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Comparison of Humic Substance- and Fe(III)-Reducing Microbial Communities in Anoxic Aquifers

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Humic substances can mediate electron transfer between microorganisms and Fe(III) minerals. Because it is unknown which microorganisms reduce humics in anoxic aquifers, we analyzed the diversity and physiological flexibility of Fe(III)-, humics-, and AQDS-reducers, which were present at up to 10^6 cells g^{-1}. No significant differences in 16S rRNA gene based diversity were found between enrichment cultures reducing ferrihydrite, humics or AQDS. Even after repeated transfers many of the enrichments retained the ability to switch to other electron acceptors. This suggests that humics- and Fe(III)-reducing microorganisms in anoxic aquifers are rather versatile and able to reduce different extracellular electron acceptors.

Keywords: DGGE, electron shuttling, Fe(III) reduction, humic acids

Introduction

Humic substances (HS) are polyfunctional organic molecules originating from the degradation of organic material (Stevenson 1994). They are present at varying concentrations in almost all aquatic and terrestrial environments (Aiken et al. 1985; Stevenson 1994) including anoxic aquifers, which are of particular interest due to their importance for the supply of drinking water. Pollutant dynamics in anoxic aquifers are influenced for instance by the reductive degradation of organic pollutants (Lee et al. 1998) or by the formation, transformation and dissolution of iron minerals, which provide reactive surface sites for pollutant transformation (Borch et al. 2010; Elsner et al. 2004) and sorption sites for contaminants such as arsenic (Hohmann et al. 2010; Kappler and Straub 2005; Tufano and Fendorf 2008). These processes can be influenced by HS (Sposito 2011), which were shown to mediate the reduction of substituted nitrobenzenes (Dunnivant et al. 1992) and chlorinated compounds (Kappler and Haderlein 2003; Van der Zee and Cervantes 2009) as well as the reduction and dissolution of different iron (oxyhydr)oxide minerals (Bauer and Kappler 2009; Lovley et al. 1998).

HS contain a variety of redox-active functional groups and can be reduced by many different physiological groups of microorganisms (Martinez et al. 2013) including Fe(III)-reducers (Lovley et al. 1996), but also fermenters (Benz et al. 1998), sulfate-reducers, halorespirers, and methanogens (Cervantes et al. 2002). Microbially reduced HS can transfer electrons to Fe(III) minerals in an abiotic electron transfer step leading to the indirect microbial reduction of the Fe(III) minerals and, most importantly, to the reoxidation of the reduced HS restoring them for further microbial reduction (Lovley et al. 1996). This so-called electron shuttling between HS-reducing bacteria and Fe(III) minerals has been shown to increase Fe(III) mineral reduction rates in batch experiments (Jiang and Kappler 2008; Lovley et al. 1996) and facilitate the reduction of otherwise inaccessible Fe(III) phases (Lovley et al. 1998). If sulfate-reducing, methanogenic, and halorespiring bacteria are involved in the HS reduction, the abiotic transfer of electrons from the microbially reduced HS to Fe (III) minerals even leads to indirect Fe(III) reduction by these microbes which cannot reduce Fe(III) minerals directly.

There have been several indications that HS reduction and electron shuttling are also taking place in the environment. Nevin and Lovley (2000) and Rakshit et al. (2009) performed microcosm experiments to determine the Fe(III) reduction rates in the presence or absence of added HS. They demonstrated that in environmental samples the addition of HS and AQDS stimulated Fe(III) reduction. Kappler et al. (2004) determined the redox state of humic acids extracted from different sediment depths from a freshwater lake and demonstrated that HS in the reduced zone were mostly in a reduced state. In combination with the high numbers of humic-reducing microorganisms quantified by the most probable number (MPN) method this indicates that HS reduction took place in the sediment.

Bacteria-reducing HS and AQDS (9,10-anthraquinone-2,6-disulfonic acid), a model compound for quinoid moieties in HS, have been isolated from a variety of environments. Most of them belonged to the Geobacteraceae and were also

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able to reduce Fe(III) (Coates et al. 1998; Snoeyenbos-West et al. 2000). However, the fact that Geobacteraceae appeared as an important HS-reducing group could be due to the use of acetate as electron donor, since Cervantes et al. (2002) reported that the HS-reducing sulfate-reducers and halorespirers in their study were not able to oxidize acetate. Kappler et al. (2004) quantified acetate- and lactate-oxidizing HS- and Fe(III)-reducers in different depths of Lake Constance sediment and found that a large fraction of the HS-reducing microorganisms were not able to reduce Fe(III).

Based on these findings, it is still unclear which microbes are reducing HS in environments such as anoxic aquifers and to which extent non-Fe(III)-reducers are involved in HS reduction. Several single strains of bacteria able to reduce the model quinone AQDS have been isolated from different environments (Cervantes et al. 2003; Coates et al. 1998) and prominent bacteria enriched in microcosms upon addition of humic acids or AQDS have been identified (Snoeyenbos-West et al. 2000). However, microbial communities reducing HS, Fe(III) or even model quinones such as AQDS in the same samples have not yet been studied systematically.

In the present study we used a combination of a cultivation-based and molecular biological approach to study the Fe(III)-, AQDS-, and HS-reducing communities in two different anoxic aquifers in terms of their abundance, diversity and physiological flexibility in order to determine if Fe(III) and HS are being reduced by the same or by different microorganisms.

Material and Methods

Aquifer Samples

Two contaminated aquifers were chosen in order to represent one aquifer with organic and one with inorganic contamination. The Weißandt-Gölzau (51°40′N 12°4′E) aquifer in Saxony-Anhalt, Germany, is a site of former coal processing industry that resulted in a high contamination with petroleum-derived hydrocarbons (Feisthauer et al. 2012). Water samples were taken from two of the installed sampling wells, G6/08 (WG1) and GWM 3/05 (WG2). Both wells are methanogenic and highly BTEX-contaminated (BTEX concentrations of 2240 and 7380 μg L^{-1}, respectively) (Table S1). Water samples were collected in sterile glass bottles filled to the top without headspace to keep anoxic conditions and closed with butyl rubber stoppers. Sediment samples were taken from liner drillings next to the respective sampling wells and sediment material was taken from the lower part of the liner with sterile spoons into sterile plastic bags. The bags were immediately put into air-tight jars to keep the samples anoxic. Sediment from sampling point WG1 was taken at a depth of 9.4–10.4 m, while sediment from WG2 was taken from 6–7 m depth. Sediment samples were taken in April 2010, water samples in October 2010.

