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Role of metal-reducing bacteria in arsenic release from Bengal delta sediments

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The contamination of ground waters, abstracted for drinking and irrigation, by sediment-derived arsenic threatens the health of tens of millions of people worldwide, most notably in Bangladesh and West Bengal[1–3]. Despite the calamitous effects on human health arising from the extensive use of arsenic-enriched ground waters in these regions, the mechanisms of arsenic release from sediments remain poorly characterized and are topics of intense international debate[4–8]. We use a microscosm-based approach to investigate these mechanisms: techniques of microbiology and molecular ecology are used in combination with aqueous and solid phase speciation analysis of arsenic. Here we show that anaerobic metal-reducing bacteria can play a key role in the mobilization of arsenic in sediments collected from a contaminated aquifer in West Bengal. We also show that, for the sediments in this study, arsenic release took place after Fe(III) reduction, rather than occurring simultaneously. Identification of the critical factors controlling the biogeochemical cycling of arsenic is one important contribution to fully informing the development of effective strategies to manage these and other similar arsenic-rich ground waters worldwide.

Potential mechanisms for the release of arsenic into ground water in Bengali shallow alluvial sedimentary aquifers have been the focus of intense debate. The oxidation of arsenic-rich pyrite in aquifer sediments has been proposed as one possible mechanism[4,5]. Other studies have suggested that the reductive dissolution of arsenic-rich Fe(III) oxyhydroxides deeper in the aquifer may lead to the release of arsenic into the ground water[2,6,7,9]. Additional factors that may add further complication to potential arsenic-release mechanisms from sediments include the predicted mobilization of sorbed arsenic by phosphate generated from the intensive use of fertilizers[10], by carbonate[11] produced via microbial metabolism[9], or by changes in the sorptive capacity of ferric oxyhydroxides[2].

Although the biotic and abiotic processes described above may all play a role in arsenic mobilization, it has been concluded that microorganisms play the defining role in catalysing the redox transformations that ultimately control the mobility of the metalloid[8]. It has also been noted recently that "there is an immediate research need for a fuller understanding of the role(s) of subsurface microbes in mobilizing arsenic in aquifers"[8]. In order to answer this important question, we used sediments from a well-characterized[12] arsenic-rich test site in the Nadia district, West Bengal, to identify the biogeochemical conditions that promote maximal arsenic release from contaminated aquifers in the Ganges delta. Samples were taken from a depth of 13 m where arsenic-rich ground waters ($>40\,\mu g\,l^{-1}$) have been found[13] (see Supplementary Information for further data on sediment and groundwater characteristics, and sampling methods). The sediments were mixed with simulated ground water and incubated at 20 °C under a range of biogeochemical conditions (Fig. 1). Our initial experiments focused on the speciation of arsenic in the pore water, and also the reduction of Fe(III), which is a significant electron acceptor in these sediments and can support the growth of organisms capable of respiring using sediment-bound As(V)[14]. Arsenic speciation in the aqueous fraction was monitored using a sensitive coupled IC-ICP-MS system[15], while Fe(II) (in the pore water and bound to sediment material) was monitored using a ferrozine based assay after extraction with 0.5 M HCl[16].

Sediments incubated under aerobic conditions showed negligible reduction of Fe(III) with time, or release of arsenic from the sediments (Fig. 1a). Incubation under anaerobic conditions, however, resulted in Fe(III) reduction concomitant with arsenic mobilization (Fig. 1b). Fe(II) concentrations in the sediment fraction increased from approximately 3.5 mmole l^{-1} to 6 mmole l^{-1} after 38 days incubation, concomitant with the release of 13 nM arsenic, principally as As(III), into the pore water. Throughout these experiments, negligible concentrations of soluble Fe(II) were released into the pore water. Addition of acetate as a potential electron donor for metal reduction and a proxy for organic matter[17] resulted in a marked stimulation in the rate of Fe(III) reduction followed by arsenic release (Fig. 1c). Initially, predominantly dissolved As(V) was detected (up to 23 nM at 15 days), followed by

