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Influence of Nutrient Concentrations on MPN Quantification and Enrichment of Nitrate-Reducing Fe(II)-Oxidizing and Fe(III)-Reducing Bacteria from Littoral Freshwater Lake Sediments

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The application of culture-dependent studies to quantify Fe-metabolizing microorganisms from the environment is a necessity, as there are so far no universal functional marker genes for application in culture-independent studies. Media composition can vary between studies, therefore, we determined the effects of three different growth media on the quantification (MPNs) and identity (via cloning and sequencing of dominant DGGE bands) of nitrate-reducing Fe(II)-oxidizers and lactate- or acetate-oxidizing Fe(III)-reducers from a lacustrine sediment: low sulphate freshwater medium (FWM), sterile filtered bicarbonate-buffered lake water (BLW) and a mixture of both (MIX). We consistently found fewer cells in the BLW than in the FWM and the MIX. The DGGE banding patterns of the microbial communities enriched in different media types clustered together according to the e^- donor and acceptor couples and not according to the medium used. Thus, although the medium composition significantly influenced the quantification and thereby conclusions on the abundance and potential significance of the targeted group within the ecosystem, biodiversity assessments through enrichment cultures were less influenced by the medium, but instead were affected by the type and concentration of the e^- donor/acceptor.

Keywords: ferric iron, ferrous iron, Lake Constance, microbial identity

Introduction

Iron is the fourth-most abundant element in the Earth's crust (Taylor and McLennon 1985) and the most abundant redox active transition metal in soils and sediments. The two most-abundant environmental redox states are the reduced ferrous (Fe(II)) and the oxidized ferric iron (Fe(III)) species. Ferrous iron is stable in the absence of O_2 and relatively soluble at acidic and neutral pH, while in the presence of molecular oxygen it is rapidly oxidized abiotically at neutral pH (Stumm and Morgan 1996; Tamura et al. 1976) or very high HCl concentrations (Porsch and Kappler 2011).

Microaerophilic iron(II)-oxidizing microbes can oxidize ferrous iron at neutral pH in microoxic environments (Edwards et al. 2003; Emerson and Moyer 1997; Kucera and Wolfe 1957). Under anoxic conditions, phototrophs are able to couple light energy to iron(II) oxidation for ATP production and carbon fixation (Ehrenreich and Widdel 1994; Widdel et al. 1993). Mixotrophic nitrate-reducing iron (II)-oxidizers are also able to couple iron oxidation to their metabolism, either through enzymatic oxidation of the ferrous iron (Chakraborty and Picardal 2013; Straub et al. 1996), or through the abiotic oxidation of iron by nitrite that can be produced during heterotrophic denitrification (Klueglein and Kappler 2013).

In its oxidized form, ferric iron is poorly soluble in solutions of neutral pH and rapidly precipitates as iron(III) oxyhydroxide mineral (Cornell and Schwertmann 2003). Ferric iron can in turn be re-reduced to ferrous iron through abiotic processes (Roden et al. 2010) or through microbial iron(III) reduction (Lovley and Phillips 1988). The microbially mediated iron cycle is widely studied and reviewed (Kappler and Straub 2005; Kendal et al. 2012; Konhauser et al. 2011, Weber et al. 2006) due to its link to many other pivotal biogeochemical cycles such as the carbon (Lovley and Phillips 1988; Thamdrup 2000) and nitrogen cycle (Chakraborty et al. 2011; Kappler et al. 2005; Straub et al. 1996). In addition, iron minerals play a large role in environmental nutrient and contaminant retention and mobility through their redox active surface areas (Borch et al. 2010; Vaughan and Lloyd 2011).

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Culture independent molecular techniques often unveil species not obtained in culture before and as a result, many genera lack a cultured representative species (Hugenholtz et al. 1998). As the genes for anaerobic nitrate-dependent iron(II) oxidation are unknown and the genes for microbial iron(III) reduction are extremely diverse, the use of molecular probes against specific functional markers genes for these metabolisms is currently not feasible. Therefore, the identification and quantification of putative iron(II)-oxidizers or iron(III)-reducers in these studies are currently to large extent done through their 16S rRNA gene sequence similarity with known iron-metabolizers, identified in culture-dependent studies. Therefore, the application of culture-dependent studies to Fe-metabolizing microorganisms is a necessity, in order to isolate and quantify relevant players from environmental samples.

Successful culture medium should represent an artificial environment that reflects the actual environmental geochemical conditions as closely as possible. Nevertheless, dating all the way back to the great plate count anomaly (Staley and Konopka 1985), it is known that not all bacteria are culturable in the laboratory (Puspita et al. 2012; Zengler 2009). In fact, the culture efficiency of lacustrine bacteria has been estimated to vary between 0.1 and 1.3% (Tamaki et al. 2005) and represents a considerable challenge for environmental microbiologists. Since the discovery of anaerobic nitrate-reducing iron(II)-oxidizers (Straub et al. 1996), mineral medium (Ehrenreich and Widdel 1994; Widdel and Bak 1992), and slight modifications thereof (Hegler et al. 2008), is widely used for their quantification, isolation and characterization (Hauck et al. 2001; Kappler et al. 2005; Kappler et al. 2010; Muehe et al. 2009; Straub and Buchholz-Cleven 1998).

It has even been shown that all anaerobic neutrophilic iron-metabolizing groups (phototrophic and nitrate-reducing Fe(II)-oxidizers and organoheterotrophic Fe(III)-reducers) are able to grow on the modified mineral medium and thus it has been used in diverse studies involving the cultivation of iron-metabolising bacteria (Hegler et al. 2008; Klueglein et al. 2013; Larese Casanova et al. 2010; Pantke et al. 2012; Piepenbrock et al. 2011). Fe(III)-reducers in the *Geobacter* genus were originally enriched and isolated on freshwater medium (FEM) (Lovley and Phillips 1986), which they and others have used in ensuing studies of these Fe(III)-reducers (Lovley and Phillips 1988; Roden et al. 2010).

Fe(III)-reducers within the *Shewanella* genus were first cultured on iron(III) using a medium defined by Fredrickson and colleagues (Fredrickson et al. 1998), which has also been used in many subsequent studies (Glasauer et al. 2002; Hansel et al. 2003). Besides the relative conservation of medium composition and nutrient concentrations, the added vitamin (Widdel and Pfennig 1981), selenite tungstate (Widdel and Bak 1992) and trace element solution (Tschech and Pfennig 1984) also remain largely unchanged.

The absence of a mixture of complex organic molecules in culture media is not thought to inhibit growth, on the contrary, it is thought that an excess of nutrients and growth substrates, often provided in artificial culture medium, hinders the growth of the majority of indigenous microbial species (Aagot et al. 2001; Zengler 2009). It has been shown that the quantification of certain organic metabolisms by the MPN method is affected by the dilution factor of the nutrients in the medium (Bussmann et al. 2001).

However, it is unknown so far what the effect of medium, and more specifically nutrient salt composition, is on the quantification and identity of iron-metabolizing enrichments. Therefore, the goal of this study was to determine the variance within the quantification of lacustrine sedimentary neutrophilic anaerobic iron(II)-oxidizing and iron(III)-reducing microorganisms, first using the established FWM, second, sterilized buffered lake water (BLW) and third, a mixture of the first two media types (MIX). In addition, enrichments were set up using these three media types to determine whether the diversity of the iron-metabolizing communities in the enrichments would be the same or media dependent. In addition to understanding the effects of media composition on bacterial enrichments, this study illustrates how the enrichment or quantification method can influence bacterial biodiversity and ecosystem assessments in general.