Furthermore, samples were taken from a shallow aquifer at Araihazar, Narayanganj, Bangladesh (23.7917°N 90.6500°E) at two sites, Lakhopura (Ar1) where the water is contaminated with 200 μg L^{-1} arsenic (As) and the As-free control site Sattavand (Ar2). Shallow aquifers in Araihazar are characterized by reducing conditions and the absence of dissolved O_2 (Zheng et al. 2005). Sediment samples were taken from a depth of 3–3.2 m with a hand auger. Water samples were taken from wells at 18 and 15 m depth for sites Ar1 and Ar2, respectively. Sediment and water samples were collected in July 2010 and filled into sterile glass bottles filled to the top without headspace to preserve anoxic conditions and closed with butyl rubber stoppers. All samples were stored at 4°C in the dark and processed within a few days after sampling.

Media and Substrates

MPN analyses and enrichments were set up in bicarbonate-buffered fresh water medium (Ehrenreich and Widdel 1994) modified from Hegler et al. (2008) (0.6 g L^{-1} KH_2PO_4, 0.3 g L^{-1} NH_4Cl, 0.025 g L^{-1} $MgSO_4 \times 7\ H_2O$, 0.4 g L^{-1} $MgCl_2 \times 6\ H_2O$, 0.1 g L^{-1} $CaCl_2 \times 2\ H_2O$, 30 mM $NaHCO_3$). The medium was prepared aseptically and anoxically in a Widdel flask. Where specified, the medium was amended with lactate/acetate as electron donor, and ferrihydrite, humic acid or AQDS as electron acceptor and 2% fresh yeast extract from sterile, anoxic stock solutions. Na-L-lactate and Na-acetate were dissolved in MilliQ-water to a concentration of 0.5 M each. Ferrihydrite was prepared by neutralization of 200 mM $Fe(NO_3)_3$ with KOH (Cornell and Schwertmann 2003), made anoxic, autoclaved and stored at 4°C in the dark for up to 6 weeks. XRD analysis of the ferrihydrite is available in our previous studies (Amstaetter et al. 2012; Piepenbrock et al. 2011). Humic acid was purchased from Aldrich and added to 50 mM phosphate buffer to a concentration of 100 g L^{-1}. AQDS (9,10-anthraquinone-2,6-disulfonic acid) was dissolved in MilliQ-water to a concentration of 20 mM. Aldrich humic acid was chosen as an inexpensive example of a humic acid with an electron accepting capacity and C/H ratio in the same range as many of the standard IHSS humic acids (Ratasuk and Nanny 2007). Fresh yeast extract was prepared from fresh baker's yeast (Leadbetter et al. 1999), filter-sterilized, made anoxic and stored at 4°C for up to 6 weeks. If not stated otherwise, all substrates were made anoxic by three cycles of vacuum (3 min) and flushing with N_2 and sterilized by autoclaving.

Enumeration of Microorganisms and Setup of Enrichment Cultures

For most-probable-number (MPN) analyses (Cochran 1950), a 1:10 dilution series, starting from 1 g sediment sample suspended in 9 mL of medium or from 10 mL of water sample, were prepared anoxically in un-amended fresh water medium (see above). Samples from the dilution series were inoculated into deep well plates (Riplate 1 mL) containing medium amended with lactate/acetate (5 mM each) as electron donor and one of three electron acceptors: 5 mM ferrihydrite for Fe (III)-reducers, 1 g L^{-1} humic acid for HS-reducers or 0.2 mM AQDS for AQDS-reducers. The inoculation into the deep well plates was performed in an anoxic glove box

(100% N_2 atmosphere) and the plates were sealed immediately to prevent outgassing of CO_2 from the bicarbonate buffer.

MPN plates were incubated in air-tight plastic bags (Anaerocult A mini, Merck Millipore, Germany) at room temperature in the dark for 6-8 weeks. Room temperature was chosen for incubation for practical reasons, i.e., to speed up microbial growth compared to the aquifer in-situ temperature of ca. 12°C. Evaluation of microbial growth was done visually for AQDS-reducers (color change from color-less to orange) and Fe(III)-reducers (color change from reddish-brown to black) and by performing a ferrozine assay or a citrate assay plus ferrozine assay (see below) for each well for Fe(III)-reducers and HS-reducers, respectively. Most probable numbers were calculated using the KLEE software (Klee 1993).

Enrichment cultures were set up in the same medium amended with the same substrates as for the MPN analysis. For each culture, 500 μL of sample were transferred from the MPN plate into culture tubes containing 4.5 mL of medium. The culture tubes were incubated at room temperature in the dark. From each plate, 3-4 enrichment cultures were initially set up using the wells with the highest dilution that showed microbial growth in the MPN analyses. Enrichment cultures were evaluated for reduction of the electron acceptor every 4–6 weeks and transferred (6% incoculum) into fresh medium. Of the 3-4 cultures set up per MPN plate, the best performing enrichment culture was chosen so that in the end one culture per MPN plate was obtained, i.e., one Fe (III)-reducer enrichment culture, one HS-reducer enrichment culture and one AQDS-reducer enrichment culture per initial aquifer sample.

After 1–2 transfers, the enrichment cultures were analyzed by DGGE (see below) and only a subset (cultures enriched from water and sediment from sites WG1 and Ar1) was chosen for long-term enrichment studies. To this end, the culture volume was increased to 25 mL, the concentration of electron acceptor was increased to 25 mM ferrihydrite, 5 g L^{-1} HS, or 1 mM AQDS, respectively, in order to favor the enrichment of respirers over lactate- or acetate-fermenting microorganisms. The incubations were shifted from room temperature to 28°C to increase the growth rates and allow faster transfer of the enrichment cultures. Slow growing enrichment cultures were amended with fresh yeast extract in some transfers to ensure survival.

Fe(III) and HS Reduction Experiments

After 11–17 transfers on the same electron acceptor, the three enrichment cultures originating from the same aquifer sample were compared for their ability to reduce Fe(III) and HS. To this end, medium bottles were amended with lactate/acetate (20 mM each) and either 15 mM ferrihydrite or 3 g L^{-1} HS and inoculated with one of the three enrichment cultures (Fe (III)-reducers, HS-reducers or AQDS-reducers) originating from the same aquifer sample. The bottles were incubated at 28°C in the dark and samples for quantification of Fe(II) and of the reducing capacity (RC) of the HS (see below) were taken every 2–3 days.

