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Microbes-Assisted Arsenate Reduction *Activity* in Bangladesh Drinking Water Wells as Revealed by Enrichment Cultivation

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ABSTRACT

Arsenate-reducing microorganisms may mobilize arsenic into groundwater. Phylogenetic DNA sequence information cannot exclusively be used to infer arsenate reduction, as the spotted homologies may not translate to biochemical activities. We here develop a cultivation-based metagenomic strategy for inferring actual activities: we complement a cultivation-independent microbial community survey covering 22 arsenic-contaminated drinking water wells in Bangladesh, with the characterization of enrichments of anaerobic arsenate reducers. All investigated samples revealed microbial arsenate reduction activity. The enriched microbial communities were phylogenetically diverse within and between the enrichments. The enrichment also unveiled species: the arsenate-reducing enrichments held 16S rRNA gene sequences closest to *Dechloromonas* sp., *Azonexus fungiphilus, Youngiibacter fragile, Methanogenic prokaryote* and *Fusibacter* sp., in addition to arsenate-reducing *Sulfurospirillum* strains NP4, b10 and dissimilatory iron-reducing *Geobacter* species. The enriched arsenate reductase gene (*arrA*) sequences were closest to sequences in known arsenate reducing *Sulfurospirillum barnesii*. This study bridges the knowledge gap between cultivation-independent and cultivation-dependent analyses. It clarifies the mechanism for arsenic mobilization and redistribution in rural drinking water wells, as a balance between bioremediation and harmful activities. The identified, potentially dissimilatory arsenate and iron-reducing microbes, constitute a risk for arsenic bioremediation. Appropriate metagenomic and dynamic analyses should help design and maintain safer, effective bioremediation.

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Introduction

Lack of access to clean water is a major concern in Bangladesh where natural contamination of groundwater by arsenic has been generating one of the largest health catastrophes in the world (Adeel 2001). The high and widespread abundance of arsenic in groundwater has led the World Health Organization (WHO) to set the guideline standard for arsenic in drinking water in Bangladesh to 50 µg/l, whereas the universal standard is 10 µg/l (Flanagan et al. 2012; WHO 2011). Long-term exposure to such trace amounts of arsenic (As) in shallow groundwater wells used for drinking, cooking and irrigation puts millions of people at risk of chronic disease (Baker et al. 2018; Cubadda et al. 2017). In Bangladesh alone, 35–80 million people are exposed chronically to arsenic in drinking water, resulting in 40,000 deaths annually (Flanagan et al. 2012). The arsenic contamination is thought to be biogeogenic, originating in the sediments in the upland Himalayan catchments, eluting into the major rivers that run through these catchments and accumulating in the Bangladesh sediments (Harvey et al. 2002; Newman 2010; Nickson et al. 2000). Transformation of arsenic species and subsequent liberation into the groundwater is regulated by the indigenous microorganisms (Zhu et al. 2017). Several studies have suggested microbial processes (dissimilatory reduction or detoxification) are substantial contributors to arsenic contamination in the aquifers of Bangladesh (Fendorf et al. 2010; Gnanaprakasam et al. 2017; Hassan et al. 2015, 2019; Mailloux et al. 2009). Microbial reduction of arsenate to arsenite directly increases the mobility, toxicity, and distribution of arsenic and subsequently limits arsenic adsorption on sediment minerals (Cui and Jing 2019; Tamaki and Frankenberger 1992). Although microbial processes are implicated in mobilizing arsenic

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from aquifer sediments into groundwater, the precise mechanism remains ambiguous (Gnanaprakasam et al. 2017).

Microbial arsenate reduction occurs through a variety of pathways. A diverse range of microorganisms contributes to arsenate reduction through Gibbs energy extraction or through intercellular detoxification processes (Malasarn et al. 2004; Saltikov and Newman 2003; Stolz et al. 2006). The 'AB' gene cluster in bacteria, containing the *ars* and *arr* operon, is used for detoxification and energy-conservation processes, respectively. Both of these processes produce arsenite [As(III)], which is usually the dominant form of aqueous arsenic in contaminated aquifers (Afkar et al. 2003; Krafft and Macy 1998; Lloyd and Oremland 2006). During detoxification, arsenate enters the cell through phosphate uptake channels (Pst or Pit), due to structural homologies with phosphate ions. Once in the cytoplasm, arsenate [As(V)] is reduced by the cytoplasmic arsenate reductase ArsC to arsenite, which is then pumped out from the cell via the cytoplasmic transmembrane protein ArsB (Mukhopadhyay and Rosen 2002; Rosen 1999). In the case of dissimilatory arsenic reduction, As(V) is used by a number of prokaryotes as the terminal electron acceptor under anoxic conditions. The reduction of the latter is mediated by the respiratory arsenate reductase Arr, a periplasmic molybdoprotein (Ji and Silver 1992; Saltikov and Newman 2003; Zobrist et al. 2000). Soluble arsenate can directly be reduced to arsenite if coupled with the oxidation of electron donors, thereby releasing Gibbs energy to support growth. Microorganisms performing this process are referred to as dissimilatory arsenate reducing prokaryotes (DARPs) (Oremland and Stolz 2003). DARPs belong to groups such as Gram positives, β-, γ-, δ-, and ε-*Proteobacteria*, thermophilic Eubacteria, *Chrysiogenes arsenatis*, *Crenarchaeota*, Firmicutes, Aquificae and Deferribacteres (Mohsin et al. 2021). These metabolically diverse groups can use a wide range of inorganic compounds as electron donors ranging from H_2 to small organic acids and sugars and further to complex aromatic substrates like benzoate and toluene. These microorganisms can have a significant impact on the mobilization of both adsorbed and dissolved forms of arsenate via the conversion of the latter to the more toxic and more soluble and hence more mobile arsenite.