Materials and Methods

Sampling Site and Sampling Procedure

Environmental samples were taken in March 2012 from the littoral zone of the Überlingersee in Lake Constance, a freshwater lake in the South of Germany on the border to Switzerland, at 47°41′42.63″N and 9°11′40.29″E. Littoral water samples were taken from surficial water in Nalgene PE bottles, by filling them completely and closing them submerged underwater excluding a gaseous headspace, push-cores were taken from the sediments at 0.65 m water depth with cylindrical polypropylene tubes. The samples were transported to the laboratory in Tübingen at 4°C and the sediment cores were processed immediately for microelectrode analysis, necessary to define the most suitable sediment layer beneath the oxygen penetration depth for inoculation of the most probably number studies (MPNs) and enrichment incubations.

Microelectrode Measurements

A high-resolution oxygen microelectrode profile was taken from an intact push-core immediately upon arrival in the laboratory with a Unisense Clark-type oxygen microelectrode with a tip diameter of 100 μm. The electrode was two-point-calibrated in air-saturated water and anoxic water. The detection limit was 0.3 $\mu mol \cdot L^{-1}$. Measurements were taken with a micromanipulator at depth intervals of 500 μm.

Analytical Methods

The water content of the sediment was determined in triplicate by weighing portions of wet sediment, drying them for 4 days at 90°C and subsequently determining the dry weight. The dissolved organic carbon (DOC) content and total inorganic carbon (TIC) content of the pore water were determined from a centrifuged portion of wet sediment, of which the supernatant was subsequently filtered with a 0.45-μm

filter (mixed esters of cellulose nitrate and acetate membrane). The DOC and TIC of the water overlying the sediment were also determined from filtered water. Samples were then analyzed in a High TOC Elementar instrument. In addition, the overlying water was analyzed for dissolved cation and anion concentrations by ion chromatography (Dionex DX-120 and the AS40 Automated Sampler). The specifications for the used anion column were Dionex Ion Pac AS23 with a dimension of 4 × 250 mm and the guard column was a Dionex Ion Pac AG23 both by Thermo Scientific. The cation column used was a Dionex Ion Pac CS12A 5 μm with a dimension of 3×150 mm and the guard column was a Dionex Ion Pac CG12A 5 μm, also both from Thermo Scientific.

Medium Preparation

Freshwater medium was prepared sterile and anoxically with MQ water containing 0.6 g KH_2PO_4, 0.3 g NH_4Cl, 0.025 g $MgSO_4 \cdot H_2O$, 0.4 g $MgCl_2 \cdot 6\ H_2O$ and 0.1 g $CaCl \cdot 2\ H_2O$. The medium was buffered with 22 mM bicarbonate buffer and a 7 vitamin solution, trace elements and selenite tungstate solution were added and the pH was adjusted to 7.10 (Ehrenreich and Widdel 1994). Water samples from Lake Constance were transferred into 1 L Schott bottles and purged with N_2 gas under constant stirring for 1 h in order to become anoxic. Subsequently they were sterile filtered (mixed cellulose ester membrane filter (Fisher Scientific)) in the glovebox under N_2 atmosphere, into sterile Schott bottles, bicarbonate buffer was added to a concentration of 22 mM, and the pH was adjusted to circumneutral (pH 7.1).

The bottles were stoppered with a butyl rubber stopper and the headspace exchanged for N_2CO_2 gas (90:10). A mix of the freshwater medium and lake water was prepared by adding sterile anoxic lake water to the above described medium salts, in the same ratio. For the mixture the media salts were diluted in 100 ml MQ water and autoclaved in an anoxic Schott bottle. The Widdel flask and bicarbonate buffer were autoclaved separately. After autoclaving, the Widdel flask was attached to an N_2:CO_2 (90:10) gasline and the medium salts, buffer and sterile filtered anoxic lake water were added. The mixture also contained a 1 ml$\cdot L^{-1}$ seven-vitamin solution, trace elements and selenite tungstate solution (Ehrenreich and Widdel 1994).

Most Probable Number Method (MPNs)

A slice of 5-mm sediment right below the oxygen penetration depth was sampled (4–8 mm depth defined by microelectrode measurements) using a subcore slicer (Gerhardt et al. 2005) and homogenized inside an anoxic glovebox. One ml of wet sediment was inoculated anoxically into tubes containing 9 ml of freshwater medium (Figure 1). From this tube a 10-fold dilution series into subsequent medium tubes was prepared anoxically (10^{-1}–10^{-12}). A dilution series was prepared in freshwater medium (FWM), buffered lake water (BLW) and in the mixture of lake water and medium (MIX).

The dilution series were inoculated into 96-well (1 ml volume each) deep well plates with the three corresponding media types and screened after 8 weeks of incubation (dark and 23°C) for mixotrophic nitrate-reducing iron(II)-oxidizers (22 mM bicarbonate buffered medium + 10 mM Fe^{2+}, 4 mM NO_3^- and 0.5 mM acetate), acetate-oxidizing iron (III)-reducers (5 mM ferrihydrite (prepared as in Cornell and Schwertmann 2003) and 5 mM acetate) and lactate-oxidizing iron(III)-reducers (5 mM ferrihydrite (prepared as in Cornell and Schwertmann 2003) and 5 mM lactate). The deep well plates were incubated anoxically in the dark at 23°C for 8 weeks. Positive wells were determined based on the spectrophotometric ferrozine assay (50 μl sample and 50 μl 1 M HCl for Fe^{2+} determination or 50 μl HAHCl for total Fe determination, and 100 μl 0.1% w/v ferrozine) (Stookey 1970) in a Flashscan 550 micro-plate reader, Analytik Jena AG, Germany with a 5% internal error. These results were then analyzed with the software program KLEE (Klee 1993).

Bacterial Fe-metabolizing Enrichments

Parallel to MPN quantifications, three parallel enrichment series for each metabolic group were set up in dilution series (10^{-1}–10^{-5}) tubes of each medium type with a 10% homogenized sediment inoculum from below the oxygen penetration depth and incubated in the dark at 23°C (Figure 1). Over time, anoxic enrichments containing ferrihydrite and the electron donor acetate or lactate were initially brick red which gradually changed to black during incubation. Anoxic enrichments containing Fe(II) as electron donor and nitrate as electron acceptor gradually changed color from colorless to green to orange. For all enrichment types the highest dilution to show growth, judged by the color, was transferred into a new dilution series (at time intervals of approximately 1–2 weeks). After approximately 10 transfers through dilution series, the enrichments were observed under the light microscope, and based on morphology uniformity, the enrichments were transferred to agar and gelrite shakes (Figure 1).

Agar and Gelrite Shakes

Agar and gelrite shakes were prepared following a slightly modified protocol from Pfennig and Trüper (1981). In addition to using 3% agar as a gelling agent in agar tubes, we also used gelrite (1%) in the separate gelrite tubes. The advantage of using gelrite as a gelling agent in agar tubes instead of agar was that the bacterial colonies were optically clearly visible and thus the colonies were more conducive to being photographed. In addition, gelrite is a stronger gelling agent than agar, which facilitated the picking of isolated colonies for transfer into subsequent medium. All the tubes were buffered with 30 mM MOPS buffer. Two parallel set-ups contained FWM or the MIX with substrates for nitrate-reducing iron (II)-oxidizers, acetate-oxidizing iron(III)-reducers and lactate-oxidizing iron(III)-reducers as in the MPNs. Dilution series were inoculated (10^{-1}–10^{-5}) after which the tubes were solidified on ice and incubated at 23°C in the dark. After visual colony formation, colonies were picked and transferred into new medium in 50 ml serum bottles. Images of the colonies were taken with a binocular light microscope.