Analytical Methods

Fe(III) reduction in the enrichment cultures and Fe(III) reduction experiments was evaluated by quantification of Fe(II). To this end, 100-μL samples were withdrawn from the culture bottles inside an anoxic glove box and transferred into 400 μL of 0.5 M HCl. After 2 h of incubation, the samples were centrifuged and Fe(II) and Fe(tot) were quantified in the supernatant using the ferrozine assay (Hegler et al. 2008; Stookey 1970). The extraction with 0.5 M HCl was chosen in order to assess the bioavailable fraction of the iron minerals present (Shelobolina et al. 2005).

HS reduction was determined as the increase of the reducing capacity (RC) of the HS, i.e., the amount of electrons that can be transferred from the HS to Fe(III) citrate (Lovley et al. 1996). To this end, 100-μL samples were taken from the bottles inside the glove box, amended with 5 mM Fe(III) citrate solution and incubated for 1 h to allow the reduction of the Fe(III) citrate by the reduced HS. After this time, the samples were stabilized with 200 μL 1 M HCl, centrifuged and Fe(II) in the supernatant was quantified by the ferrozine assay. Because non-reduced HS already have a low RC towards Fe(III) citrate, the RC of a non-reduced control was measured at each sampling time and subtracted from the RC of the analyzed samples so that the increase in RC (ΔRC) was plotted over time.

Molecular Biological Analyses and Data Evaluation

For molecular biological analysis, complete bottles (25 mL) of enrichment cultures were sacrificed, centrifuged for 15 min at 4°C, 7000 rcf. The pellets were washed once in sterile phosphate buffered saline (PBS) buffer and frozen at −20°C until further analysis. DNA extraction was performed with the UltraClean DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Bacterial 16S rRNA gene copy numbers were quantified by quantitative PCR using SsoFast Eva Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) and general bacterial primers 341F (Muyzer et al. 1995) and 797R (Nadkarni et al. 2002), on an iQ5 real-time PCR cycler (Bio-Rad Laboratories GmbH, Munich, Germany). The 20-μL reaction mix contained: 10 μL Eva Green Supermix, 1.5 pmol primer 341F, 4.5 pmol primer 797R, 6.8 μL of water, and 2 μL of template DNA (0.1 to 1 ng/μL). Due to inhibition effects of the HS, the DNA extracts had to be pre-diluted 1:10 or 1:100 before qPCR. The cycler program was as follows: 98°C for 2 min, 40 cycles of 5 s at 98°C and 12 s at 60°C. A plasmid containing a *Thiomonas sp*. 16S rRNA gene fragment was used as standard and data was analyzed with the iQ5 optical system software, version 2.0 (Bio-Rad). Gene copy numbers were divided by the average number of bacterial ribosomal RNA operons of 4.2 as derived from the Ribosomal RNA Operon Copy Number Database (Klappenbach et al. 2001) to get an estimate of the total cell numbers in the enrichment cultures.

For denaturing gradient gel electrophoresis (DGGE), a fragment of bacterial 16S rRNA genes was amplified by PCR using the primers 907R (DeLong et al. 2006) and 341F-GC

(Muyzer et al. 1993) and separated on a 6% acrylamide gel with a denaturing gradient of formamide and urea ranging from 35 to 60% as previously described (Hegler et al. 2012). For the PCR, 2 μL of DNA extract were added to 48 μL of master mix containing 0.2 μM of each primer, 200 μM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 1 × PCR buffer, 1.25 U Go Taq DNA polymerase (Promega, Germany) and for samples containing HS also 50 mM betain and 0.5 μL of 99.5% (v/v) dimethylsulfoxid (DMSO). DGGE banding patterns were analyzed with the GelCompar II software, version 6.0 (Applied Maths) by manual band matching followed by calculation of the Dice coefficient of similarity and construction of dendrograms by the unweighted-pair group method with arithmetic mean (UPGMA). Significance of the clusters of DGGE banding patterns was evaluated by Jackknife resampling as available in the GelCompar II software package. Similarity values as well as band intensities were exported to a spreadsheet program (MS Excel) and different diversity indices were calculated (see below). Differences between the cultures enriched from the same samples on the different electron acceptors and the different transfers were analyzed by the paired Wilcoxon signed ranks test with the JMP® 10.0.2 software package (SAS Institute Inc., Cary, North Carolina, USA).

Calculation of Diversity Indices

The Shannon-Weaver index of diversity was calculated from the DGGE banding pattern according to Nübel et al. (1999) by the following formula:

$$H' = -\sum_{N}^{i=1} a_i ln(a_i) \quad [1]$$

with N the number of bands and a_i the relative intensity of each band. The functional organization (Fo) after Marzorati et al. (2008) was determined by ranking the bands according to their intensities and constructing a Pareto-Lorenz curve. The cumulative normalized intensity at a cumulative normalized number of bands of 0.2 gives the value for Fo. A high Fo value indicates an enrichment culture in which the most prominent 20% of the bands account for a large part of the cumulative intensity, i.e. an enrichment culture with an uneven distribution of bands (Marzorati et al. 2008). The Range-weighted richness (Rr) according to Marzorati et al. (2008).was calculated by the formula:

$$Rr = N^2 \times D_g \quad [2]$$

where N is the number of bands in the pattern and D_g is the denaturing gradient spanned by the pattern, i.e., the difference in denaturant concentration between the first and the last band of the pattern.

Results

Quantification of Fe(III)- and HS-Reducing Microorganisms in Anoxic Aquifers

In order to assess the potential for humic substances (HS) and Fe(III) reduction in anoxic aquifers, we first performed MPN studies with four water and four sediment samples from two anoxic aquifers to estimate the number of HS-, Fe(III)- and AQDS-reducing microorganisms (Fig. 1). MPN studies using a mixture of lactate and acetate as electron donor showed that Fe(III)-reducing, HS-reducing and AQDS-reducing microorganisms were present in almost all samples with the exception of one water sample where we did not observe growth of Fe(III)-reducers (Ar1) and one sediment sample where we did not observe growth of HS-reducers (WG2).

Estimated population densities ranged from 10^1–10^6 cells (g water-saturated sediment or water)$^{-1}$. In five of the eight samples, the MPNs of HS-reducers were higher than MPNs of Fe(III)-reducers by 2–4 orders of magnitude. In the other three samples no significant difference between the numbers of HS-reducers and Fe(III)-reducers could be observed. The MPNs of HS-reducers, however, were never smaller but always at least equal to the numbers of Fe(III)-reducers. The MPNs of AQDS-reducers were similar to the MPNs of HS-reducers in five out of the eight samples.