We hypothesized that a diverse range of cultivable arsenate-reducing microorganisms is active in arsenic contaminated groundwaters in Bangladesh. In a cultivation-independent survey of 22 drinking wells in Bangladesh we confirmed an abundance of nucleic acids homologous or identical to arsenate reductase (*arrA*) genes of anaerobic arsenate reducers (Hassan et al. 2015). However, cultivation-independent nucleic-acid-based approaches have limitations (Lin et al. 2007) and organisms detected on the basis of nucleic acid traces of arsenic related genes, may not be enzymatically active in arsenic biotransformation. In the present study, we developed a cultivation approach to test for arsenic biotransformation activity.

We here report that we could indeed detect arsenate reduction after cultivation enrichment. This suggests a widespread risk of arsenic dissemination in subsurface groundwater and sediments, due to arsenate reducing prokaryotes. We discuss how bioremediation of arsenic in Bangladesh drinking water wells requires a systems approach informed by monitoring the microbial ecology also in enrichment cultures.

Materials and methods

Field sampling

Between August 2011 and March 2012, a total of 22 groundwater samples were collected from shallow and deep tubewells in the Jessore, Satkhira and Comilla districts in Bangladesh. Anaerobic groundwater samples were collected in sterile serum glass bottles by letting the bottles overflow, after 3 volumes of standing water in each tubewell had been removed by hand pumping. Bottles with groundwater samples for enrichment were capped with small headspace and transferred to the laboratory, where they were stored for less than 24 h at 4 °C. Details on the hydrochemistry of these samples were reported previously (Hassan et al. 2015).

Enrichment of anaerobic dissimilatory arsenate-reducing microorganisms

Strictly anaerobic techniques were employed for the activity analysis and enrichment of anaerobic arsenate-reducing microorganisms. The anoxic minimal medium was largely based on the experience of Santini et al. with some modifications (Santini et al. 2002). The medium contained 20 mM NaCl, 4.0 mM KCl, 2.8 mM NH_4Cl, 1.5 mM KH_2PO_4, 0.2 mM Na_2SO_4, 20 mM $MgCl_2.6H_2O$, 1.0 mM $CaCl_2.2H_2O$, 0.05% $NaHCO_3$, 20 mM Na-lactate, 3.0 mM Na-acetate, 3.0 mM $Na_2HAsO_4.7H_2O$ ('arsenate'; Sigma-Aldrich, Germany), 1.0 ml/l trace element solution, and 1.0 ml/l vitamin solution (medium 141, DSMZ). Additionally, 0.1 mM of L-cysteine was used as oxygen scavenger. Anaerobic medium was prepared in serum vials sealed with butyl rubber septa and crimped with aluminum caps. The basal medium was flushed with a mix of N_2 and CO_2 (80/20%). All media were sterilized for 15 min at 121 °C without the phosphate, bicarbonate, vitamins, trace elements, and arsenate: these components were added after the media had cooled to room temperature. Subsequently, pH was adjusted to 7.00 with 1.0 mM sterilized H_2SO_4. Arsenate-reducing enrichments were performed on six groundwater samples out of 22 (Table 1). One milliliter of sample was inoculated into an anaerobic serum vial containing 9 ml of medium. These cultures were tenfold serially diluted up to 10^{-3} in the same medium and incubated in the dark at 28 °C for 2–3 weeks. Anaerobic serum vials with media without inoculation served as negative controls. After the 2–3 weeks of incubation, the cultures were quick-screened for the reduction of arsenate in enrichment media using a qualitative test where potassium permanganate ($KMnO_4$) was used as oxidizing agent for any arsenite produced (Fan et al. 2008): 20 µl of a 0.01 M $KMnO_4$ solution was added to 1.0 ml of the culture. A clear to yellowish color

Table 1. Cultivation of arsenate-reducing microorganisms initiated from six drinking water wells in Bangladesh.

Location		Physicochemical parameters				Arsenate reducing enrichments		
Sample ID	Name of the village	Depth (m)	pH	$[As_{total}]$ (μM)	NO_3^-(mg/l)	Dilution factor at which arsenite was still produced	Dilution factor at which arsenite was no longer produced	*arrA*
A1	Assasuni sadar	14	6.8	1.6	24	100	1,000	–
N1	Nagda	146	8	0.06	0.05	10	100	–
NA1	Nawapara1	23	6.9	0.09	0.08	10	100	+
T1	Tarali	49	6.7	3.3	0.06	10	100	+
NA2	Nawapara2	49	6.8	3.5	3	100	1,000	+
K1	Kaliganj sadar	29	6.8	0.1	25	100	1,000	+

The well is described by sample code or ID in terms of name of the village in which the well is located. The Table gives the full name of the village, well depth and physicochemical parameters. All villages are in the Satkhira district. The final column indicates the results of molecular searches for specific functional genes indicative of potential for anaerobic arsenate biotransformation, i.e., *arrA* (arsenate reductase gene): +: detected, –: not detected.

of the supernatant was taken to indicate a 'positive culture' able to reduce the purple/pink permanganate to yellowish MnO_2, i.e., to evidence the presence of at least 0.2 mM of arsenite. This color developed either within a minute or not at all, i.e., the test evidences the presence of arsenite produced during the 2–3 weeks incubation, not the capacity of the microorganisms to produce arsenite or to reduce permanganate during the quick assay. A persisting pink color was taken as evidencing the persistence of the arsenate, which does not react with permanganate, suggesting 'negative growth of arsenate reducing microbes.' Oxidation of arsenite in the presence of the oxidizing agent $KMnO_4$ takes place by the following reaction:

$$3As^{3+} + 2MnO_4^- + 8H^+ \rightarrow 3As^{5+} + 2MnO_2 + 4H_2O$$

The culture with the highest dilution factor (i.e., 10^n) that showed such permanganate reduction ability was subsequently diluted at that same dilution factor into fresh medium and incubated again for 2–3 weeks. This procedure was repeated three to four times. For the molecular analyses specified below, each arsenate-reducing positive culture from the highest total dilution (i.e., 10^{5n}; see below) was vacuum filtered over a 45-mm-diameter, 0.2-µm-pore-size nitrocellulose membrane filter (Millipore, Billerica, MA, USA) and the residue was frozen at −20 °C until DNA isolation.