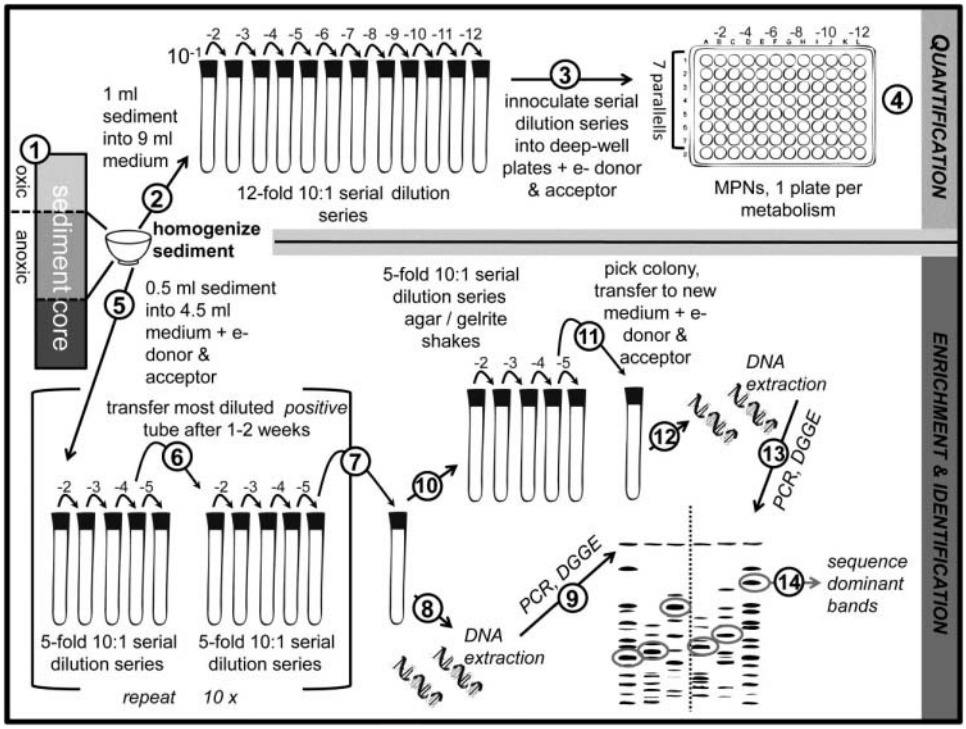

Fig. 1. Schematic representation of the experiments for quantification and identification of Fe-metabolizing microorganisms performed with each medium type (thus the above scheme was performed three times, once for BLW, once for FWM and once for the MIX medium).

1. The oxygen penetration depth of the sediment core was determined using microelectrode analysis. The sediment layer right beneath the oxygen penetration depth was cut from the core with a core slicer (Gerhardt et al. 2005) and homogenized anoxically.
2. First, 1 ml of the homogenized sediment was inoculated into a 12-fold serial dilution series containing only medium, no electron donors or acceptors.
3. The dilution series was inoculated into deep well plates for MPN analyses. Three deep-well plates were necessary for each media type, as we investigated three metabolisms: nitrate-reducing Fe(II)-oxidizers, acetate-oxidizing Fe(III)-reducers and lactate-oxidizing Fe(III)-reducers. The deep well plates contained the necessary electron donors and acceptors.
4. The deep well plates were incubated in the dark at 23°C for 8 weeks. Positive wells were optically identified and the results for the quantification were processed with the software program KLEE. The results are shown in Figure 3.
5. In parallel to, but independently from, MPN inoculations enrichments for the three investigated iron-metabolisms were set up. For this purpose, three series of 5-fold dilution series were set-up (each containing the necessary electron donor and acceptor) and incubated in the dark at 23°C for 1–2 weeks.
6. The most diluted positive tube was transferred to a new 5-fold dilution series. This process was repeated for approximately 10 transfers into a new dilution series.
7. After approximately 10 transfers, the most diluted tube to show growth in the series was selected as the "end stage enrichment" judged by morphology uniformity and dead/live staining under a light microscope.
8. From the end stage enrichment tube a 1-ml sample was taken for DNA extraction.
9. PCR and DGGE analyses to investigate the community composition.
10. From the same end stage enrichment tube a 0.5 ml sample was inoculated into agar and gelrite shakes (5-fold serial dilution series).
11. After 3 weeks of incubation in the dark at 23°C colonies were picked from the highest dilution and re-suspended in medium with the appropriate electron donor and acceptor.
12. A 1 ml sample was taken from the transferred agar and gelrite shakes from which DNA extractions were performed.
13. PCR and DGGE from the extracted gelrite and agar shake DNA yielded a distinct banding pattern on a DGGE gel.
14. The DGGE gel contains information on the community composition of the end stage enrichments and the gelrite/agar shakes. The most dominant bands from the DGGE band patterns were cloned and sequenced and compared to published sequences in the NCBI database to identify the most closely related bacterial species.

Molecular Analyses

DNA was extracted from 1 ml samples from the end-stage enrichment tubes (before transfer into agar and gelrite shakes) and agar and gelrite shakes with an ultra-clean microbial DNA isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) (Figure 1). Polymerase chain reactions (PCR) were performed as in Emmerich and colleagues (2012) with general bacterial 16S rRNA genes group-specific primers 341GCF (Muyzer et al. 1993) and 907R (Lane 1991), to obtain PCR products for Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al. 1993).

To analyze the richness of the enriched bacterial community structure, we used a 6% acrylamide gel with a denaturing gradient of formamide and urea ranging from 35 to 60%. The banding patterns produced by the DGGE gradient were analyzed with the software package GelCompar II (version 6.0 Applied Maths). Manual band matching was performed, followed by Dice's coefficient of similarity calculations and the construction of dendrograms by the unweighted-pair group method with arithmetic mean (UPGMA). Jackknife resampling was applied to evaluate the significance of the DGGE band clusters. The functional organization (Fo) was calculated by ranking the band intensities from high to low and constructing Pareto-Lorenz curves (Marzorati et al. 2008). The range-weighted richness (Rr) was calculated using the following formula (Marzorati et al. 2008):

$$Rr = N^2 \times D_g$$

where N represents the total number of bands in the pattern and D_g represents the percentage of the denaturing gradient that is covered by the bands.

The most prominent bands from the DGGE gel were excised, diluted with 200 μl water and incubated for 2 h at 4°C. Subsequently a PCR was run from 2 μl water with primers 341F and 907R, where the thermocycle PCR program was the same as for DGGE. The resulting PCR products were loaded on a 1% agarose gel, the bands were excised and cleaned with the Wizard PCR clean-up system (Promega Laboratories) and ligated with the Qiagen PCR cloning kit. The products from this kit were transformed into DH5α competent *E. coli* cells and plated on LB medium with ampicillin, EPTG and XGal for the blue/white assay. White colonies were picked and screened for their products. Overnight cultures were prepared from colonies with the correct inserts in 5 ml liquid LB medium and ampicillin. Plasmid isolations were conducted from these cultures and sent to GATC Biotech (Konstanz, Germany) for sequencing. The resulting sequences were trimmed using the software program Geneious R6. The resulting sequences were entered into the basic local alignment search tool (BLAST) to identify each individual sequence (Altschul et al. 1990).