No distinct difference in MPNs could be observed between the As-contaminated and the corresponding non-contaminated samples from the Araihazar aquifer. In the samples from the Araihazar aquifer, MPNs were always higher in the sediment than in the corresponding water samples. However, the same trend could not be observed in Weißandt-Gölzau aquifer samples.

Community Composition of Enrichment Cultures

To compare the microbial communities reducing Fe(III) and HS in the different aquifer samples, enrichment cultures were

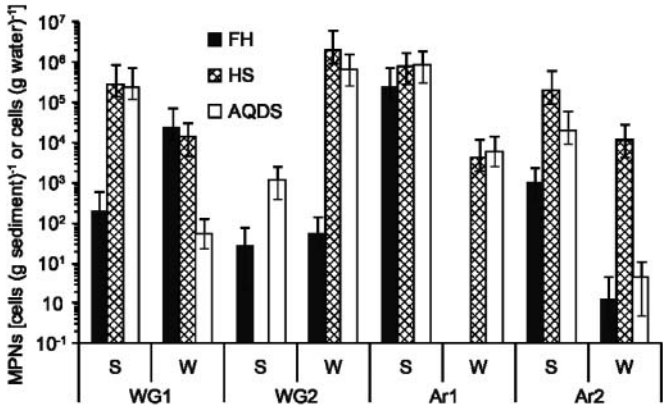

Fig. 1. Most probable numbers (MPNs) of Fe(III)-reducing (FH), humic substance-reducing (HS) and AQDS-reducing microorganisms in sediment (S) and water (W) samples from Araihazar aquifer, Narayanganj, Bangladesh and Weißandt–Gölzau aquifer, Saxony–Anhalt, Germany. Ar1 = As-contaminated site Lakhopura, Ar2 = As-free site Sattavand. WG1 and WG2 = BTEX-contaminated sites.

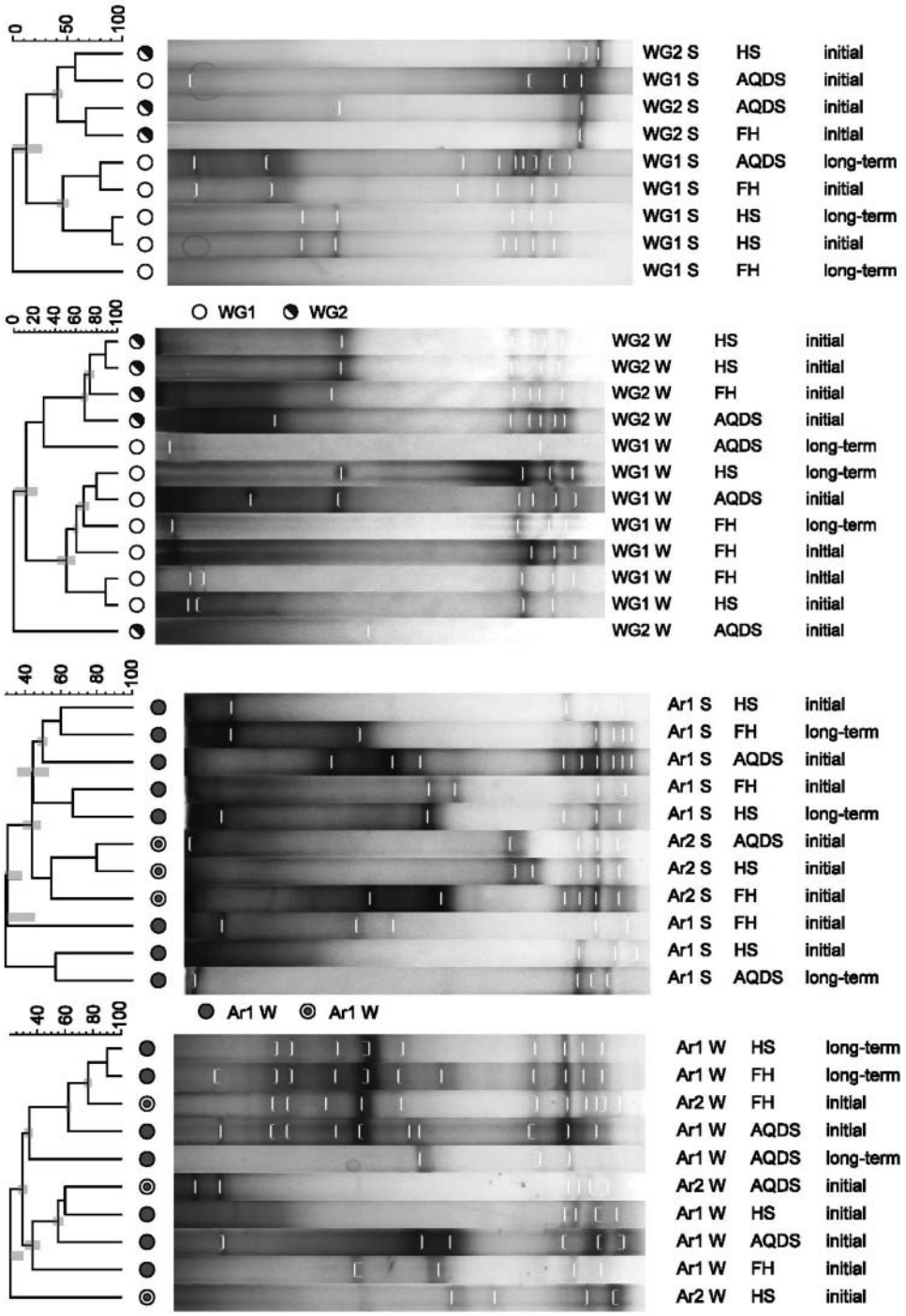

Fig. 2. DGGE banding patterns, identified bands and UPGMA dendrograms of the different initial and long-term enrichment cultures. Open and half-filled circles at the bottom of the dendrograms indicate the different origin of the cultures (different aquifer samples they were enriched from) in order to illustrate the clustering according to origin.

set up from the MPN plates with a mixture of lactate and acetate as electron donor and either ferrihydrite, HS or AQDS as electron acceptor. We transferred the inocula from the highest positive dilution in the MPN plates 1–2 times to fresh growth medium in order to enrich for microorganisms able to reduce the respective electron acceptor before the community composition of the enrichment cultures was analyzed by DGGE.