Rationale behind the enrichment experiments

The enrichment experiments establish for which value of n ($n = 1$, 2, or 3) a 10^n-fold dilution of the sample is able to produce more than 0.2 mM of arsenite from the arsenate in three weeks, whereas a 10^{n+1}-fold dilution was not able to do so. Subsequently, a culture is only considered to be 'positive at dilution factor 10^n' if a 10^{4n} times further dilution (carried out serially in four steps) was still able to produce the arsenite in a two to three weeks incubation. This excludes the interpretations that the initial sample contained sufficient arsenite, or sufficient cell numbers of arsenate reducing microorganisms that themselves, i.e., without multiplication by growth, were responsible for the observed arsenate reduction after final subculture. Accordingly, the interpretation of these enrichment experiments is that during each 2–3 weeks' incubation the arsenate reducing microorganisms were engaged in growth energized by the reduction of arsenate by lactate or acetate. Consequently, these experiments, if positive for any value of n, are taken to demonstrate (i) that microorganisms in the culture are active in anaerobic reduction of arsenate to arsenite and (ii) that these microorganisms are able to grow on the basis of respiration with arsenate as electron acceptor. Moreover they lead to a sample that is manifold (10^{5n}) enriched in terms of such microorganisms as compared to other organisms that are indifferent with respect to the energy potential of the anaerobic combination of lactate (or acetate) and arsenate.

The magnitude of n is an indication of the specific growth rate of the organisms under the laboratory conditions: that rate should be sufficient to overcome, in a period of 2–3 weeks, the 10^n-fold dilution, but not the 10^{n+1}-fold dilution. Writing t_d for the doubling time (in days) of the organisms, this implies that $10^n < 2^{18/t_d} < 10^{n+1}$: For $n = 2$ their doubling time should range between 1.8 and 2.7 days and for $n = 1$ between 2.7 and 5.4 days.

DNA extraction

DNA was extracted using the soil DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad, CA, USA) according to manufacturer's instructions. DNA was stored at −20 °C until required for further analysis.

DGGE profiling of enrichment cultures

Partial 16S rRNA gene sequences were amplified using the bacterial primer set 357 F–GC clamp and 907r (for PCR conditions, see Supplementary Table S1). Each PCR reaction was carried out in a 25-µl (total volume) mixture containing 12 µl of GoTaq (Promega; USA) ready Master Mix, 1 µl of each primer (0.4 µM final concentration), 8 µl of nuclease free water (Promega; Madison, WI, USA) and 3 µl of undiluted DNA as template.

Denaturing gradient gel electrophoresis (DGGE) was carried out using a DcodeTM Universal Mutation Detection System (BIORAD Laboratories, CA, USA). PCR product was loaded onto a 1 mm thick 8% (w/v) polyacrylamide (ratio of acrylamide to bis-acrylamide, 37.5:1) gel containing a linear

gradient of 30–55% of urea–formamide. The running conditions were 200 V at a constant temperature of 60 °C in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA, pH 8.0) for 4 h. The gels were stained in 1× TAE buffer containing 1 µg/ml of ethidium bromide and visualized using a UV transilluminator. To aid normalization of and comparison between gels, a DGGE marker (M12) consisting of 12 bands was added to the external lanes of the gels, as well as to lanes in between every four samples. All gels fingerprinting a particular type of enrichment were run on the same day. The average between-gel similarity of the marker lanes was 95%, with 3% standard deviation.

Bands were excised using sterile wide-mouth-blunt tips. Excised DNA bands were suspended in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored overnight at 4 °C. One microliter of suspension was used as template in the aforementioned PCR, using primers without GC clamp. Products were checked on 1.5% agarose gels and sequenced (Macrogen, Amstelveen, The Netherlands).

Arsenate respiratory reductase (arrA) gene-based analysis of arsenate-reducing communities

Three primer sets were used for the amplification of the arsenate respiratory reductase gene *arrA* (Kulp et al. 2006; Lear et al. 2007; Malasarn et al. 2004) or close homologues thereof (see below). However *arrA* gene was not detected by PCR using these different primer sets except one. Resulting partial *arrA* gene fragments were amplified using the primers ArrAfwd and ArrArev (Supplementary Table S1). Amplification often yielded faint products, consisting of multiple bands. Bands with the expected size for the *arrA* fragment (~160–200 bp) were cut from a 2% low-melting-point agarose gel using wider mouth-blunt tips. The bands were re-amplified and subjected to RFLP using enzyme R*sa*I and gel-electrophoresis on 3% (w/v) agarose gels.

Putative *arrA* amplicons (i.e., *arrA* PCR products) were cloned into *Escherichia coli* JM109 using the pGEM-T-vector system (Promega; Madison, WI, USA). Transformants were checked for correctly sized cloned inserts by a PCR with pGEM-T-specific primers T7 and Sp6 (Supplementary Table S1) (Mead et al. 1986). Products with the expected size (~300–340 bp) were digested with R*sa*I in order to screen clone libraries and compare the profiles of cloned fragments to the RFLP profiles of the enrichments. Three clone libraries (i.e., for locations NA2, T1 and K1) were constructed and the clones were screened by RFLP (on average 21 clones per enrichment; range 18 to 24). Based on differences in restriction profiles, the clones were classified into RFLP types. At least one representative clone per RFLP type and per enrichment was sequenced.

Phylogenetic analysis

Sequences were aligned and manually edited with ClustalW using default settings. Primer regions were removed in view of six sequence-ill-defined bases at the ends. Phylogenetic analyses were performed with MEGA 4 (Tamura et al. 2007). Nucleotide distance analyses were performed through Maximum Composite Likelihood computation and trees were constructed using the neighbor-joining method with a bootstrap value of 1000 replicates. Gene sequences (partial primer sequences retained) have been deposited in GenBank under accession numbers KU685222 to KU685235 (16S rRNA genes for anaerobic arsenate reducers), and in the DDBJ database under accession numbers LC122352 to LC122357 (*arrA*).