Scanning Electron Microscopy (SEM)

Fixed culture samples (3% paraformaldehyde) for SEM were pipetted onto Al stubs covered with sticky graphite tape, critical point dried and stored anoxically. After subsequent sputtering under vacuum for 60 sec with a 6–8 nm thick Pt layer (SCD 005/CEA 035 sputter, BAL-TEC, 35 mm working distance, 30 mA, 0.05 mbar argon), images were taken with an electron microscope (SEM LEO-1450 VP,LEO Electron Microscopy Ltd.) with an acceleration voltage of 15 kV at a working distance of 8–9 mm.

Results

The Geochemical Depth Profile of Lake Constance Littoral Sediments

A depth profile of sliced littoral sediment cores from Lake Constance was analyzed to determine the oxygen penetration depth, pore water content and high spatial resolution of bioavailable (0.5 M HCl extractable) iron. This was done in order to determine the sediment depth where nitrate-reducing Fe (II)-oxidizers and Fe(III)-reducers would most likely be active. The pore water content was used to convert the measured values from the MPNs in cells/ml to cells/g dry weight sediment.

We determined an initial pore water content of approximately 50% that decreased with depth and stabilized after 12.5 mm to 20% (Figure 2A). The iron content peaked at a depth of 7.5 mm at a concentration of 1.46 g Fe per g dry weight sediment (Figure 2B). The pore water contained a native carbon source of which 7.55 ± 0.14 mg $\cdot$ L^{-1} was organic carbon and 50.37 ± 0.84 mg $\cdot$ L^{-1} was inorganic carbon which is equivalent to 4.2 mM bicarbonate (assuming all inorganic carbon is bicarbonate). The overlying bulk water contained slightly less carbon in which the organic carbon content was 2.20 ± 0.2 mg $\cdot$ L^{-1} and the inorganic carbon content

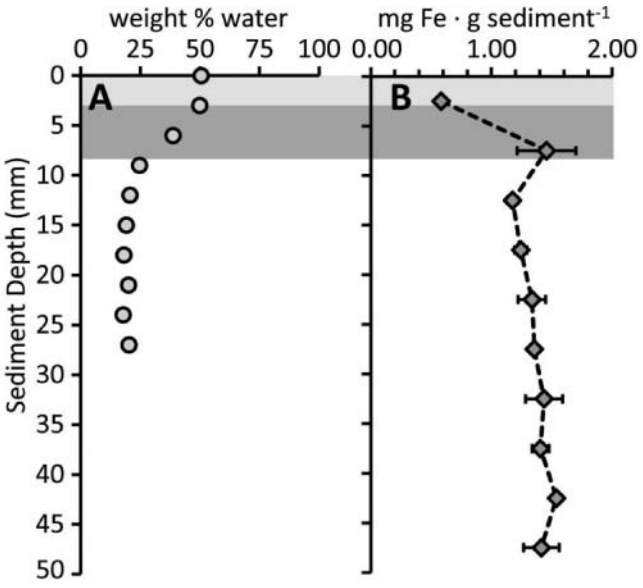

Fig. 2. Oxygen penetration depth (3 mm light grey shaded area) and the sediment depth from which the MPN study and the enrichments were inoculated (dark grey, shaded area) are depicted. A) Depth pore water profile of the sediments; B) Depth profile of total (sum Fe(II) and Fe(III)) bioavailable (0.5 M HCl extractable) iron.

was 31.30 ± 0.18 mg $\cdot$ L^{-1}, or 2.6 mM bicarbonate. The native inorganic carbon source of the bulk water was not taken into consideration when adding 22 mM of bicarbonate buffer to the sterilized lake water, therefore, the buffer concentration in the BLW was slightly higher at 24.6 mM than the LWM and the MIX which contained 22 mM bicarbonate buffer.

Nutrient Compositions of the Natural Lake Constance Water and the Media: FWM and the MIX

Under the assumption that a successful medium must very closely represent the geochemistry of the natural environment, the anions and cations in all three media types were measured and compared to one another and to the natural lake water (Lake Constance Water) (Table 1). Between the natural Lake Constance water and the FWM, the FWM contained the highest concentration of all cations save calcium (0.74 mM), which was mildly higher in the lake water (1.16 mM). Trace amounts of nitrite (0.02 mM) and nitrate (0.04 mM) were also detected in the Lake Constance water but absent in the medium. The FWM contained much higher salt concentrations than the natural Lake Constance water (Table 1), in the case of NH_4^+ and PO_4^{3-} as much as approximately 500 times more.

Quantification of Nitrate-Reducing Fe(II)-Oxidizers and Fe (III)-Reducers by the MPN Method

The MPN quantifications of the nitrate-reducing Fe(II)-oxidizers in the sediment lay within an order of magnitude varying between $6.0 \times 10^2 \pm 1.9 \times 10^2$ cells $\cdot$ g^{-1} in the BLW, $6.1 \times 10^3 \pm 1.9 \times 10^3$ cells $\cdot$ g^{-1} in the FWM and the MIX yielded an intermediate number of $4.0 \times 10^3 \pm 1.3 \times 10^3$ cells $\cdot$ g^{-1} (Figure 3A). The different quantity of Fe(II)-oxidizers in the BLW compared to the other two media types was significant based on a *t*-test ($p < 0.001$), and yielded the lowest number of Fe(II)-oxidizing cells of all three media types. The difference between the FWM and the mixture was only significant, and thereby the least significant difference between two media types in this study (Figure 3A).

The MPNs of acetate-oxidizing Fe(III)-reducers yielded $2.4 \times 10^6 \pm 5.5 \times 10^5$ cells $\cdot$ g^{-1} in the BLW, $1.1 \times 10^{10} \pm 2.8 \times 10^9$ cells $\cdot$ g^{-1} in the FWM and the MIX yielded $5.4 \times 10^8 \pm 1.7 \times 10^8$ cells $\cdot$ g^{-1} (Figure 3B). The cell numbers quantified in these three media types differed significantly based on a *t*-test ($p < 0.001$) with numbers that varied over 4 orders of magnitude (Figure 3B). Like for the nitrate-reducing Fe(II)-oxidizers, the most acetate-oxidizing Fe(III)-reducing cells were quantified in the FWM and the least in the BLW.

Consistent with the other tested metabolisms (Figures 3A, 3B), the BLW enriched the lowest number of lactate-oxidizing Fe(III)-reducers; $8.4 \times 10^5 \pm 2.4 \times 10^5$ cells $\cdot$ g^{-1}. The MIX generated the highest number of cells at $1.5 \times 10^8 \pm 3.5 \cdot 10^7$ cells $\cdot$ g^{-1} and the FWM yielded a slightly lower number by one order of magnitude of 1×10^7 cells $\cdot$ g^{-1} (Figure 3C). The quantifications of the lactate-oxidizing Fe(III)-reducers bore significantly different cell numbers in all the tested media types based on a t-test ($p < 0.001$) (Figure 3C).