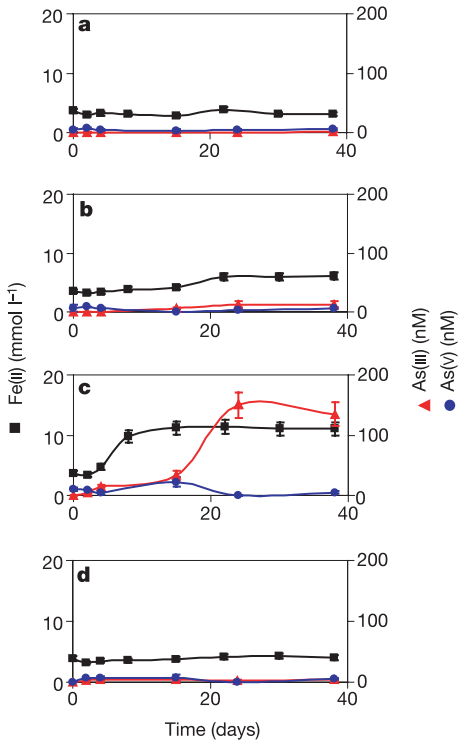

Figure 1 Reduction of Fe(III), and mobilization of arsenic in microcosms containing Bengali sediments incubated under a range of biogeochemical regimes. **a**, Aerobically; **b**, anaerobically; and **c**, anaerobically with 4 g l^{-1} sodium acetate as a proxy for organic matter. 'Abiotic' control sediments with added acetate were autoclaved before incubation (**d**). Black squares, Fe(II); red triangles, As(III); purple circles, As(V). Each point and error bar represents the mean and standard deviation of three replicate experiments.

predominately dissolved As(III) which reached concentrations of 150 nM after 24 days. Taking into account the much lower water/sediment ratios in the aquifer (1:8 wt./wt. for a typical porosity of 25% and assuming a sediment density equal to that of quartz) compared to that in our microcosm experiments (2:1 wt./wt.), this As(III) concentration would be equivalent to 2.4 μM or 180 μg L^{-1}, broadly comparable with the higher groundwater arsenic concentrations found in this and similar shallow aquifers in Bengal. Increased concentrations of As(III) in pore waters of these sediment experiments is consistent with microbial reduction of As(V). X-ray absorption spectroscopy showed that the predominant (>90%) oxidation state of arsenic in the sediment both before and after metal reduction had been stimulated by acetate was As(V)[18] suggesting that not all of the As(V) was bioavailable for microbial reduction, possibly through its association with recalcitrant crystalline Fe oxides. The observed co-existence of an As(V) dominant solid phase assemblage with an As(III) dominated aqueous phase suggests preferential sorption of As(V) on surfaces of the solid phase assemblage. This is consistent with a number of studies of As sorption on iron oxyhydroxides, (reviewed in ref. 2) although the relative strengths of sorption of As(III) and As(V) is the subject of debate[19]. The reduction of Fe(III) and the release of arsenic were clearly decoupled in the sediments that were stimulated with acetate, with Fe(II) concentrations in the sediment fraction approaching the maximum values measured (11 mmoles l^{-1}) after only 8 days incubation, while the highest concentrations of As(III) were not recorded until the sediments were incubated for another two weeks (Fig. 1c). That Fe(III) reduction and arsenic release through As(V) reduction are decoupled is not unexpected. Bioavailable terminal electron acceptors will be used in sequence governed by the energy yield, derived from the relevant oxidation/reduction potentials. The reduction of Fe(III) followed by reduction of As(V) observed in our experiments can be rationalized in terms of the higher oxidation/reduction potential for $Fe(OH)_3/Fe^{2+}$ at the initially low Fe^{2+} concentrations of our experiments (+241 mV for $(Fe^{2+}) = 10^{-8}$ M) compared to the oxidation/reduction potential for As(V)/As(III) (+14 mV for equimolal concentrations of As(V) and As(III); calculated from data of refs 20,21; see Supplementary Information).