Statistical analysis

Quantitative analysis of DGGE profiles was performed with GelCompar II (Applied Math, Belgium) (van Verseveld and Röling 2004). Similarities between profiles were calculated using the Pearson correlation coefficient, and visualized by the 'unweighted paired group clustering method with arithmetic means' (UPGMA). The observed clusters of enrichments were related to hydrochemical characteristics of the groundwater from which these enrichments were derived, using non parametric analysis of variance (Kruskal-Wallis) as described in our previous study (Hassan et al. 2015).

Results

Characterization of arsenate-reducing enrichments

A total of six groundwater samples from six different locations were used to initiate arsenate-reducing enrichments. In all cultivations initiated with 1 in 10 dilutions of the samples from the various locations, microbial arsenate reduction by the lactate or acetate in the anaerobic medium had occurred after 3 weeks of incubation as judged by the permanganate reduction assay of arsenite (Table 1). For half of these locations, arsenate reduction was even observed at the 10^{-2} dilution, though not at the 10^{-3} dilution. We observed that the cultivations maintained their arsenate reduction capability during four serial transfers at their dilution factor, each step having a 2–3 weeks incubation time. As discussed under Materials and Methods, the rationale of these experiments was that during the serial transfers the cultures were being enriched in microorganisms engaging in growth energized by lactate (or acetate) respiration with arsenate as the electron acceptor. Accordingly any sample taken from the last of the series of dilutions and incubations was considered to be an arsenate-reducing enrichment. The six arsenate-reducing enrichments obtained, one for each location, were then characterized further.

In line with the enrichment strategy, subsequent 16S rRNA gene-based DGGE analysis of the six anaerobic arsenate-reducing enrichments revealed relatively simple profiles with 2–3 dominant bands and some 4 less-dominant bands per enrichment. Still, diversity between enrichments resulted in eleven different banding positions in six profiles (Figure 1(a)). A total of 14 dominant bands were 16S-rDNA sequenced. Half of these (7 bands) were most closely related to *Betaproteobacteria*, followed by *Firmicutes* (4 bands), *Epsilon-* (2 bands) and *Delta-proteobacteria* (1 band). More

