Microbial ecology of arsenic-mobilizing Cambodian sediments: lithological controls uncovered by stable-isotope probing

Marina Héry,1*† Athanasios Rizoulis,1 Hervé Sanguin,2‡ David A. Cooke,3 Richard D. Pancost,4 David A. Polya1 and Jonathan R. Lloyd1
1School of Earth, Atmospheric and Environmental Sciences, Williamson Research Centre for Molecular Environmental Science and 2Faculty of Life Sciences, University of Manchester, Manchester, UK.
3Faculty of Engineering and Environment, Northumbria University, Newcastle upon Tyne, UK.
4Organic Geochemistry Unit, The Cabot Institute, Bristol Biogeochemistry Research Centre, School of Chemistry, Cantock’s Close, Bristol University, Bristol, UK.

Summary

Microbially mediated arsenic release from Holocene and Pleistocene Cambodian aquifer sediments was investigated using microcosm experiments and substrate amendments. In the Holocene sediment, the metabolically active bacteria, including arsenate-respiring bacteria, were determined by DNA stable-isotope probing. After incubation with 13C-acetate and 13C-lactate, active bacterial community in the Holocene sediment was dominated by different Geobacter spp.-related 16S rRNA sequences. Substrate addition also resulted in the enrichment of sequences related to the arsenate-respiring Sulfurospirillum spp. 13C-acetate selected for ArrA related to Geobacter spp. whereas 13C-lactate selected for ArrA which were not closely related to any cultivated organism. Incubation of the Pleistocene sediment with lactate favoured a 16S rRNA-phylotype related to the sulphate-reducing Desulfovibrio oxamicus DSM1925, whereas the ArrA sequences clustered with environmental sequences distinct from those identified in the Holocene sediment. Whereas limited As(III) release was observed in Pleistocene sediment after lactate addition, no arsenic mobilization occurred from Holocene sediments, probably because of the initial reduced state of As, as determined by X-ray Absorption Near Edge Structure. Our findings demonstrate that in the presence of reactive organic carbon, As(III) mobilization can occur in Pleistocene sediments, having implications for future strategies that aim to reduce arsenic contamination in drinking waters by using aquifers containing Pleistocene sediments.

Introduction

In South and South East Asia, where high concentrations of arsenic in groundwater are causing a humanitarian disaster (Smith et al., 2000), aquifer sediments have been studied largely in terms of their geological, mineralogical and geochemical characteristics (Acharyya et al., 2000; Berg et al., 2001; 2007; Polya et al., 2003; Ravenscroft et al., 2005; 2009; Zheng et al., 2005; Charlet and Polya, 2006; Buschmann et al., 2007; Rowland et al., 2008). However, studies based on arsenic-rich South East Asian sediments support the consensus that the oxidation of organics coupled to the reduction of As(V) sorbed to mineral surfaces plays a major role in As(III) mobilization in anoxic aquifers (e.g. van Geen et al., 2004; Islam et al., 2004; Héry et al., 2010). Metal-reducing bacteria conserve energy by using metals including Fe(III) and the metalloid As(V) (arsenate) as electron acceptors. Respiratory arsenate reduction is catalysed by an enzyme of which the arrA gene is a key component, and known As(V)-respiring bacteria are affiliated with many different bacterial genera (Oremland and Stolz, 2005). Profiling of the bacterial structure based on 16S ribosomal RNA (rRNA) gene phylogeny and the study of arsenate reductase genes in arsenic-rich sediments have emphasized the enrichment of metal-reducing Geobacter spp. (Islam et al., 2004; Lear et al., 2007; Rowland et al., 2007), which have the potential to respire both Fe(III) and As(V) (Lloyd et al., 2011). Furthermore, the probable influence of Geobacter in arsenic biogeochemistry in groundwater

Received 26 July, 2013; accepted 20 January, 2014. *For correspondence. E-mail marina.hery@univ-montp2.fr; Tel. (+33) (0) 67 42 74 74; Fax (+33) (0) 67 14 47 74. Present addresses: †Hydrosciences UMR 5569, CNRS, Universités Montpellier I and II, IRD, Place Eugène Bataillon, CC MSE, 34095 Montpellier cedex 5, France; ‡CIRAD, UMR LSTM, Campus International de Baillarguet, 34398 Montpellier cedex 5, France.