The DGGE fingerprints (Figure 2) of the different enrichment cultures were analyzed by calculating a similarity matrix based on Dice's coefficient of similarity followed by UPGMA cluster analysis. Due to the difficulty of comparing DGGE fingerprints located on different gels and because the two field sites where fundamentally different with regard to the identity of the contamination (organics in the one case versus arsenic in the second one), only cultures enriched from

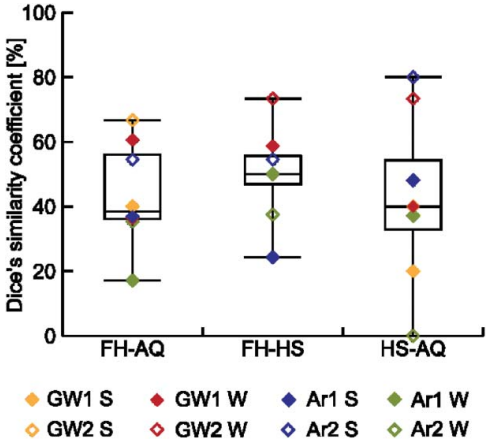

Fig. 3. Dice's similarity coefficients of pairs of cultures enriched from the same aquifer sample using different electron acceptors. FH = Fe(III)-reducers, AQ = AQDS-reducers, HS = humic-substance reducers. Boxplots indicate the median, 25% and 75% quartile, minimum and maximum of the coefficient values.

water or sediment from the same aquifer were compared to each other. In the resulting dendrograms, the fingerprints clustered mainly according to the samples the cultures were originally enriched from (Figure 2). Jackknife resampling yielded a correct classification of 70–91.7% of the enrichment cultures for three of the four DGGE gels analyzed. Only the water samples from Araihazar aquifer did not cluster according to sampling site (Jackknife 28.6%) but instead clustering was random and neither followed the sampling site nor the electron acceptor. The cluster analyses did generally not reveal any clustering of the cultures according to the electron acceptor they were enriched on (Jackknife 33–47% for all gels).

To find out whether Fe(III) and HS are being reduced by the same bacterial groups in our investigated anoxic aquifers, we wanted to know how similar the communities of Fe(III)-reducers and HS-reducers were that have been enriched from the same sample. To this end, we calculated the Dice's coefficients of similarity of the enrichment cultures of Fe(III)- and HS-reducers from the same sample. These values were then compared to the similarity of the HS- and AQDS-reducers and to the similarity of the Fe(III)- and AQDS-reducers (Figure 3).

The analysis did not show any significant difference between the similarities of either of the combinations of enrichment cultures. That means the similarity of the HS-reducer enrichments to the corresponding AQDS-reducer enrichments was not significantly different from the similarity of the HS-reducer enrichments to the corresponding Fe(III)-reducer enrichments (paired Wilcoxon, $s = -5$, $n = 8$, $p = 0.4688$). This indicates that based on DGGE banding pattern comparisons, the microbial cultures enriched with all three electron acceptors were equally similar to or different from each other.

Diversity of the Microbial Enrichment Cultures

Since it was shown that HS can also be reduced by physiological groups of microorganisms such as fermenters or halorespirers (Benz et al. 1998; Cervantes et al. 2002) that are not able to reduce Fe(III) directly, we wanted to know whether the HS-reducer enrichment cultures were more diverse than the enriched Fe(III)-reducer cultures. In order to answer this question, three different indices of diversity were calculated from the DGGE banding patterns of the enrichment cultures (Figure 4).

Fig. 4A shows that there was no significant difference between the Shannon–Weaver index (H') of the HS-reducer enrichments and the corresponding Fe(III)-reducer enrichments of the first or second generation (paired Wilcoxon, $s = -3$, $n = 8$, $p = 0.7422$). Additionally, the functional organization was not significantly different between the cultures enriched on different electron acceptors (Figure 4B), i.e., no increased evenness of the HS-reducer enrichments compared to the Fe(III)-reducer enrichments was observed (paired Wilcoxon, $s = -1$, $n = 8$, $p = 0.9453$).

As a third index of diversity, we calculated the range-weighted richness (Rr) (Figure 4C). Unlike the other two diversity indices, the Rr was significantly lower for the HS-reducer enrichments than for the other two enrichment cultures (FH and AQDS) from the same sample (HS-FH: paired Wilcoxon, $s = -15$, $n = 8$, $p = 0.0391$; HS-AQDS: paired Wilcoxon, $s = -15$, $n = 8$, $p = 0.0391$). Since H', which also takes into account the number of bands in the pattern, did not show any significant differences, the differences measured in the Rr can be attributed to differences in the denaturing gradients spanned by the patterns, i.e., differences in the diversity of the GC-content of the 16S rRNA genes. The HS-reducing enrichment cultures thus are less diverse in terms of GC-content of the 16S rRNA genes than the Fe(III)- and AQDS-reducing enrichment cultures.

Diversity of the Enrichment Cultures after Long-Term Enrichment

After the initial DGGE analysis, the three different physiological types of enrichment cultures reducing either Fe(III), HS or AQDS were further transferred to fresh growth medium several times. After more than 10 transfers on the same medium containing the same electron acceptor, DNA was extracted and the diversity of the enrichment cultures was analyzed. The goal of these experiments was to see if the repeated transfers resulted in any further enrichment, i.e., a decrease of diversity in the enrichments. Due to the different substrate turnover and growth rates of the different enrichment cultures, the number of transfers that had taken place during this time varied between 11 and 17 (Figure S1 and Table S2). The diversity indices of the long-term enrichment cultures are also shown in Figure 4 denoted as "long-term."

As can be seen in Figure 4, none of the three diversity indices differed significantly between the initial and long-term enrichment cultures (H': paired Wilcoxon, $s = -5$, $n = 12$, $p = 0.7334$; Rr: paired Wilcoxon, $s = 3$, $n = 12$, $p = 0.8501$; Fo: paired Wilcoxon, $s = 0$, $n = 11$, $p = 1$). This indicates that the evenness and richness of the enrichment cultures did not significantly change over several transfers but stayed at the same level as calculated for the initial transfers from the MPN plates.