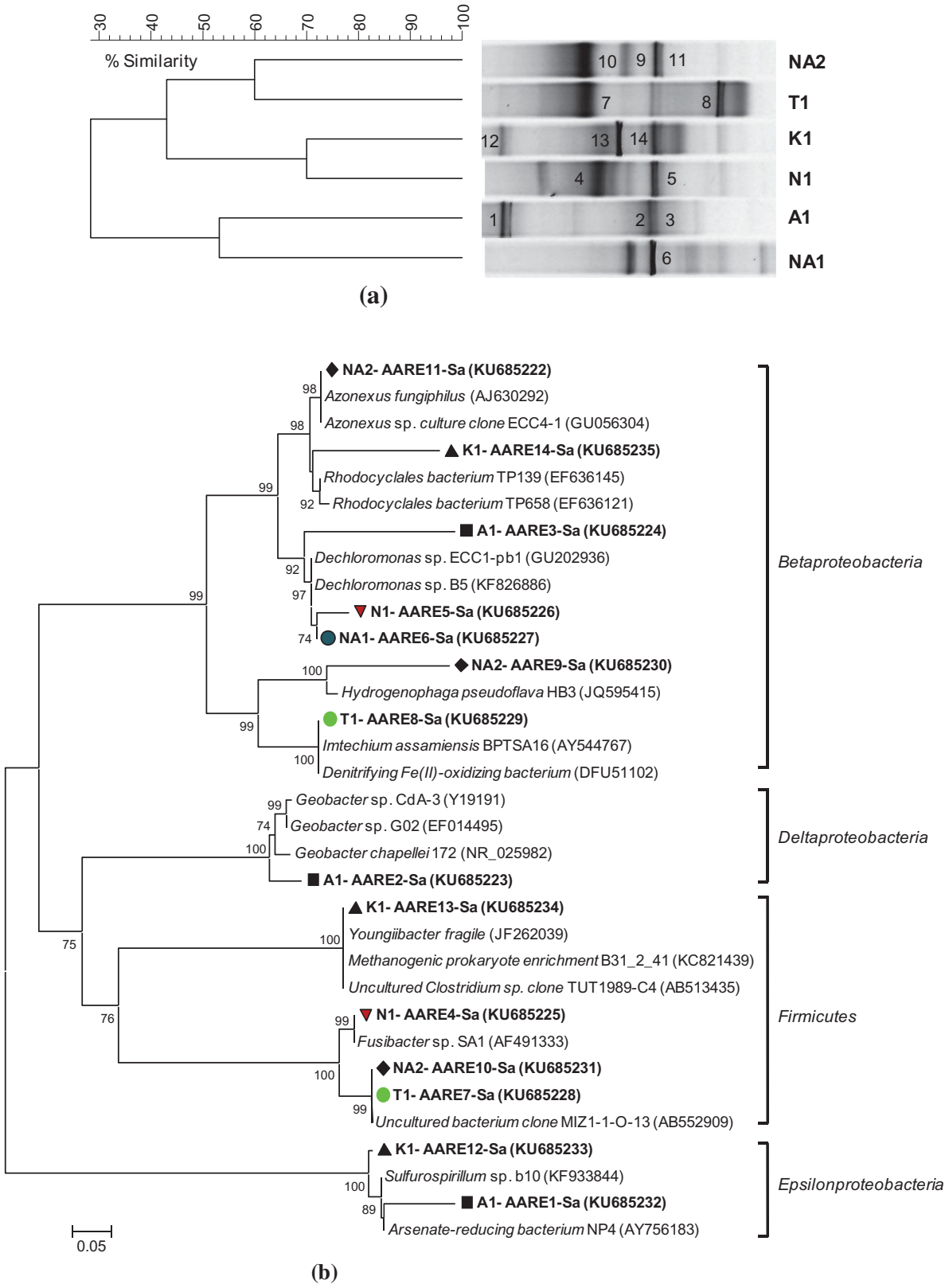

Figure 1. Comparison of anaerobic arsenate reducing enrichments from 6 water wells in Bangladesh in terms of their molecular genetics. (a) UPGMA cluster analysis of bacterial 16S rRNA gene-based DGGE profiles (30–55% denaturant gradient) of six anaerobic arsenate-reducing enrichments, using Pearson correlation analysis as measure of identity. The enrichment IDs refer to the location of the drinking water well (see Table 1 for details). Numbers refer to the position of excised bands. (b) Phylogenetic analysis of 16S rRNA gene sequences retrieved from the DGGE bands excised from (a). Sequences are indicated by enrichment ID, the number of the excised band as shown in (a) and the district in which the well is located (e.g., A1-AARE-5-Sa refers to village code A1 = Assasuni sadar, AARE refers to type of enrichment = anaerobic arsenate-reducing enrichment, 5 refers the number of the band excised as in (a), the lane for A1, and Sa refers to the district code = Satkhira). Enrichment sequences are accompanied by a colored symbol, specific for each of the six enrichments. The other sequences (including *italics*) we used for analysis from the GenBank data base (http://www.ncbi.nlm.nih.gov/Genbank/index.html) are as reference strains/sequences. The tree was constructed with the neighbor-joining method, and the numbers for interior branches indicate the bootstrap values (%) for 1000 replications. The scale bar represents 5% sequence divergence.

specifically, several sequences were closely related to genera implicated in arsenate reduction: sequences of band 1 in enrichment A1 and band 12 in K1 were most closely (96–99% nucleotide identity) related to sequences in the arsenate reducing *Sulfurospirillum* strains NP4 and b10 (Figure 1(a,b)). Band 2 in the fingerprint of enrichment A1 showed 98% identity to *Geobacter* species. Several other band sequences (4 in N1, 10 in NA2 and 7 in T1) were closely related to *Fusibacter* sp. The sequences of several bands were mostly closely related to genera that are known to contain anaerobic arsenite oxidizing species: bands labeled 3 in A1, 5 in N1 and 6 in NA1 embodied 93–99% identity to *Dechloromonas* sp. ECC1-pb1 (Sun et al. 2010), while the sequence of band 11 in NA2 was 100% identical to *Azonexus fungiphilus*.

Dissimilatory arsenate reductase genes (*arrA*; arsenate respiratory reductase, a functional gene often responsible for arsenate reduction) were retrieved by *arrA*-gene-specific PCR from only four of the six anaerobic arsenate-reducing enrichments (see Table 1); and there were signs that the amplified gene was not quite identical to the targeted *arrA*-gene (see under Materials and Methods). All of the *arrA* sequences showed either a single RFLP profile per enrichment or a profile that differed by at most 20%, indicating very limited microbial beta diversity (diversity between different habitats) of anaerobic arsenate-reducing enrichments (data not shown). This is paradoxical to (but not in conflict with) the phylogenetic analysis of 16S rRNA genes which revealed diverse microbial communities within single enrichments Figure 1(b): various microbial 'species' were enriched that appear to host highly similar *arrA*-like genes.

Although the enrichments were for anaerobic respiration using arsenate as electron acceptor and lactate or acetate as electron donor, the arsenate reductase responsible for the final step in that respiration need not have the precise cognate *arrA* sequence. Indeed, our PCR results suggested that already in the four ArrA positives, the probes did not quite match or the gene length was not precisely the same as that of the cognate *arrA* gene. We gather that in the other two cases the gene responsible for the selected arsenate respiration, was even less homologous and not picked up by our PCR probes and conditions.

Three of the enriched *arrA* gene fragments were successfully cloned and sequenced. Phylogenetic analysis revealed that these *arrA* sequences grouped into two different phylotypes based on a 90% nucleotide sequence identity cutoff (Figure 2). The *arrA* sequences from the NA2 enrichment were closely related (92–95% nucleotide identity) to uncultured bacterial clones ArrA34, ArrA38 and ArrA54 (retrieved from a fixed-bed bioreactor treating synthetic groundwater containing 0.3 mg/l arsenate (Upadhyaya et al. 2012) and virtually identical (98–100%) to an *arrA* sequence previously detected in groundwater from the NA1 location in Bangladesh (Hassan et al. 2015; Figure 2). The *arrA* sequences retrieved from the T1 and K1 enrichments clustered with both *Sulfurospirillum barnesii* (75–80% nucleotide sequence identity) and an *arrA* sequence detected in groundwater from the NA2 location (Figure 2). We did not observe any obvious correspondence between identities based on *arrA* sequences and identities based on bacterial 16S rRNA sequences retrieved from the same arsenate reducing enrichments. The exception was the 16S rRNA gene based detection of *Sulfurospirillum* in enrichment K1, which corresponded to the *arrA*-based analysis of the same enrichment.

Discussion

Microbial communities and diversity in arsenate-reducing enrichments

We had hypothesized that in arsenic-contaminated groundwaters in Bangladesh, a diverse range of microorganisms reduces arsenate. We therefore deployed an enrichment strategy of anaerobic dissimilatory arsenate-reduction. Traditionally, this strategy is used to amplify microorganisms with some desired capability that enables them to grow. Here we applied the strategy as the generator of evidence for or against the presence of dissimilatory arsenate reduction *activity* of such organisms. As discussed under Materials and Methods, the idea behind this is that for amplification to take place it is not sufficient for organisms to host DNA that is homologous to arsenate reductase genes: the organism should also profit from that DNA by using the corresponding biochemical activity to drive its proliferation. We already had the evidence that genes homologous to known arsenate reductase genes were present in the microbial populations in the drinking water (Hassan et al. 2019). However, there was no evidence yet that these homologous genes were indeed active in arsenate reduction nor that these genes enabled proliferation. Now we have evidence of both activity and coupled proliferation.