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has been highlighted recently by a transcriptomic approach (Giloteaux et al., 2013).

The use of 13C-labelled organic substrates and DNA stable-isotope probing (DNA-SIP) can reveal the active members of the microbial community, and it has been used to study a wide range of microbial processes in various environments (reviews by Neufeld et al., 2007a; Uhlík et al., 2009). However, for arsenic-rich sediments, DNA-SIP has only been used once to characterize 13C-acetate utilizing arsenate-respiring bacteria (Lear et al., 2007). Furthermore, the majority of microcosm studies with arsenic-rich sediments have been carried out using acetate as the organic carbon proxy to enhance microbial activity (e.g. van Geen et al., 2004; Islam et al., 2004; Gault et al., 2005; Héry et al., 2010). Thus, it is not clear whether incubation with different organic substrates can affect the structure of the microbial community and its potential for As(III) mobilization.

Most previous microbial ecology studies of arsenic-rich sediments have focused on sediments collected from Holocene aquifers, mainly because Pleistocene aquifers are generally thought to be less affected by arsenic contamination (Acharyya et al., 2000; Ravenscroft et al., 2005; Zheng et al., 2005) and are also more difficult to access. The use of waters from deeper Pleistocene aquifers (well switching) may represent a viable strategy for limiting arsenic uptake by at-risk populations, as shown in Bengal (van Geen et al., 2003; Polya and Charlet, 2009; Burgess et al., 2010). Recent studies have suggested that biogeochemical mobilization of As(III) may occur to a lesser extent in Pleistocene sediments (Sutton et al., 2009; Al Lawati et al., 2012). Another study showed that the addition of arsenate respiring Shewanella spp. to Pleistocene sediments is necessary to trigger the release of As(III) (Dhar et al., 2011). However, the actual potential for indigenous microbial communities to mobilize As(III) under anoxic conditions, when stimulated by a simple organic substrate that can act as electron donor, needs to be explored further. Therefore, in this study the structure of the whole bacterial community and of the As(V)-respiring bacteria were determined using culture-independent techniques in two arsenic-rich Cambodian sediments (i.e. containing higher As content than the crustal average): a Holocene and Pleistocene sediment with contrasting mineralogical and geochemical characteristics, depositional history, and organic matter composition and abundance. In addition, the Holocene sediment was incubated with both 13C-acetate and 13C-lactate, to investigate and compare the impact of two different short-chain organic acids on the microbial community structure and reductive As(III) mobilization. The use of 13C-labelled substrates combined with DNA-SIP facilitated the profiling of the active members of the bacterial community.

The aims of this study were (i) to characterize and compare the bacterial community-metabolizing acetate or lactate and the As(V)-respiring bacteria in a Holocene and a Pleistocene sediment, (ii) to investigate the effect of different organic carbon amendments on bacterial community structure and As(III) mobilization, and (iii) to investigate the potential for As(III) mobilization in Holocene and Pleistocene sediments. A better understanding of the biotic and abiotic factors controlling arsenic mobilization and immobilization in South East Asian aquifers, including the lithological characteristics of the sediments, is ultimately required to provide insight into appropriate strategies to reduce the exposure to arsenic through the consumption of contaminated groundwater.

Table 1. Arsenic speciation in the solid phase (based on X-ray Absorption Near Edge Structure spectra) of Holocene sediments and of Pleistocene sediments before and after incubations with lactate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation period (d)</th>
<th>% arsenic in form of (XANES)</th>
<th>Fit index %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Arsenate</td>
<td>Arsenite</td>
</tr>
<tr>
<td>Holocene sediment</td>
<td>–</td>
<td>7</td>
<td>85</td>
</tr>
<tr>
<td>Pleistocene sediment</td>
<td>–</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>P + lactate</td>
<td>77</td>
<td>96</td>
<td>4</td>
</tr>
</tbody>
</table>

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below detection limits). Detailed analysis of the organic biomarker distribution, which further highlights the large differences between the two sediments, is provided in Supporting Information Appendix S1.