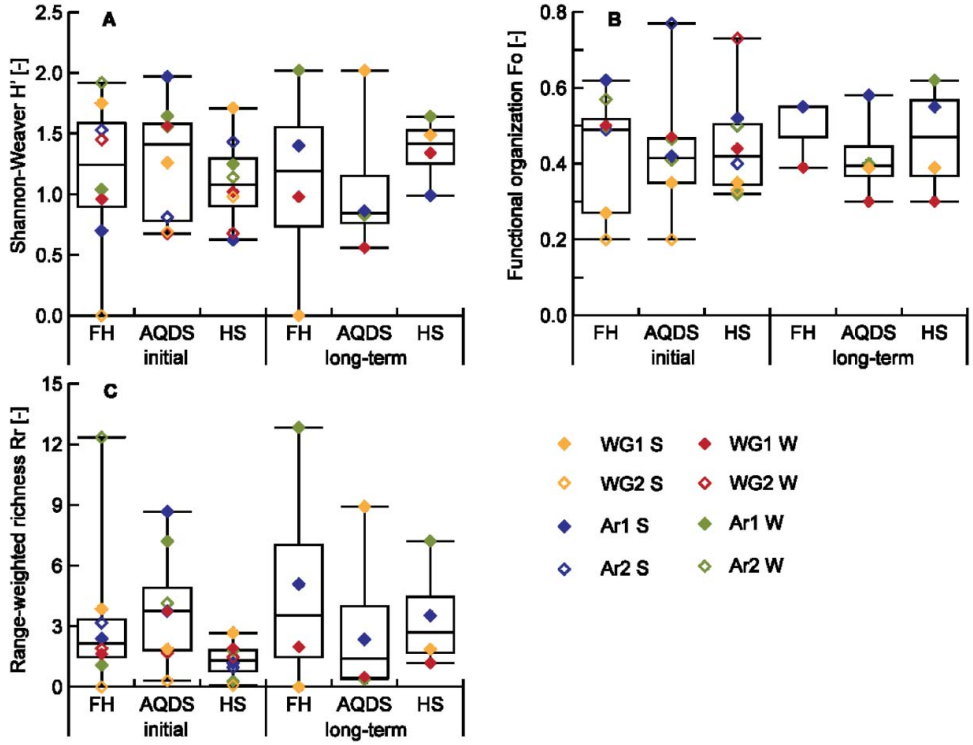

Fig. 4. Diversity of the cultures enriched from the different aquifer samples after 1–2 transfers (initial) and after more than 10 transfers (long-term) using Fe(III) (FH), AQDS or humic substances (HS) as electron acceptors. (A) Shannon–Weaver index of diversity (H'), (B) Functional organization (Fo) and (C) Range-weighted richness (Rr). Boxplots indicate median, 25 and 75% quartiles, minimum and maximum of the distributions.

Physiological Flexibility of the Enrichment Cultures

Finally, we determined if the enrichment cultures that were transferred more than 10 times on the same electron acceptor (either ferrihydrite, HS or AQDS) still retained the ability to reduce HS and Fe(III). To this end, we set up reduction experiments with the long-term enrichment cultures from sites WG1 and Ar1 to compare HS and Fe(III) reduction performance by the three cultures enriched from the same aquifer sample. In addition to the amount of electrons transferred to Fe(III) or HS over time, total cell numbers were quantified at the beginning and at the end of the experiments by qPCR. The results are shown in Figure 5 (experiments with the three enrichment cultures from the sediment sample from the anoxic aquifer Ar1) and Figure S2 (all other experiments).

The HS reduction experiments showed that all four tested long-term Fe(III)-reducer enrichment cultures enriched from water and sediment from sites WG1 and Ar1 were able to reduce HS, even after being transferred 11–16 times with only ferrihydrite as electron acceptor (Figure 5A, Figure S2). In some cases, as shown in Figure 5A, the Fe(III)-reducer enrichments reduced HS even faster and to a higher extent than the corresponding HS-reducer enrichment. Because both the Fe(III)- and the HS-reducing enrichment culture

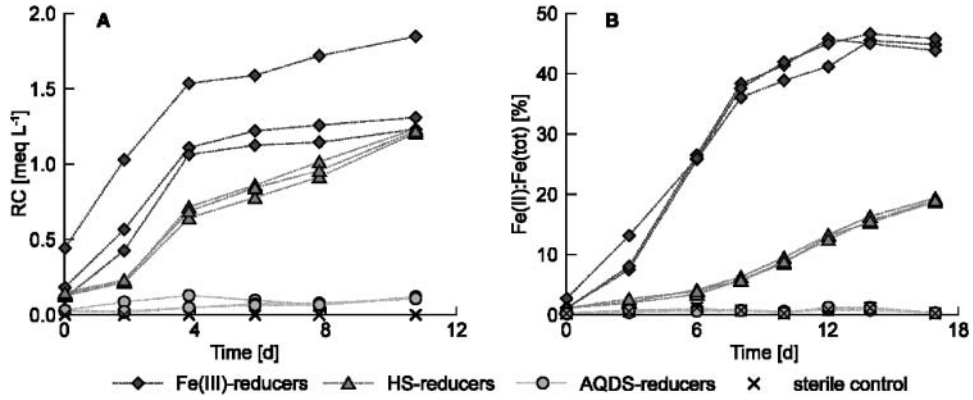

Fig. 5. Humic substance (A) and ferrihydrite (B) reduction by the three enrichment cultures obtained from the sediment sample of site Ar1. Identical symbols show development of triplicate bottles.

from Ar1 sediment contained a total cell number of about 3×10^5 cells mL^{-1} in the beginning of the HS reduction experiment, the observed differences cannot be attributed to differences in cell numbers. During HS reduction by the Fe(III)-reducing enrichment cultures from sediment and water samples from site WG1, the cell numbers increased from 10^4 to 10^5 and from 10^6 to 10^7 cells mL^{-1}, respectively, showing that the enriched Fe(III)-reducers were able to grow by HS reduction.

In contrast, the HS-reducer enrichment cultures did not perform as well in the Fe(III) reduction experiments. Three out of four HS-reducer enrichment cultures reduced ferrihydrite, however, with a considerably lower rate than the corresponding Fe(III)-reducer enrichments (Figure 5B, Figure S2). Nevertheless, the slow Fe(III) reduction by the HS-reducer enrichments was in some cases still accompanied by cell growth. For example, an increase from 10^5 to 10^7 cells mL^{-1} could be measured during ferrihydrite reduction by the HS-reducer enrichment culture from the Ar1 sediment sample.

The performance of the AQDS-reducer enrichment cultures varied the most. Two enrichment cultures did not reduce any Fe(III) (Figure 5B, Figure S2), while the other two enrichment cultures reduced Fe(III) even faster and to a higher extent than the corresponding Fe(III)-reducer enrichments (Figure S2). Interestingly, also the performance of the AQDS-reducers in the HS reduction experiments varied. While two AQDS-reducer enrichments reduced HS at a similar rate and extent as the corresponding HS-reducer enrichments (Figure S2), the other two AQDS-reducer enrichment cultures transferred only very few electrons to HS (Figure 5A).