Decoupling of the reduction of Fe(III) and As(V) may, therefore, reflect adaptation of the respiratory pathways in the microorganisms in the sediments, through dynamic changes in the species that are metabolically active in the microbial community, or through altered expression of the relevant metal reductases in key anaerobes constituting this community. No methylated arsenic species (monomethylarsonic acid or dimethylarsinic acid) were detected (limit of detection, 7 nM as arsenic). Finally, control sediments were autoclaved and then incubated in the presence of acetate. Concentrations of Fe(II), As(V) and As(III) did not increase in these control sediments (Fig. 1d), confirming a role for microorganisms in the reduction of Fe(III) and subsequent mobilization of arsenic.

Having identified incubation conditions that promoted arsenic mobilization, the microbial communities present under the different biogeochemical conditions imposed were analysed, using both cultivation-dependent and molecular (polymerase chain reaction; PCR) techniques.

Initial experiments used a DNA profiling technique that amplified the variable length intergenic spacer region between the genes encoding 16S and 23S rRNA in bacteria (ARISA analysis[22]). Using this technique, we noted from ARISA banding patterns (not shown) that there were only minor changes upon a shift from aerobic to anaerobic cultivation conditions, while the addition of acetate resulted in a marked shift in the population, suggesting that microbial metal reduction in our samples was limited by available electron donor. Additions of as little as 1 mM acetate also resulted in a similar shift in the microbial communities as shown by ARISA analysis, with corresponding enhanced levels of As(III) measured in the pore waters (data not shown). An approximately 500 base pair (bp) region of the 16S rRNA gene was amplified by PCR using broad specificity bacterial primers, cloned and typed using restriction fragment length polymorphism (RFLP) analysis. Of the 46 clones analysed from the starting sediment material, 28 gave distinct banding patterns, suggesting a diverse community of bacteria in the sediments. The majority of these 'RFLP-types' were affiliated with known members of the γ- and β-Proteobacteria (30% and 24% of the clone library respectively; Fig. 2, with a detailed phylogenetic analysis presented as Supplementary Information). In the γ-Proteobacteria, the majority of the clones corresponded to sequences derived from *Pseudomonas* species (26% of total clone library), facultative anaerobes able to respire using alternative electron acceptors including nitrate, but not As(V) or Fe(III)[8,23]. Other sequences detected that were consistent with anaerobic conditions in the sediments included those related closely to known *Clostridium* species (9% of the clone library), which are involved in the fermentation of organic matter, and can reduce a range of metals[23] including As(V)[8]. Specialist dissimilatory metal-reducing bacteria were also detected; 11% of the clones were affiliated to *Geobacter* type sequences in the δ-Proteobacteria, which have been shown to dominate zones of Fe(III) reduction in the subsurface[24] and reduce a wide range of high valence metals with the notable exception of As(V)[23]. Finally, it is conceivable that the sample used in this study may have been obtained at the interface between oxic and anoxic regions of the aquifer, as 13% of the clone library aligned with sequences from *Nitrosolobus* species, which are aerobic ammonia-oxidizing bacteria of the β-Proteobacteria[25].

When metal (Fe(III) and As(V)) reduction was stimulated by the addition of acetate, there was a marked shift in the population (Fig. 2). The dominant sequences detected in the clone library (70% of the 30 clones analysed) were affiliated with members of the family *Geobacteraceae* in the δ-Proteobacteria. Similar shifts in microbial communities have been noted in other studies in which the reduction of metals was stimulated in the subsurface[24]. In comparison, *Pseudomonas*-type sequences (γ-Proteobacteria) corresponded to only 3% of the clone library after stimulation with acetate. The marked increase in numbers of sequences of known Fe(III)-reducing bacteria was mirrored in the most probable number (MPN) counts