As we had hypothesized, nearly all wells did house arsenate reduction *activities* that could be amplified 100 thousandfold or 10 billionfold in enrichment cultivation, suggesting that virtually all of the investigated wells contained genes encoding enzymes that catalyze dissimilatory arsenate reduction. This makes it likely that these genes were active *in situ*, because also there arsenate is present. Of course, the results confirm that the wells themselves offer a potential of cultivatable arsenate-reducing microorganisms.

In an important way, the evidence was overwhelming to us: We found *multiple* arsenate-reduction-related nucleic acids to be amplified in addition to the ones (the functional *arrA* gene) already identified in our previous cultivation-independent study (Hassan et al. 2015). This suggests that our enrichment strategy offers a third advantage (in addition to the evidence for activity and the obtention of microbial biomass with arsenate reduction activity), i.e., an increase in signal-to-noise ratio leading to a better appreciation of the diversity of the arsenate reducing population: the species diversity in the arsenate-reducing enrichments included microorganisms that had not been identified as arsenate reducers on the basis of our previous cultivation-independent 16S rRNA-gene based study (Hassan et al. 2015).

These findings suggest that enrichment may be a strategy for exploring the potential for bioremediation of arsenic that

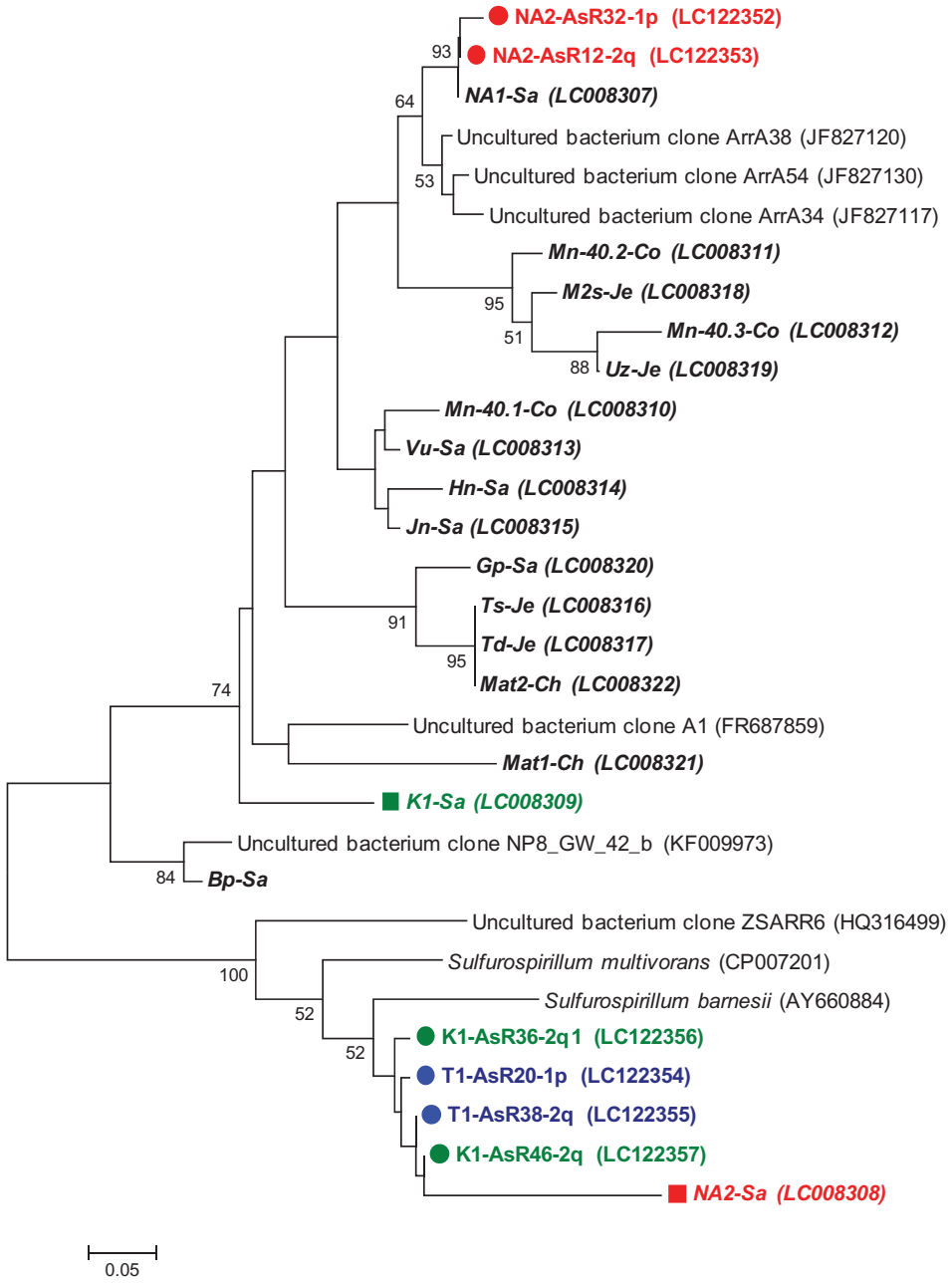

Figure 2. Unrooted neighbor-joining tree of partial nucleotide sequences (140 unambiguously aligned positions) of dissimilatory arsenate reductase genes (*arr*A) retrieved from anaerobic arsenate-reducing enrichments, initiated with groundwater from the Satkhira district in Bangladesh. The colored circles indicate the enrichments, with different colors referring to the various drinking water wells from which the enrichments were initiated. IDs in italics with and without colored squares indicate sequences derived directly from groundwater samples, without intermediate culturing (Hassan et al. 2015). The tree was constructed with the neighbor-joining method, and bootstrap values (1000 replications) are indicated at the interior branches. The scale bar represents 5% sequence divergence. Each clone is represented by the respective enrichment name and an additional number to distinguish between different RFLP types in the same enrichment. The other sequences (including *italics*) we used as reference strains/ sequences for analysis. They were derived from from the GenBank data base (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

resides in the water wells themselves (Crognale et al. 2017; Hassan et al. 2019). They also highlight that such a strategy should be accompanied by metagenomic and computational analyses. The enrichment may also contribute to the understanding of the possibilities of arsenic transformation and removal through the production of biogenic amorphous ferric iron oxyhydroxide in arsenic polluted water leading to co-precipitation of the arsenic with the iron (Hassan et al. 2016).