Discussion

To assess which groups of microorganisms are reducing humic substances (HS) in anoxic aquifers, we studied Fe(III)-reducing and HS-reducing microbial communities in eight different aquifer samples in terms of their abundance, diversity, and physiological flexibility. We found that HS-reducing microorganisms were present in seven out of our eight samples (Figure 1). Even the one sample in which no HS-reducers could be detected (sediment sample WG2) contained AQDS-reducers. Considering the findings of Coates et al. (1998) that all AQDS-reducers isolated in their study were also able to reduce HS, and taking into account the highly reducing conditions at the site (Table S1), HS-reducers would be expected in this sample.

Given that in this sample the Fe(III)- and AQDS-reducers were also present at relatively low numbers (2.8×10^1 and 1.1×10^3 cells mL^{-1}, respectively), the number of HS-reducers in sample WG2 sediment might have been just below the detection limit of the MPN method. MPNs of HS-reducers in the other samples ranged from 10^3 to 10^6 cells g^{-1} and thus agreed well with cell numbers previously published for the same physiological types of microorganisms in other environments. Coates at al. (1998) found 10^4–10^6 cells g^{-1} wetland sediment using acetate as electron donor and AQDS as electron acceptor and Kappler et al. (2004) quantified 10^6–10^7 cells of HS-reducers mL^{-1} in different depths of Lake Constance sediment.

Comparison of HS- and Fe(III)-Reducing Microbial Communities

The physiological flexibility experiments showed that all of the Fe(III)-reducer enrichment cultures, even after long-term enrichment with only ferrihydrite as electron acceptor, were still able to reduce HS (Figure 5). This suggests that most if not all Fe(III)-reducers present in our aquifer samples are also able to reduce HS. This is in agreement with a study by Lovley et al. (1998), who showed that out of twelve tested cultures of known neutrophilic Fe(III)-reducing bacteria, all of them were also able to reduce HS and AQDS. Absence of HS reduction has so far only been described for an acidophilic Fe(III)-reducer (Emmerich and Kappler 2012), which is not surprising since Fe (III) is more soluble at acidic pH while humic acids are mostly precipitated and thus present as solid-phase at this low pH.

The numbers of HS-reducers quantified by the MPN technique were in the same range or even higher than the numbers of Fe(III)-reducers in the same sample (Figure 1). This indicates that, besides Fe(III)-reducers, the HS-reducing microbial communities must additionally include some microorganisms that are not able to reduce Fe(III) directly as it was shown previously for some fermenters (Benz et al. 1998), halorespirers and methanogens (Cervantes et al. 2002). Especially methanogens can be expected to be abundant in the samples from the Weißandt-Gölzau aquifer that showed methanogenic redox conditions (see methane concentrations provided in Table S1). More support for the involvement of microorganisms other than Fe(III)-reducers in HS reduction comes from the physiological flexibility experiments with the HS-reducing enrichment cultures which showed that HS-reducing enrichments reduced ferrihydrite only slowly compared to the corresponding Fe(III)-reducer enrichment cultures (Figure 5).

The slow ferrihydrite reduction could partly be due to the presence of low concentrations of HS which were transferred with the inocula and could have blocked the ferrihydrite surface by sorption to reactive surface sites (Piepenbrock et al. 2011), or to the need of a physiological adaptation of the microorganisms to ferrihydrite reduction, i.e., the induction of specific terminal reductases. Nevertheless, the slow Fe(III) reduction by the HS-reducing enrichment cultures indicates that the HS-reducer enrichment cultures contained some microorganisms, which were not able to reduce ferrihydrite in addition to some HS-reducers, which were indeed able to reduce Fe(III). For the microorganisms that are unable to reduce ferrihydrite directly, electron shuttling, e.g., via HS, is the only way to reduce Fe(III) minerals. The same observation was made by Kappler at al. (2004), who found only 10^4–10^6 cells mL^{-1} of Fe(III)-reducers but 10^6–10^7 cells mL^{-1} of HS-reducers in Lake Constance sediments.

Based on the MPNs and the physiological flexibility experiments, one would expect a different species composition of the HS- and Fe(III)-reducing enrichment cultures and a higher species diversity among HS-reducers. However, the comparison of the 16S DGGE banding patterns of the initial enrichment cultures indicated that the similarity between the HS-reducer and the corresponding Fe(III)-reducer enrichments was as high as the similarity between the HS-reducer and the respective AQDS-reducer enrichment (Figure 3). Even though

this is only a relative description based on DGGE banding patterns which does not give any indication about the absolute similarity nor the actual species composition of the enrichment cultures, it suggests that the microbial communities of the HS- and Fe(III)-reducers are not as different as expected based on the physiological flexibility experiments. This is consistent, however, with a finding that was reported by Coates at al. (1998), who isolated AQDS-reducers from different environments using acetate as electron donor and found that all of the isolates could also reduce Fe(III).

Also in terms of diversity (Shannon–Weaver index, functional organization), no difference between the HS-reducing and the corresponding Fe(III)-reducing enrichment cultures could be observed (Figure 4). This result is surprising considering that the HS-reducer enrichments were expected to comprise all Fe(III)-reducers (Lovley et al. 1998) and additionally some sulfate-reducers or fermenters, which are not able to reduce Fe (III) directly (Benz et al. 1998; Cervantes et al. 2002). However, our results indicate that the HS-reducer enrichments probably contain some different species than the Fe(III)-reducer enrichments (as suggested by the slower ferrihydrite reduction) but overall are not characterized by a higher species diversity. This is also confirmed by the lower range-weighted richness of the HS-reducers compared to the Fe(III)-reducers (Figure 4C).

This difference in species composition between HS- and Fe(III)-reducing enrichment cultures could be caused by two effects: i) the random effect of the sub-sampling and dilution series, and ii) the 1-2 transfers of the enrichment cultures in growth medium containing only one electron acceptor (either HS, FH or AQDS) prior to the DGGE analysis. Therefore, from our data it cannot be clarified whether the observed differences between the Fe(III)- and HS-reducing enrichments are due to structural differences between the Fe(III)- and HS-reducing communities *in situ* or whether the differences are a consequence of the enrichment process. It also has to be considered that only a small fraction of the total microbial diversity will grow in batch cultures (e.g., Ferrari et al. 2008) and that only populations that make up at least 1% in relative abundance will be detected by DGGE (Muyzer et al. 1993). Therefore, our DGGE analysis only considered a certain fraction of the total microbial community capable of reducing HS and Fe(III) in the aquifers. A more direct, activity-based analysis of the HS- and Fe(III)-reducing microbial communities in the environment would require knowledge on specific genes and/or enzymes involved in only HS- or Fe(III) reduction, which are not available until now.