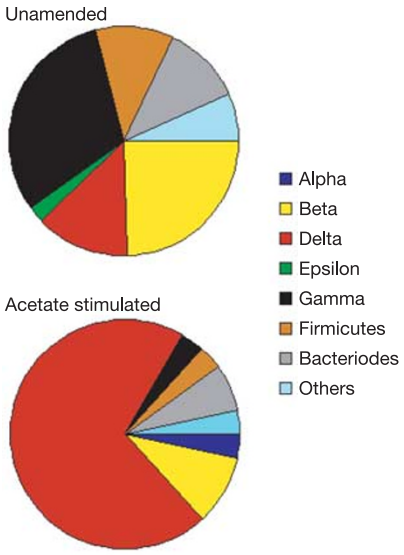

Figure 2 Shifts in the microbial community of the sediment from the Nadia district, West Bengal, after stimulation of anaerobic metal reduction by acetate. 16S rDNA clone libraries were amplified by PCR from untreated sediment (top), and after incubation under anaerobic conditions with added acetate (bottom). Charts show phylogenetic affiliation of the clones. Alpha, beta, delta, epsilon and gamma represent proteobacterial divisions.

on these experiments. Numbers of Fe(III)-reducing bacteria were shown to increase after stimulation with acetate: the following counts per gram sediment were recorded; 55 (aerobic), 220 (anaerobic) and 2.3×10^3 (anaerobic stimulated with acetate). It should be noted that although these counts are satisfactory for comparing the relative amounts of Fe(III)-respiring organisms in the different treatments, MPN counts can underestimate cell numbers by several orders of magnitude.

The organisms that catalyse the reductive mobilization of arsenic remain to be identified irrevocably, and we do not discount the importance of bacterial strains not represented in our clone libraries for this transformation. However, the lack of a coherent phylogenetic grouping or a conserved functional gene for this group of diverse microorganisms[8] makes it impossible, at present, to search for these organisms using PCR-based techniques, and a novel MPN-based technique for enumerating As(V)-reducing bacteria[26] proved irreproducible in our hands. Nevertheless, it remains highly probable that the Fe(III)-reducing bacteria, maintained on the relatively high concentrations of Fe(III) in the sediments, played a major role in the subsequent reduction and release of arsenic from sediments once the bioavailable Fe(III) had been used as an electron acceptor. Indeed, of the 16 species of As(V)-respiring organisms currently in pure culture, none are obligate As(V) reducers[8]. All are 'opportunists' capable of respiring using other electron acceptors, for example Fe(III)[8]. It should be noted that measurements of the metal fractions extracted from our test sediments (before mixing with ground water in our microcosm experiments), using 0.2 M NH_4 oxalate[27], showed that they contained 0.7% bioavailable Fe in amorphous and poorly crystalline Fe hydrous oxides, compared to only 1.8 μg As per g.

To confirm whether Fe(III)-reducing bacteria could play a role in the release of arsenic from the West Bengal sediments, a stable enrichment culture of Fe(III)-reducing bacteria was obtained by resubculturing in Fe(III)-containing medium 4 times, washed in artificial ground water and introduced into heat sterilized sediments at a cell concentration similar to those noted in the MPN experiments. The stable culture of Fe(III)-reducing bacteria was indeed able to mobilize arsenic from the sediments, with 94 nM As(III) and 7 nM As(V) noted after 41 days in the pore waters in the presence of added acetate (Fig. 3). Negligible arsenic mobilization was noted in uninoculated sterile controls.

These studies offer direct evidence that anaerobic metal-reducing bacteria play a role in the formation of toxic, mobile As(III) in sediments from the Ganges delta. Our results also suggest that the capacity for arsenic release was severely limited by the availability of electron donor in the sediments from our test site. Although our experiments are microcosm-based and not a direct simulation of the hydrogeological parameters and organic carbon flux *in situ*, we suggest that our results support theories that the delivery of surface-derived organic carbon[28] into subsurface communities (driven, for example, by irrigation pumping[9]) may have a dramatic role in enhancing arsenic mobility in shallow ground waters in the Ganges Delta. Further studies are now warranted on the detailed mechanisms of arsenic release by metal-reducing bacteria isolated from arsenic-contaminated sediments, alongside field-based studies assessing the impact of such transformations *in situ*. □