Overall, the observations complement our previous findings (Hassan et al. 2015): they confirm and even extend the appreciation of the diversity of microorganisms with potential for arsenate reduction in the investigated Bangladeshi aquifers. As we did not isolate and characterize individual strains, not all the observed microorganisms may themselves be capable of arsenate reduction; only the communities where they are in, should be. The enriched arsenate-reducing communities do not merely correspond to the ones that we already detected in our cultivation-independent studies (Hassan et al. 2015). The species identified by our 16S rRNA gene-based analyses of microbial communities in arsenate-reducing enrichments were confined to several

members of *Beta-*, *Delta-*, *Epsilon-proteobacteria* and *Firmicutes*, whereas the previous cultivation-independent study of the 6 samples investigated here, were mostly *Betaproteobacteria*, and very few *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* related 16S rRNA gene sequences: our expectation of a reduced overall diversity after enrichment, was incorrect; the diversity remained substantial, but was different.

Which organisms did we enrich then? Surprisingly, arsenate-reducing enrichment-derived 16S rRNA gene analysis revealed few sequences closely related to the known arsenate-reducing *Sulfurospirillum* strains NP4 and b10 or to dissimilatory iron-reducing *Geobacter* species. Other genera known to contain anaerobic arsenite *oxidizing* species surfaced: *Dechloromonas* sp. ECC1-pb1 and *Azonexus* sp. ECC4-1 (Sun et al. 2010). *Dechloromonas* sp. has been identified both in arsenite oxidizing and arsenate reducing enrichments (Suhadolnik et al. 2017). This might be due to a metabolic flexibility of *Dechloromonas* sp. that can contribute both anaerobic-arsenite oxidation and anaerobic-arsenate reduction. Recently, we also demonstrated the presence of *Dechloromonas* sp. ECC1-pb1 related 16S rRNA genes in an anaerobic arsenite-oxidizing enrichment (Hassan et al. 2019). We found rare sequences most closely related to known arsenate reducing strains *Sulfurospirillum* b10 and NP4. Arsenate-respiring *Sulfurospirillum* may play a role in arsenic release in South-East Asian aquifer sediments (Héry et al. 2008). Other sequences inside the *Firmicutes* phylum most closely related to *Clostridium* and *Fusibacter* were also in low abundance. It has been reported that the *Clostridium* genus can contribute arsenate reduction (Suhadolnik et al. 2017). A more recent report has demonstrated that *Firmicutes* were one of major phyla of microbial communities in microbe mediated anaerobic arsenate reduction (Luo et al. 2020).

In view of the interactions e.g., in terms of co-precipitation (Hassan et al. 2016) between arsenic and iron, a limitation of this study was that it focused on arsenic, not on both iron and arsenic: we did not test for the presence of more *Geobacter* species by using *Geobacteraceae* specific primers to analyze the arsenate-reducing enrichments. *Geobacteraceae* have often been demonstrated to constitute the most abundant iron reducers in iron-reducing subsurface environments (Lovley et al. 2011), and this included arsenic-contaminated sediments in West Bengal, India (Héry et al. 2010; Islam et al. 2004). We also encountered *Geobacteraceae* in all our iron-reducing enrichments in line with our previous cultivation-independent analysis of the same arsenic contaminated drinking water samples (Hassan et al. 2015; 2016). This should be a motive for further cultivation studies focusing on the interactions between arsenic and iron.

Acquisition and diversity of arrA genes in arsenate-reducing enrichments

The arsenate reductase (*arrA*) gene diversity was and remained low in the enrichments investigated here; other studies on arsenic-rich aquifers revealed higher alpha-diversity (Giloteaux et al. 2013; Héry et al. 2010; Mirza et al. 2014; Song et al. 2009). In our case the lack of observed *arrA* gene diversity could be thanks to our enrichment approach, and/or due to environmental heterogeneity and to methodological limitations, such as observed by others: Mirza et al. reported that most of the available primer sets are insufficient for the amplification of diverse *arrA* genes (Mirza et al. 2017) and that *arrA*-containing arsenate-reducing prokaryotes are diverse depending on the biogeography and biogeochemical condition. Escudero and coworkers found that some of the arsenic metabolizing genes were untypeable by using the newly designed and available primer sets in the literature. They concluded that this could be due to environmental heterogeneity and to primer mismatches affecting the efficiency of the PCR (Escudero et al. 2013). Other studies also reported that the investigation of arsenic-related functional genes through PCR approaches as well as through metagenome sequencing is currently difficult in ecological studies. This is probably due to high microbial diversity and to a primer coverage that may not be sufficient to capture high gene diversity (Crognale et al. 2017; Fahy et al. 2015). As a consequence, in our previous cultivation-independent survey, the majority of the detected *arrA*-like gene sequences belonged to yet uncharacterized arsenate-reducing species (Hassan et al. 2015). It will be important to follow up the *arrA*–like genes by using the newly designed degeneracy primer sets which should have a wider genetic coverage. This may then provide more robust information on the biodiversity of arsenate-reducing microorganisms in diverse environmental conditions.