Community Composition and Diversity of the Enrichments of the Same Sample in Different Media Types

DGGE bands were generated from the end stage enrichments (right before inoculation into agar and gelrite shakes) and of colonies picked from selected agar and gelrite shakes (Figure 1) to compare the community composition of the nitrate-reducing Fe(II)-oxidizers and the acetate- and lactate-oxidizing Fe(III)-reducers in the different media types, FWM, BLW and MIX from the same environmental littoral sediment sample (Figure 4). As a means of judging the diversity and the richness of the samples, we applied calculations described originally by Marzorti et al. (2008) formulated for environmental samples and very diverse communities.

Table 1. Anions and cations in the three media types measured by ion chromatography

	Freshwater Medium (FWM)	Lake Constance Water (BLW)	Medium/Water Mix (MIX)
Cations	mM	mM	mM
Na^+	22.63 ± 0.33	0.29 ± 0.01	22.39 ± 0.00
NH_4^+	5.47 ± 0.00	0.01 ± 0.00	5.17 ± 0.00
K^+	4.68 ± 0.00	0.04 ± 0.01	4.50 ± 0.00
Mg^{2+}	3.66 ± 0.23	0.39 ± 0.04	2.76 ± 0.10
Ca^{2+}	0.74 ± 0.04	1.16 ± 0.50	1.95 ± 0.16
Anions			
F^-	bdl	bdl	bdl
Cl^-	11.04 ± 0.45	0.23 ± 0.03	11.03 ± 0.66
NO_2^-	bdl	0.02 ± 0.00	bdl
Br^-	bdl	bdl	bdl
NO_3^-	bdl	0.04 ± 0.02	0.03 ± 0.01
PO_4^{3-}	4.91 ± 0.14	0.01 ± 0.01	5.12 ± 0.18
SO_4^{2-}	0.96 ± 0.05	0.35 ± 0.00	0.38 ± 0.04

Data are given for triplicate measurements with standard deviations. (bdl = below detection limit).

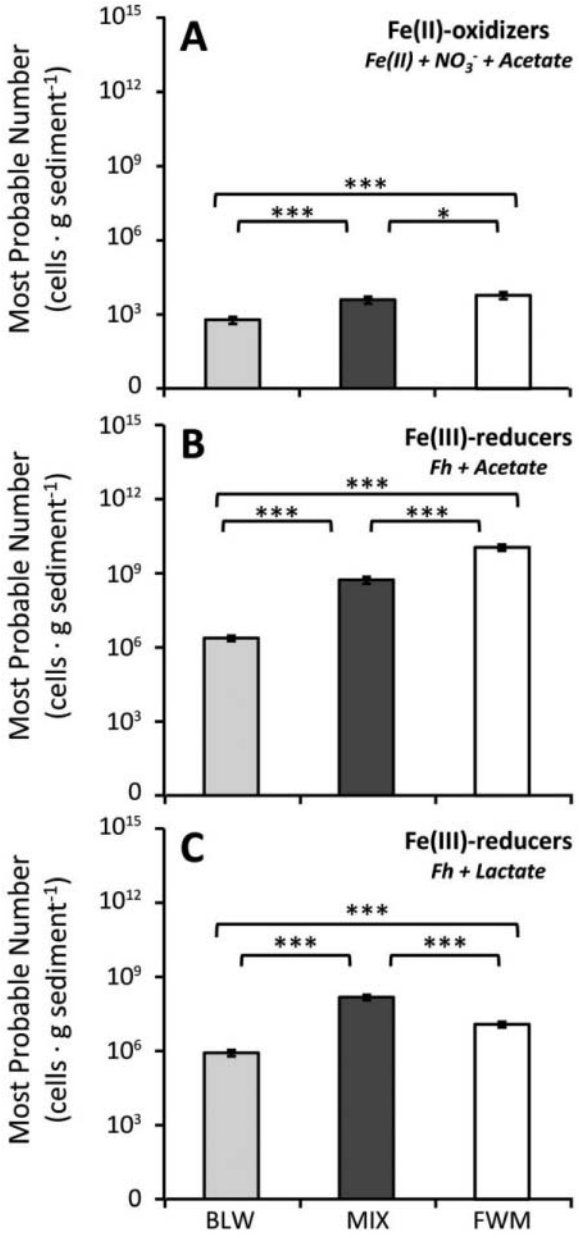

Fig. 3. Most Probable Number study of iron-metabolizers cultured in three different media types: Buffered Lake Water (BLW), Lake Water and Freshwater Medium (MIX) or Freshwater Medium (FWM). A) Mixotrophic nitrate-reducing Fe(II)-oxidizers, B) acetate-oxidizing Fe(III)-reducers and C) lactate-oxidizing Fe(III)-reducers. Stars indicate whether the difference between the numbers obtained from two media types is significant according to a *t*-test, where $p < 0.001$ = highly significant (***), $p < 0.01$ = very significant (**), $p < 0.1$ = significant (*).

Nevertheless we believe that although these banding patterns originated from enrichments, which exhibit a far lower diversity than environmental samples, this still represents a very good way to compare the richness of the samples and the functional organization and to potentially isolate the determining factor and answer the following question: Is the diversity of the enrichments determined by the added electron donors and acceptors or by the nutrient concentration in the medium? A dendrogram indicating the similarity between the banding patterns of the different samples is depicted in Figure 5. First, as all Jacknife nodes lay between 72 and 100%, this indicated that the branching of the dendogram was very reliable.

Second, the banding patterns were grouped into three subgroups; two groups of iron(III)-reducers, where one was mainly composed of the MIX samples and the other contained BLW FWM and MIX samples, and one group of iron (II)-oxidizers from all media types. Colonies picked from the gelrite and agar shakes shared a 100% band pattern similarity. The two groups of iron(III)-reducers were only 30% similar whilst the Fe(II)-oxidizers were overall 65% similar.

The functional organization (Fo) overall ranged between 24 and 44 with an average value of 36 (Table 2). The range-weighted richness (Rr) was highest in the BLW at 47.9 for lactate-oxidizing Fe(III)-reducers and 39.2 for the acetate-oxidizing Fe(III)-reducers (Table 2). The Rr was lowest in the MIX with values ranging from 2.7 to 26.9 (both lactate-reducing Fe(III)-reducers). The Rr in the FWM ranged from 11.1 to 37.1, and it was the same for all agar shakes and the gelrite shake at 17.6.

Excision of DGGE bands (Figure 3) yielded 16S rRNA gene sequence information with high similarity to known species able to oxidize or reduce iron species. Fe(II) oxidation could not be maintained in the BLW over longer periods of time and could not be recovered from older tubes (*data not shown*). Overall, only a single strain from the Fe(II)-oxidizing nitrate-reducing medium was identified by cloning and sequencing the dominant DGGE bands (Figure 1 and Figure 4), while six genera from the iron(III)-reducing media were identified. The bacterial species are not all known Fe-metabolizers, but have been shown to be capable of using a diverse array of electron donors and acceptors (Table 3).

Two colonies picked from gelrite shakes were investigated further with light microscopy and scanning electron microscopy (SEM). The nitrate-reducing Fe(II)-oxidizing strain identified from dominant DGGE bands from the picked colony (Figure 1) showed a sequence similarity of 99.5–100% to *Acidovorax defluvii* strain BSB411 (Schulze et al. 1999). Light microscopy showed that this strain forms red colonies, which are darker in the center and become progressively lighter towards the outer rim (Figure 6B). SEM images showed that the obtained culture of this strain contained encrusted cells in Fe(III) minerals and non-encrusted cells simultaneously (Figure 6C). An Fe(III)-reducing colony picked from a gelrite shake showed 99.7–100% sequence similarity to *Dechloromonas hortensis* strain MA-1 (Wolterink et al. 2005) (Figure 6DEF). Light microscopy revealed that these bacteria form small black colonies (Figure 6E). SEM microscopy showed that the bacteria are non-encrusted and have an oblong rodlike shape (Figure 6F).