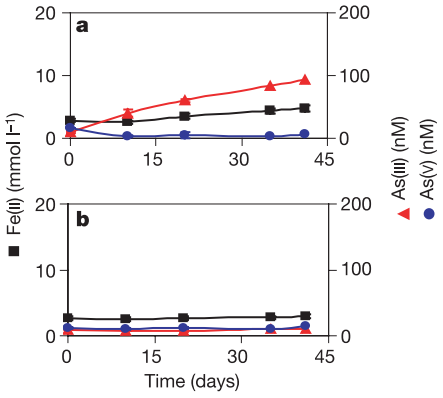

Figure 3 Reduction of Fe(III), and mobilization of arsenic in microcosms containing heat-sterilised Bengali sediments. **a**, Inoculated with an enrichment culture of Fe(III)-reducing bacteria. **b**, Control microcosms were not inoculated with the enrichment culture. Black squares, Fe(II); red triangles, As(III); purple circles, As(V). Each point and error bar represents the mean and standard deviation of three replicate experiments.

Methods

Sediment incubations

Sediment was collected from Chakdaha block, Nadia district in West Bengal (GPS location 23° 04′ 55″ N / 88° 30′ 44″ E) using a reverse circulatory drilling method but with samples recovered without the use of drilling fluid to minimize the possibility of contamination with non-indigenous microorganisms. The sample was transported and stored at 4 °C under N_2 in a sealed transparent plastic tube to minimize microbial activity before use (see Supplementary Information for a more detailed description of sampling and storage protocols). About 15 g of sediment was mixed with 30 ml artificial ground water based on the constituents of water samples from the study site ($MgCl_2$, 0.34 mM; KH_2PO_4, 0.01 mM; $NaHCO_3$, 0.51 mM; K_2CO_3, 0.025 mM; $MgSO_4$, 0.03 mM; KNO_3, 0.001 mM; and $CaCO_3$, 1.85 mM; adjusted to pH 7 using HCl). All glassware was prewashed with 1 M HCl and ultraclean water before use. The bottles were sealed with butyl rubber stoppers, and incubated under aerobic conditions (stoppers pierced with three hypodermic syringe needles), anaerobic conditions (N_2 atmosphere), and anaerobic with added electron donor (4 g l^{-1} acetate). Control experiments comprised autoclaved sediments amended with acetate, incubated under N_2. Sediments were incubated in the dark at 20 °C.

Analytical techniques

Fe(II) was quantified spectrophotometrically after reaction with ferrozine, with total Fe measured after reaction with hydroxylamine[16]. Arsenic speciation was analysed by IC-ICP-MS[15]. Samples (1 ml) were removed from the bottles in an anaerobic cabinet and passed through a 0.45 μm filter. As(V) and As(III) were separated using a Cetac ANX3206 anion exchange column, housed in a Metrohm 790 Personal IC unit, which was interfaced to a VG PlasmaQuad II ICP-MS. The aqueous mobile phase was 1.7 mM $NaHCO_3$ / 1.8 mM Na_2CO_3. Standard reference materials were included in each analytical run; the arsenic concentrations determined were found to agree well with those certified. The limited sample volumes available precluded the determination of other key analytes in the microcosms.

Amplification of 16S rDNA

Sediment samples (0.25 g) were pretreated with 2 ml sodium oxalate solution (0.3 M), passed through a 0.25 μM filter and DNA extracted using a Fast DNA spin kit (UltraClean, Soil DNA Isolation Kit, MO BIO Laboratories). A fragment of the 16S rRNA gene, approximately 520 bp, was amplified by PCR from samples using the broad-specificity primers 8f and 519r[24] using an iCycler (BioRad). Purified DNA (5 μl) and 2.5 μl of 25 μM primer stocks were added to the reaction mix to a final volume of 50 μl. The purity of the amplified product was determined by electrophoresis of 10 μl samples in a 1.0% agarose Tris-borate-EDTA (TBE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light.