Several iron reducers and especially *Geobacter* species known to reduce both Fe(III) and As(V), could play a critical role in arsenic release (Gnanaprakasam et al. 2017). They possess the respiratory and cytoplasmic arsenate reductase genes *arrA* and *arsC* respectively, which can reduce arsenate to arsenite as well as detoxify the cell (Dang et al. 2017; Kudo et al. 2013;; Ohtsuka et al. 2013; Osborne et al. 2015). Other studies have shown that *Geobacter*-related dissimilatory arsenate reductase (*arrA*) genes were abundant in several anaerobic arsenate-rich aquifers (Giloteaux et al. 2013; Héry et al. 2010). Previously we tested the presence of the arsenate reductase gene (*arrA*) in iron-reducing enrichments and revealed that almost half of the environments contained *arrA* genes (Hassan et al. 2016). This indicates that *Geobacter* and *S. barnesii* related *arrA* sequences co-occurred in iron-reducing enrichments. In the case of our arsenate-reducing enrichments, similar species-related functional and ribosomal genes are consistent with the previous evidence (Hassan et al. 2016): sequences we found were most closely related to those of uncultured bacterial clones and different species of *Sulfurospirillum*. In the present study we did not conduct microbe mediated aerobic arsenate reduction of the same samples that we analyzed under anoxic conditions. Although not only anaerobic respiratory arsenate reductase (ArrA) but also cytoplasmic arsenate reductase (ArsC) may contribute to arsenate reduction under aerobic conditions (Corsini et al. 2010), species with

only ArsC should not have been enriched in our cultivations.

Implications for the mobilization of arsenic

The mobilization of arsenic in subsurface environments has enormous toxic consequences for millions of people in Bangladesh who are vulnerable through their use of groundwater as drinking water, for cooking, and for irrigation (Huq et al. 2006). Currently available remediation technologies have major disadvantages such as that they are often expensive and result in secondary exposure to and environmental pollution with arsenic, e.g., through inadequate handling and disposal of arsenic-binding water filters (Gonzaga et al. 2006). Accordingly, van Halem and coworkers introduced subsurface arsenic removal (SAR) technology in Bangladesh, i.e., an *in situ* removal of iron and arsenic from the mobile water (van Halem et al. 2010). It comprises the injection of oxygenated water into aquifers so as to oxidize ferrous iron abiotically and therewith precipitate ferric iron, which adsorbs, co-precipitates and thereby immobilizes arsenic. The activity of iron-oxidizing microorganisms that also oxidize arsenite could potentially enhance SAR efficiency by further lowering residual concentrations of arsenic. Arsenite oxidizing microbes produce the less toxic arsenate, which is also less mobile as arsenate is more strongly absorbed by oxidized and precipitating iron minerals. However, the nature of the minerals formed during microbial iron oxidation, which varies between crystalline ferric iron oxyhydroxide and amorphous iron phosphate minerals (Kish et al. 2016; Li et al. 2015), may have important implications for arsenic mobility during anaerobic iron reduction. Dissimilatory arsenate reducing prokaryotes are mostly facultatively anaerobic heterotrophs. They have the specific enzymatic system to reduce arsenate to arsenite whilst attached to the solid phase either on surfaces of Fe(III) or $Al(OH)_3$ minerals. Subsequently the toxic form of arsenic (i.e., arsenite) is released and some of the released arsenite can re-adsorb to unreacted Fe(III). Other dissimilatory arsenate reducing prokaryotes can also act as iron-reducers such as *Sulfospirillum barnesii*, which can release both As(III) and Fe(II) from ferrihydrite that was initially co-precipitated with As(V) (Oremland and Stolz 2005). Also *Geobacter* species play a major role in microbe-mediated arsenic release from metal hydroxides in the subsurface. This release raises arsenic concentrations in drinking water to levels that are high enough to cause major health problems (Dang et al. 2017). It has also been reported that *Geobacter* sp. OR-1 possesses arsenic islands (Ehara et al. 2015) which are encoding *arrB* genes as well. These genes are flanked by *ars* operons and they play a pivotal role in utilization of arsenate ferrihydrite as an electron acceptor and thus play a role in arsenic respiration. Groundwater in Bangladesh also contains a wide range of anaerobic iron reducers (Hassan et al. 2015; 2016) which may then become active and release arsenic as a result of the reduction of precipitated and arsenic-adsorbing ferric (oxyhydr)oxides. Specialized arsenate reducers and iron reducers possessing arrA (Hassan et al. 2016) may enhance the release of arsenic by reducing arsenate to more mobile and more toxic arsenite.

Barrier for bioremediation of arsenic in drinking waters

The microbial community differed between enrichments from different groundwater wells. Counter to our expectation, the observed diversity was higher after (this paper) than before (Hassan et al. 2015) enrichment for arsenate-reduction-based growth. This shows that it is important to complement cultivation-independent surveys with enrichment studies, which evidence functional activities. Our experiments (Table 1) support our hypothesis that arsenate-reducing microorganisms are widely distributed in the arsenic-contaminated aquifers in Bangladesh and are active as such when provided with the proper conditions. Independent of whether biological arsenic remediation from Bangladesh groundwater through conversion to arsenate would be conducted *in situ* or *ex situ*, much attention should be paid to anaerobic conditions, which could revamp arsenite from precipitated arsenate (Hassan et al. 2015). Keeping the subsurface aerobic and maintaining the iron in its ferric (oxidized form), is expensive however. To monitor the microbial activities related to arsenate reduction more detailed laboratory studies are required. By focusing on individual strain isolation, tolerance limits, capability to enhance the dissolved arsenate [As(V)] concentration through column experiments that mimic natural electron-donor and -acceptor conditions, such studies may help assess both the potential for and the threats compromising, the natural or activated bioremediation of arsenic in the subsurface groundwater. Combinations of the bioengineering and synthetic biology techniques with the quantitative analysis and modeling of the ecosystem, should enable the management of arsenic (im)mobilization with the perspective of sustainable development of safe drinking water for rural poor people.

Disclosure statement

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