Substrate utilization and As(III) release during microcosm incubations

Anaerobic microcosms were set up with Holocene (H) sediment amended with 10 mM $^{13}$C-lactate or 10 mM $^{13}$C-acetate. After incubation under anaerobic conditions, a clear succession of substrate utilization was observed. In the ‘H $^{13}$C-lactate’ microcosms, the largest proportion of the lactate (84%) was degraded rapidly within 7 days (Fig. 1A), and the degradation of lactate was followed by an increase in the concentration of acetate up to 9.6 mM on day 28, before it was largely degraded by day 52 (Fig. 1A). The concentration of acetate in the ‘H $^{13}$C-acetate’ treatment mostly decreased between days 28 and 43 (Fig. 1B).

In the microcosms set up with the Pleistocene sediment amended with 10 mM lactate, lactate utilization within the first 28 days of incubation was accompanied with the production of 3 mM acetate (Fig. 1C). By the end of the incubation period (77 days), most of the lactate was degraded (84%), while acetate had accumulated in the microcosms (11 mM; Fig. 1C). Contrary to lactate, acetate was not degraded in the Pleistocene sediment over the time period used in this study (data not shown). In all abiotic controls (sterilized by autoclaving), no significant degradation of the added substrates (lactate or acetate) or formation of acetate was observed during the incubation period (Fig. 1A–C), indicating that utilization of these organic substrates was microbially driven.

In the microcosms set up with the Pleistocene sediment amended with 10 mM lactate, lactate utilization within the first 28 days of incubation was accompanied with the production of 3 mM acetate (Fig. 1C). By the end of the incubation period (77 days), most of the lactate was degraded (84%), while acetate had accumulated in the microcosms (11 mM; Fig. 1C). Contrary to lactate, acetate was not degraded in the Pleistocene sediment over the time period used in this study (data not shown). In all abiotic controls (sterilized by autoclaving), no significant degradation of the added substrates (lactate or acetate) or formation of acetate was observed during the incubation period (Fig. 1A–C), indicating that utilization of these organic substrates was microbially driven.

In the microcosms set up with Holocene sediment and amended with $^{13}$C-lactate or $^{13}$C-acetate (Fig. 1A and 1B respectively), As(III) release was not observed during the incubation period. Thus, arsenic speciation in the solid phase of these incubations was not examined. In contrast,
low As(III) release occurred in the aqueous phase of the ‘P + lactate’ microcosms (up to 0.16 μM/12 ppb; Fig. 1C), and it was accompanied by a small increase in the As(III) content in the solid phase (4%; Table 1).

**Phylogenetic structure of bacterial communities**

In the 16S rRNA gene clone library constructed from the Holocene sediment (73 clones), 21 operational taxonomic units (OTUs) were identified at 97% similarity level, with none of the genera representing more than 20% of the sequences. The bacterial community of the Holocene sediment was dominated by β-Proteobacteria (60%), Firmicutes (21%) and δ-Proteobacteria (8%) phyla, with most of the sequences affiliated to the Hydrogenophaga (29%), Acidovorax (16%), Acetobacterium (8%) and Geobacter (8%) genera (Fig. 2). In addition, one sequence was closely related (99% similarity) to the dissimilatory arsenate-reducing strain *Sulfurospirillum halorespirans* PCE-M2 (GenBank accession number: AF218076) (Luijten et al., 2003).

After incubations with 13C-labelled substrates, DNA-SIP fractionation resulted in the separation of the heavy and light DNA fractions, evident by the distinct denaturing gradient gel electrophoresis (DGGE) profiles produced by the different DNA fractions (Fig. 3). It was therefore possible to identify the active members of the microbial communities in these microcosms by 16S rRNA gene cloning and sequencing of the heavy (13C-labelled) DNA fraction. After incubation with 13C-lactate, most of the sequences in the heavy DNA fraction belonged to the Geobacter (71% of the sequences), *Sulfurospirillum* (15%) and