Discussion

Media Effects

The BLW most closely represents the natural environmental nutrient concentrations in the lake water out of the three

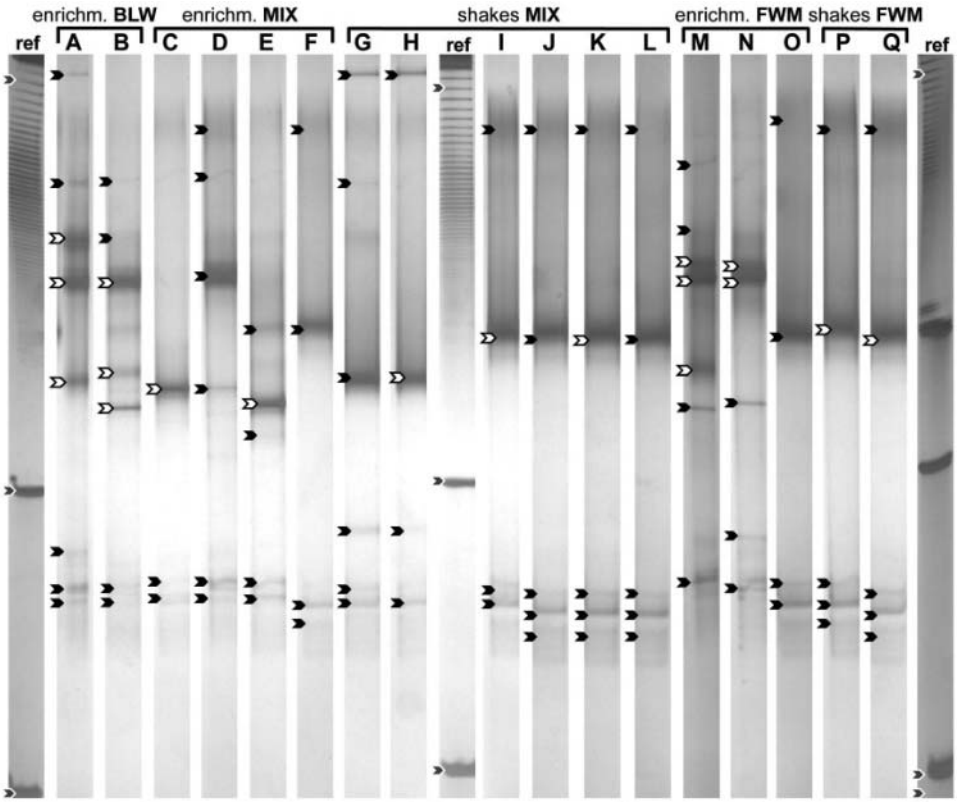

Fig. 4. Denaturing Gradient Gel Electrophoresis (DGGE) of iron-metabolizing enrichments from anoxic littoral Lake Constance sediments. All arrows (➤) indicate bands considered in the analysis, open arrows (➢) indicate bands excised for sequencing, grey arrows (➤) indicate reference bands. Each lane represents a PCR product from end stage enrichments (enrichm.) (Figure 1) or from a culture grown from a picked colony from a gelrite or agar shake (shakes) as follows, where *t* indicates the transfers since inoculation from the original sample for enrichments (Figure 1, step 5) and from picked colony from agar/gelrite shakes for shakes (Figure 1, step 11): *Enrichments BLW* A) t11 lactate-oxidizing Fe(III)-reducing, B) t10 acetate-oxidizing Fe(III)-reducing *Enrichments MIX,* C) t14 lactate-oxidizing Fe(III)-reducing transferred, D) t14 lactate-oxidizing Fe(III)-reducing into FWM since t9, E) t11 acetate-oxidizing Fe(III)-reducing, F) t7 nitrate-reducing Fe(II)-oxidizing. *Shakes MIX*; G) agar t3 lactate-oxidizing Fe(III)-reducing 4th transfer, H) agar t4 lactate-oxidizing Fe(III)-reducing 5th transfer, I) agar t3 nitrate-reducing Fe(II)-oxidizing, J) gelrite nitrate-reducing Fe (II)-oxidizing, K) gelrite t4 nitrate-reducing Fe(II)-oxidizing, L) gelrite t4 nitrate-reducing Fe(II)-oxidizing. (J,K and L originate from colonies from the same gelrite shake tube) *Enrichments FWM*: M) t9 lactate-oxidizing Fe(III)-reducing, N) t7 acetate-oxidizing Fe(III)-reducing, O) t10 nitrate-reducing Fe(II)-oxidizing. *Shakes FWM*: P) agar t3 nitrate-reducing Fe(II)-oxidizing, Q) gelrite t4 nitrate-reducing Fe(II)-oxidizing.

medium types (Table 1). As it has been shown before that low nutrient concentrations yield high numbers of targeted bacteria in MPN studies of heterotrophs (Bussmann et al. 2001), and low amounts of nutrient salts are thought not to inhibit growth of native species (Aagot et al. 2001; Zengler 2009) this provided an indication that the evenness of the grown Fe-metabolizing populations may be highest in the BLW. However, the lowest number of Fe-metabolizers was consistently found in the BLW compared to the MIX and the LWM (Figure 3).

This discrepancy with previously published higher MPN numbers for low nutrient grown lacustrine bacteria (Bussmann et al. 2001) could be due to a difference in metabolic pathways between heterotrophic and Fe-metabolizing microorganisms, or due to a higher stress caused by the potentially toxic ferrous iron to cells in low-salt medium (Carlson et al. 2013; Poulain and Newman 2009), exacerbated by a lack of competition of other divalent cations present in the high-salt medium for diffusion into cells. The diversity of the BLW enrichment, however, was the highest of all three media types with an Rr between 39.2 and 47.9 (Table 2). A higher degree of richness was cultivated under low nutrient conditions because the nutrient concentrations are more environmentally relevant and thereby sustain and support a larger fraction of the natural microbial population.

The artificial FWM generated a higher number of cells for acetate-oxidizing Fe(III)-reducers and Fe(II)-oxidizing nitrate-reducers, but the MIX yielded a higher number for the lactate-oxidizing Fe(III)-reducers. The nutrient concentrations in these media were extremely high compared to the natural lake water (Table 1), in some cases the difference was even ca. 500-fold. Only a few specialized organisms can deal with such a high nutrient supply and will outgrow the other bacteria swiftly, biasing the outcome to their genera.