RFLP analysis

PCR products were purified using a QIAquick PCR purification kit (Qiagen) and ligated directly into the cloning vector pCR 2.1 (Invitrogen) before transformation into *Escherichia coli* TOP 10 competent cells. White transformants that grew on LB agar containing ampicillin (100 μg ml^{-1}) and 40 μl of 40 mg ml^{-1} of X-Gal were screened for an insert using PCR. Primers were complementary to the flanking regions of the PCR insertion site of the pCR 2.1 cloning vector. The PCR method was: initial denaturation at 94 °C for 4 min, melting at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min; 35 cycles, with a final extension step at 72 °C for 5 min. The PCR products were purified using a QIAquick kit and treated with the restriction endonucleases, *Sau*3A and *Msp*I. The restriction enzyme digests were separated using a 3% metaphor agarose TBE gel.

DNA sequencing and phylogenetic analysis

Nucleotide sequences were determined by the dideoxynucleotide method by cycle sequencing of the purified PCR products. An ABI Prism BigDye Terminator Cycle Sequencing Kit was used in combination with an ABI Prism 877 Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems). Sequences (typically 500 bp) were analysed against the NCBI (USA) database using BLAST program packages and matched to known 16S rRNA gene sequences. Gene sequences were aligned using the ClustalX software package and corrected manually. The TREECON package[29] was used for distance analysis using the Jukes and Cantor correction and bootstrap resampling (100 times) and a phylogenetic tree constructed from the distance matrix via neighbour joining[30].

Estimation of cell numbers

MPN counting was performed as follows. A tenfold dilution series was prepared from sediment samples, using tubes containing a freshwater medium[16] with 20 mM acetate as the electron donor and 56 mM Fe(III)-citrate as the electron acceptor. The tubes were incubated at 20 °C and scored on a weekly basis for the reduction of Fe(III) using the ferrozine assay[16]. The numbers of positive tubes were tabulated and the most probable number of Fe(III)-reducing bacteria estimated by comparison with reference MPN tables.

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Why large-scale climate indices seem to predict ecological processes better than local weather

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Large-scale climatic indices such as the North Atlantic Oscillation[1] are associated with population dynamics[2], variation in demographic rates[3] and values of phenotypic traits[4,5] in many species. Paradoxically, these large-scale indices can seem to be better predictors of ecological processes than local climate[5–8]. Using detailed data from a population of Soay sheep[9,10], we show that high rainfall, high winds or low temperatures at any time during a 3-month period can cause mortality either immediately or lagged by a few days. Most measures of local climate used by ecologists fail to capture such complex associations between weather and ecological process, and this may help to explain why large-scale, seasonal indices of climate spanning several months can outperform local climatic factors. Furthermore, we show why an understanding of the mechanism by which climate influences population ecology is important. Through simulation we demonstrate that the timing of bad weather within a period of mortality can have an important modifying influence on intraspecific competition for food, revealing an interaction between climate and density dependence[11] that the use of large-scale climatic indices or inappropriate local weather variables might obscure.

The impact of climatic variation on ecological processes has been the focus of discussion in ecology for nearly a century[12]. After a period when ecologists believed that complex dynamics were determined primarily by density-dependent intrinsic processes[13], recent work has shown that climatic variation can have an important role—either directly or through its interaction with density[14,15]. It has emerged that large-scale seasonal indices of climate, such as the North Atlantic Oscillation (NAO, defined as fluctuations in sea-level air pressure between the Atlantic sub-polar low-pressure zone centred around Iceland and the sub-tropic high-pressure zone centred around the Azores[1]), are remarkably good predictors of ecological variation—often better than local weather variables[4–6,16–18] (but see refs 19–22 for examples where local weather predicts ecological processes well). The strong performance of large-scale climatic indices in predicting ecological processes is often difficult to reconcile with the proximal physiological processes that underpin them[10]. For example, in the food-limited population of Soay sheep (*Ovis aries*) considered here, there are two mechanisms by which winter weather influences mortality rates: first, by generating energetic costs on animals in poor condition, and second, by moderating vegetation productivity and available grazing for sheep during winter[14,23,24]. In this system one may therefore expect short-term local climatic averages to predict mortality rates better than large-scale indices estimated over multiple months, yet some studies[3,6,25] found that the NAO predicted mortality rates better than indexes of local monthly weather and explained approximately 20–30% of the variation in mortality. These results have presumably