrap support >55 ( $n = 1000$ ). The scale bar shows 10% distance.

### CB- and redox gradient-associated microbiomes

In column sediments and especially also in the agar pillars, the Desulfobulbaceae detected in our study were accompanied by distinctive associated microbiomes. It is hypothesized that other microbes can access CB filaments as an electron sink under electron acceptor limitation (Vasquez-Cardenas et al. 2015). However, specific interactions such as direct interspecies electron transfer between CB and other microbes have not yet been proven. The observed enrichment of CB together with putative fermenters and other sulfide-adapted microbes leads us to assume that the agar pillar offers conditions that promote such interactions between those groups of microorganisms. Members of the phylum Firmicutes, including Ruminococcaceae (especially for streambed sediments) and other Clostridiaceae (for aquifer sediments), were enriched in agar pillar samples compared with the surrounding sediment. For streambed sediments, enriched taxa also included

members of the Bacteroidetes and Spirochaetes, many also typical fermenters (Güllert et al. 2016). Members of the Aeromonadaceae, which are typically nonfermentative, were most abundant for the Alatsee anoxic agar pillar, and have been shown to be capable of extracellular electron transfer (Conley et al. 2018).

Although electron transfer from other microbes to CB has not been experimentally proven so far, it may be a viable strategy for electroactive bacteria to access CB filaments as an electron sink. Fermenters may energetically benefit from the presence of a conductive, electron-accepting structure. For example, the need for an electron sink to overcome unbalanced fermentations can be satisfied in electrofermentations by utilizing an electrode (Moscoviz et al. 2016, Vassilev et al. 2021). In anaerobic digestion systems, an increase in redox mediator-secreting bacteria has been observed (Guo et al. 2020) and the growth of *Clostridium aceto-butylicum* and *Desulfovibrio vulgaris* in coculture has been described

to involve biological nanotubes (Benomar et al. 2015). Moreover, the abundance of Clostridia coincided with the presence of CB in polluted river sediments (Liu et al. 2021). A recent study with polluted marine sediments (Marzocchi et al. 2020) showed that CB filaments can enhance hydrocarbon degradation, potentially serving as living electrodes for pollutant degraders. Our study extends this perspective also for fermenters and CB in surface water sediments.

## Conclusions

We report clear signatures of LDET and respective CB populations in all of the three investigated freshwater sediments. These include biogeochemical evidence, such as the extension of a suboxic zone during incubation, the development of a measurable EP, the microscopic visualization of CB filaments and genus-level affiliation of near-full-length 16S rRNA gene sequences. The agar pillar is introduced as a selective niche preferentially colonized by freshwater CB from surface water sediments. Potentially, it could serve as a viable strategy for the enrichment of CB also from other, e.g. marine, habitats. For all sites, abundance patterns of Desulfobulbaceae CB were seemingly linked to the presence of fermenters, suggesting possible interactions of e-SOx populations with heterotrophic microbes. Lastly, utilizing the phylogenetic precision of near-full-length 16S rRNA gene amplicon sequencing, we were able to show that CB from the investigated surface water sediments, placed within the freshwater *Ca. Electronema* spp., were phylogenetically distinct and could represent new species-level taxa within that genus. These findings add to our current understanding of occurrence and ecology of CB in freshwater systems.

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## Supplementary data

Supplementary data are available at [FEMSEC](#) online.

## Author contributions

CS, DK and TL designed the experiments. CS executed the experiments, with specific contributions from DK, SK, MS, AS and LRD. CS analysed the data, with particular contributions from DK, SK and ARS. CS and TL wrote the manuscript with additions and revisions contributed from all authors.

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**Conflict of interest statement.** None declared.

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