As these nutrient concentrations are not as environmentally relevant, this means that the most complex medium

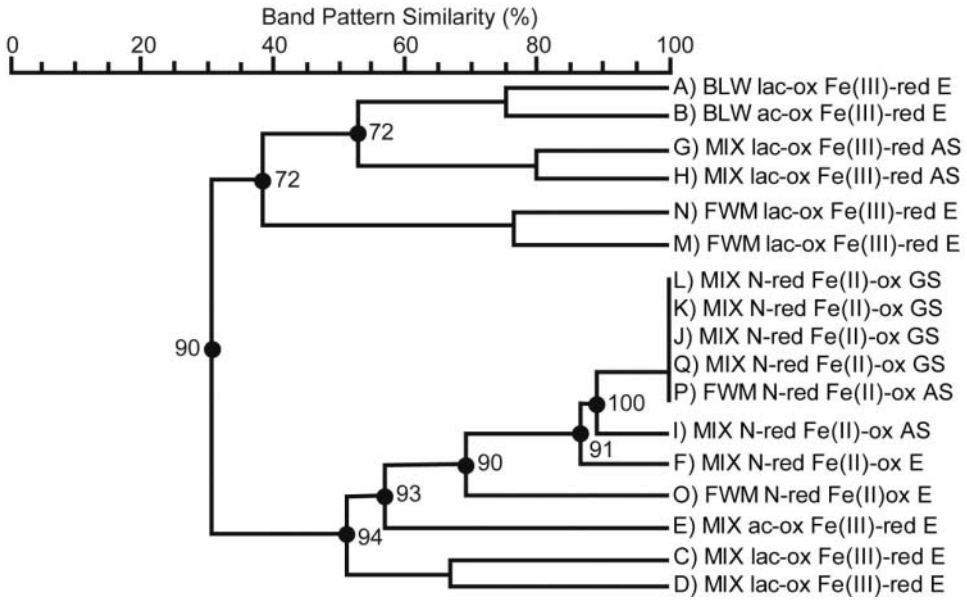

Fig. 5. Dice's similarity dendrogram comparing the similarity of DGGE based band patterns from enrichments and agar/gelrite shakes of acetate- or lactate-oxidizing Fe(III)-reducers (ac-ox Fe(III)-red and lac-ox Fe(III)-red respectively) and nitrate-reducing Fe (II)-oxidizers (N-red Fe(II)-ox), where GS = gelrite shake, AS = agar shake and E = enrichment culture.

Table 2. Functional organization and the range weighted richness calculated from the DGGE band patterns according to Marzorati et al. (2008)

Sample	Functional Organization Fo (%)	Range-weighted richness (Rr) Arbitrary unit
Buffered Lake Water (BLW)		
Enrichments		
A) t11 (lactate-oxidizing Fe(III)-reducing)	32	47.9
B) t10 (acetate-oxidizing Fe(III)-reducing)	34	39.2
Mixture (MIX)		
Enrichments		
C) t14 (lactate-oxidizing Fe(III)-reducing, in FWM since t9)	44	2.7
D) t14 (lactate-oxidizing Fe(III)-reducing)	37	24.0
E) t11 (acetate-oxidizing Fe(III)-reducing)	40	9.6
F) t7 (nitrate-reducing Fe(II)-oxidizing)	38	11.1
Agar / Gelrite Shakes		
G) agar t3 (lactate-oxidizing Fe(III)-reducing)	42	29.9
H) agar t4 (lactate-oxidizing Fe(III)-reducing)	37	12.0
I) agar t3 (nitrate-reducing Fe(II)-oxidizing)	33	10.7
J) gelrite t4 (nitrate-reducing Fe(II)-oxidizing)	39	17.6
K) gelrite t4 (nitrate-reducing Fe(II)-oxidizing)	37	17.6
L) gelrite t4 (nitrate-reducing Fe(II)-oxidizing)	42	17.6
Freshwater Medium (FWM)		
Enrichments		
M) t9 (lactate-oxidizing Fe(III)-reducing)	28	37.1
N) t7 (acetate-oxidizing Fe(III)-reducing)	36	11.1
O) t10 (nitrate-reducing Fe(II)-oxidizing)	24	11.1
Agar/Gelrite Shakes		
P) agar t3 (nitrate-reducing Fe(II)-oxidizing)	36	17.1
Q) gelrite t4 (nitrate-reducing Fe(II)-oxidizing)	36	17.6

Diversity indices Fo and Rr according to Marzorati et al. (2008).
(Fe(III)red Lac ox) = 5 mM Fh reducing and 5 mM lactate-oxidizing bacteria.
(Fe(III)red Ac ox) = 5 mM Fh reducing and 5 mM acetate-oxidizing bacteria.
(NO_3^-red Fe(II)ox) = 10 mM $FeCl_2$ oxidizing and 4 mM $NaNO_3^-$ reducing bacteria with 0.5 mM cosubstrate acetate.
t indicates the number of transfers the culture has undergone into a new dilution series.

Table 3. Identity of Fe-metabolizing species determined by sequencing of dominant DGGE bands.

Bacterial Species	Sequence Similarity (%)	Origin	e-donor	e-acceptor	Isolation Reference
Fe(II)-oxidizing nitrate-reducer					
Acidovorax defluvii strain BSB411	99.49–100	activated sludge (DE)	acetate	nitrate	Schulze et al. 1999
Fe(III)-reducing acetate/lactate- oxidizer					
Cupriavidus basilensis strain DSM 11853 (formerly Ralstonia basilensis)	99.15–99.66	Freshwater pond sediments (Fr)	Toluene, benzene, chlorobenzene, phenol, mono- and dichlorophenol isomers	H_2O_2, O_2	Steinle et al. (1998)
Dechloromonas aromatica RCB strain RCB	99.15*	River sediments (USA)	Hydrocarbons (benzene) chlorobenzoate, toluene, and xylene	nitrate, (per)chlorate	Coates et al. (2001)
Dechloromonas hortensis strain MA-1	99.66–100	Pristine garden soil	acetate	(per)chlorate,	Wolterink et al. (2005)
Geobacter argillaceus strain G12	97.62–98.13	Sedimentary kaolin strata (USA)	Ethanol, butanol, butyrate, glycerol, acetate, lactate, pyruvate	Sulphur, Fe(III), manganese, nitrate	Shelobolina et al. (2007)
Geobacter pelophilus strain Dfr2	97.63–98.14	Freshwater mud (DE)	H_2, formate, acetate, pyruvate, succinate, ethanol	Fh, Mn(IV), S^0, fumarate	Straub and Buchholz-Cleven (2001)
Geobacter psychrophilus strain P35	97.62–98.30	Groundwater aquifer (USA)	ethanol, lactate, malate, pyruvate and succinate	Fe(III), malate, fumarate	Nevin et al. (2005)
Pelobacter propionicus DSM 2379 strain	92.63–98.13	Freshwater mud (DE)	*Ferments*: 2,3-butanediol, acetoin, lactate		Schink (1984)
Rhodoferax ferrireducens T118 strain DSM 15236	94.92*	Subsurface sediments (USA)	Acetate, lactate, malate, propionate, pyruvate, benzoate, succinate	Fe(III)	Finneran et al. (2003)
Ralstonia pickettii strain ATCC 27511	99.83*	Clinical specimens (USA)	Glycerol, glucose, galactose, fructose, acetate, lactate, pyruvate	O_2, NO_3^-	Ralston et al. (1973)

Shown is the best BLAST hit of at least 5 clones per most dominant DGGE band. *Based on a single clone.