Suitability of AQDS as a Proxy for HS in Microbial Enrichments

AQDS (9,10-anthraquinone-2,6-disulfonic acid) is a model quinone which is often used as a proxy for quinoid moieties in HS in studies of HS reduction and HS electron shuttling (e.g., Coates et al. 1998; Lovley et al. 1998). However, AQDS differs from HS in some important aspects: Previous studies on microbial Fe(III) reduction in the presence of HS and AQDS indicated that AQDS stimulates microbial Fe(III) reduction even at very low concentrations while low HS concentrations decreased the reduction rates (Piepenbrock et al. 2011). This is probably due to the sorption of HS to the Fe(III) minerals surface, while AQDS does not show strong sorption to Fe(III) minerals (Wolf et al. 2009). Furthermore, high concentrations of AQDS were shown to have a toxic effect on some microorganisms (Shyu et al. 2002). Therefore, we wanted to determine how suitable AQDS is as a model compound for HS in quantification and enrichment of HS-reducers from environmental samples by comparing the abundance, diversity and physiological flexibility of HS- and AQDS-reducers from the same aquifer material.

MPNs of AQDS- and HS-reducers were similar in five out of our eight aquifer samples. However, in two samples, we detected considerably lower numbers of AQDS-reducers than HS-reducers (Figure 1). The physiological flexibility experiments showed that two of the four studied AQDS-reducer enrichments transferred only few electrons to HS (Figure 5A), while the other two enrichments reduced HS to the same extent as the corresponding HS-reducer enrichments. The most striking result, however, was the finding that the similarity (Dice coefficient determined from the DGGE banding patterns) between the HS- and corresponding AQDS-reducer enrichment cultures was not higher than the similarity between the HS- and Fe(III)-reducers or between the Fe(III)- and AQDS-reducer enrichment cultures.

Taken together, these findings indicate that AQDS is reasonably well suitable as a surrogate for HS (i.e., for quinoid moieties in HS) with respect to enrichment and quantification of HS-reducers. This is not necessarily expected given the difference in redox potential between HS and AQDS (Aeschbacher et al. 2011; Wolf et al. 2009) and the potential toxicity of AQDS at high concentrations (Shyu et al. 2002). On the other hand, the similarity of HS- and AQDS-reducing enrichment cultures agrees well with the finding that both HS and AQDS are being reduced to a large extent at the microbial cell surface and not in the cytoplasm (Gescher et al. 2008). It also fits to a study by Coates et al. showing that a number of different AQDS-reducer enrichments were all able to reduce HS (Coates et al. 2001). Nevertheless, results obtained with AQDS as model of HS should always be interpreted very carefully, since our results indicated that cultures enriched using AQDS instead of HS as electron acceptor are by no means identical. In fact, cultures enriched with AQDS as electron acceptor were not more similar to HS-reducers than enrichment cultures reducing ferrihydrite. Thus, unambiguous information about HS-reducing microorganism can only be obtained if HS are being used for the quantification and enrichment of the microorganisms. Ideally, HS should be used which are similar to the organic matter present in the environment (i.e., they need to be isolated from the environment studied), since different HS samples vary greatly in structure and composition (Stevenson 1994) and two enrichment cultures using two very different kinds of HS are also expected to differ with respect to community composition.

Implications for Microbial HS Reduction and Electron Shuttling in Anoxic Aquifers

The comparison of anoxic aquifer enrichment cultures reducing either HS, AQDS, or ferrihydrite showed that all three

electron acceptors selected for microbial communities that were not identical, but overall showed a high similarity in terms of DGGE banding patterns as well as physiology. Furthermore, it was striking that none of the three diversity indices calculated showed a decrease in diversity from the initial enrichment cultures compared to the long-term enrichment cultures. This indicates that even continued transfers using only one particular electron acceptor (either HS, FH, or AQDS) did not lead to a high degree of functional specialization in the enrichments, but sustained the growth of a diverse microbial community with the ability to reduce HS, Fe(III), or ADQS.

This agrees well with the finding that the ability to reduce Fe(III) is widespread among different phylogenetic groups of bacteria and archaea (Thamdrup 2000; Weber et al. 2006). The metabolic versatility among known Fe(III)-reducers is also reflected in their broad electron donor spectrum including hydrogen, short- and long-chain fatty acids, alcohols and aromatic compounds (Thamdrup 2000). Many Fe(III)-reducers can also use a variety of alternative electron acceptors such as oxygen, nitrate, uranium or electrodes (Lovley et al. 2004). Even some fermenters, sulfate-reducers and methanogens have been shown to reduce some Fe(III), even though it is not clear if they gain energy from the process (Lovley et al. 2004).

Just like the phylogeny of the Fe(III)-reducers, the enzymes used for microbial Fe(III) reduction are highly variable between different species and seem to be unspecific in many cases (Richter et al. 2012). The main requirement seems to be the expression of any type of *c*-type cytochromes which can transfer the electrons to the cell surface (Bird et al. 2011), and which are used not only for Fe(III) reduction but also for the reduction of other extracellular electron acceptors such as Mn(IV) oxides or electrodes (Richter et al. 2011). HS and AQDS reduction by the Fe(III)-reducers *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 have also been shown to proceed via the same protein complexes used for direct Fe(III) reduction (Gescher et al. 2008; Lies et al. 2005; Voordeckers et al. 2010). The fact that all three electron acceptors are reduced by the same enzymes at least in dissimilatory Fe(III)-reducing microorganisms, might be one reason for the high similarity observed between our enrichment cultures.

These considerations indicate a scenario in which HS as well as Fe(III) are being reduced in anoxic aquifers by diverse groups of microorganisms using a variety of functionally more or less redundant proteins and cytochromes for the electron transfer to extracellular electron acceptors. If and to what extent the electron flow towards Fe(III) minerals proceeds via electron shuttling HS is thus expected to depend rather on the geochemical conditions, i.e., the availability of Fe(III) mineral surfaces and the concentration of HS, than on the microbial community.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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