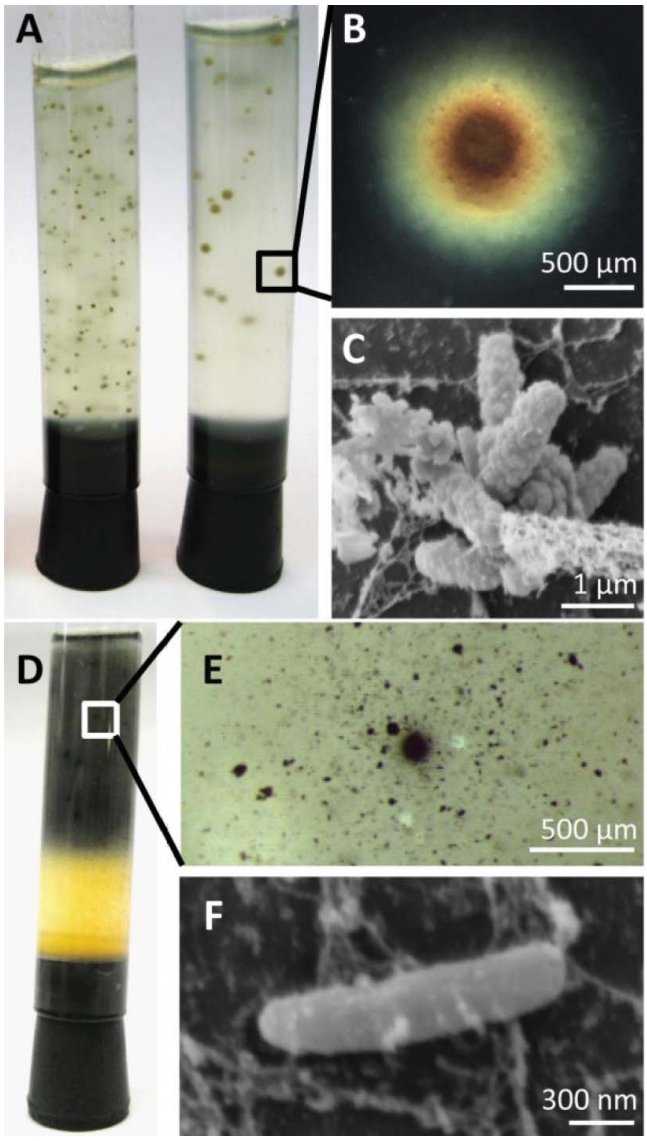

Fig. 6. Images of a nitrate-reducing Fe(II)-oxidizing bacterium with a 99.5–100% sequence similarity to *Acidovorax defluvii* strain BSB411 (A-C) and an acetate-oxidizing Fe(III)-reducing bacterium with a 99.7–100% sequence similarity to *Dechloromonas hortensis* strain MA-1 (D-F) originating from transfers from agar/gelrite shakes. A) Agar shakes containing mixotrophic Fe (II)-oxidizing NO_3^- -reducing medium with acetate as additional carbon source. B) Binocular light microscope image of a growing colony. C) Scanning electron micrograph of the Fe(II)-oxidizing bacterium. D) Agar shake containing Fe(III)-reducing acetate-oxidizing medium. E) Binocular light microscope image of a growing colony. F) Scanning electron micrograph of the Fe(III)-reducing bacterium.

(MIX) was expected to yield the lowest diversity. The complexity of the different media tested here followed the following sequence MIX > FWM > BLW, which corresponded to an increase in diversity, as determined by the Rr (Table 2). As the MPN numbers were very high in the case of the FWM and the MIX, and the richness indices were lower than in the BLW (Table 2), it is most likely that the high concentrations of nutrients in combination with certain electron donors and acceptors enforced growth of certain specialized groups of organisms able to grow under these conditions.

The functional organization of the community enriched in all three media types was within the same range with Fo values of 32–44 (Table 2), indicating that the media nutrient composition may not be the determining factor in the function of the enriched microbial communities. Thus, when only taking into consideration the differences in media nutrient composition, the Fe-metabolizing quantification by the MPN method is significantly affected, however, their overall diversity as assessed by DGGE analyses is determined by the identity of the electron donors and acceptors, not by the nutrient composition of the media. This is further confirmed by the clustering of the Dice's similarity dendrogram according to electron donors and acceptors, not according to the nutrient concentrations in the media (Figure 5). To exploit certain electron donors and acceptors, an organism needs to have certain genes and the ability to express them. Providing certain substrates exerts a strong selective force on a natural community as only the organisms that possess the respective genes will be able to profit and out-compete others.

Biases

When comparing the relative differences in the numbers of Fe (II)-oxidizers and Fe(III)-reducers for the different media, different quantifications were obtained (Figure 3). Total cell numbers in the environment are often quantified through culture-independent methods such as qPCR or FISH (Amann and Fuchs 2008; Smith and Osborn 2009). This means that when culture-dependent data (like MPNs) are used in combination with culture-independent data (like qPCR), special care needs to be taken with drawing conclusions on the relative abundance and significance of the physiological Fe(II)-oxidizing and Fe(III)-reducing groups in a certain habitat, as the numbers of the Fe-metabolizers will differ with several orders of magnitude depending on the medium salt concentrations. This means that there is a certain bias within these combined experimental setups caused by the choice of medium. Thus, for better comparability between studies with respect to quantification, it would be beneficial if all experiments would be conducted using the same medium.

Although the overall MPN numbers may vary between the different media types, the relative abundance of the different physiological groups of iron-transforming microorganisms is approximately the same in the different media types. This means that the nutrient composition in studies assessing microbial iron redox cycling may not be so important, as the relative abundance is not so strongly affected. With respect to the general role of Fe(II)-oxidizers and Fe(III)-reducers for iron redox cycling, this would not change interpretation of the data.

Community Composition

The ability to oxidize and reduce iron redox species in the environment may be more wide spread than thought before

and thus not restricted to certain groups defined as iron-metabolizers (Li et al. 2011; Straub et al. 1996, 2004). This is further illustrated by the outcome of this study, where only four out of the ten enriched species with iron as sole electron donor or acceptor, had previously been identified as iron-metabolizing species (Table 3). The enriched iron(II)-oxidizing bacterium *Acidovorax defluvii* strain BSB411 lies within the same genus as a well-known iron(II)-oxidizing bacterium isolated from the same environment under similar conditions, namely *Acidovorax* BoFeN1 sp. (Kappler et al. 2005). No new species were isolated in this study, all excised and sequenced dominant bands from the DGGE (Figure 4) resulted in species that were already previously described (Table 3).

Currently used common media thus resulted in the enrichment of previously known bacterial groups/taxa. With respect to isolating a new Fe(II)-oxidizer or Fe(III)-reducer, a different approach than using conventional medium should be attempted. Turning away from the standard media and finding more creative ways of recreating natural conditions in the laboratory in order to enrich and isolate new species may more likely result in the discovery of a new Fe-metabolizer.

Conclusions

This study contests that the best way to culture a species is to most closely simulate the geochemistry of the environment. It may depend on the goal of your study; do you want to assess the richness or the evenness of your bacterial population? The best approach to tackling these questions currently still lies in a careful combination of culture-dependent (MPNs) and culture-independent (total cell numbers) studies, as long as the functional gene markers for iron-metabolisms remain unclear. For better comparability between studies with respect to cell quantifications capable of an Fe(II)-oxidizing or Fe(III)-reducing metabolism, it would be advantageous to conserve the medium recipe between experiments. Though in order to isolate a novel Fe-metabolizing species, a decrease in salt concentrations in the medium recipe may target a wider microbial population, and thereby more likely result in the isolation of a hitherto unknown species.

We found that the nutrient composition of growth media has an extremely significant effect on the quantification of iron-metabolizing bacteria. In contrast, the medium composition did not so much affect the diversity of the Fe-metabolizing enrichments. Although the medium composition will significantly influence the quantification and thereby conclusions on the significance of the targeted group within the ecosystem, biodiversity assessments through enrichment cultures will mostly be determined by the choice of electron donor and acceptor, and not so much by the medium salt concentrations, trace elements and vitamins.

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