![Fig. 2. Phylogenetic comparison at the (A) phylum/class and (B) genus level based on bacterial 16S rRNA gene sequencing in Holocene (H) and Pleistocene (P) sediments, the heavy DNA fractions of the ‘Holocene sediment + 13C-lactate’ (Hlac) and ‘Holocene sediment + 13C-acetate’ (Hace) incubations, and the lactate-amended Pleistocene sediment (Plac). Details about the treatments are shown in Table 2. Only genera representing more than 1.3% of the total number of clones (seven sequences and above) are shown.](image-url)
Dechloromonas (10%) genera of the δ-, ε- and β-Proteobacteria respectively (Fig. 2). Similarly, most of the sequences in the heavy DNA fraction of the ‘H + ^{13}C\text{-lactate’ treatment were affiliated to the Geobacter (66%) and Sulfurospirillum (5%) genera. However, phylogenetic analysis of the Geobacter-related 16S rRNA gene sequences in this study showed that distinct Geobacter phylotypes were selected depending on the treatment (Fig. 4). In the Holocene sediment prior to incubation, most (five out of six) Geobacter 16S rRNA gene sequences were closely related (99% ID similarity) to environmental sequences from West Bengal As(III) mobilizing sediments (Héry et al., 2010), and one sequence was related to Geobacter pickeringii G13 (DQ145535). Incubation with ^{13}C-lactate led to the dominance of sequences closely related (98% ID similarity) to Geobacter chapelleii 172 (U41561) and Pelobacter propionicus DSM 2379 (CP000482). In contrast, incubation with ^{13}C-acetate led to the enrichment of a phylotype (31% of the population) related to G. pickeringii G13 (DQ145535) and another phylotype (26% of the population), which shared 98% similarity with P. propionicus DSM 2379 (CP000482) and 97% ID similarity with many Geobacter spp., including Geobacter uraniireducens RI4 (EF527427), G. chapelleii 172 (U41561) and Geobacter toluenoxydans TMJ1 (EU711072) (Fig. 4). The sequenced DGGE bands following DNA-SIP fractionation (Fig. 3) were largely in agreement with the results from the 16S rRNA gene clone libraries, as the dominant bands in the heavy DNA fractions of the ‘H + ^{13}C\text{-lactate treatment belonged to the Geobacter, Sulfurospirillum and Dechloromonas genera, and those of the ‘H + ^{13}C\text{-acetate treatment were affiliated to the Geobacter and Sulfurospirillum genera.}

The Pleistocene sediment was characterized by a complex bacterial community, as 61 OTUs were identified (out of 82 sequences, at 97% OTU similarity level), with none of them representing more than 7% of the sequences. Despite the high number of OTUs present, there was no clear overlap between the sequences of the Holocene and the Pleistocene sediments. The dominant bacterial phyla were Actinobacteria and α-Proteobacteria (20% of the sequences each), with β-, γ-, δ-Proteobacteria, Acidobacteria and Firmicutes also present (Fig. 2). Bacteria detected in this sample included those affiliated with the following taxa: Propionivibrio (five sequences), Aeromonas (five), Acidobacteria Gp6 (four) and Gp21 (two), Pelagibius (two) and Enterobacter (two). The following were also detected (one sequence each): Pseudomonas, Sphingomonas, Pseudolabrys, Methylobacterium, Microvirga, Delftia, Tunebacillus, Euzelyba and Dietzia. The existence of sequences closely related (99% and 98% ID similarity respectively) to the obligate aerobe Methylobacterium oxalidis (AB607860) and to the strictly anaerobe Propionivibrio dicarboxylicus CreMa1 (NR_026477) indicates that the Pleistocene sediment may have consisted of different micro-niches, which were inhabited by both aerobic and anaerobic organisms. After addition of lactate to the Pleistocene sediment and incubation for 77 days (‘P + lactate’ treatment), 97% (66 out of 68) of the sequences were closely related (99% ID similarity) to the sulphate- and nitrate-reducing δ-Proteobacterium Desulfovibrio oxamicus DSM 1925 (DQ122124).
Diversity of arrA gene sequences

The phylogenetic analysis of the translated amino acid ArrA sequences showed that the Holocene sediment (prior to incubation) was dominated by sequences closely related to environmental ArrA sequences detected previously in West Bengal arsenic-rich sediments (Héry et al., 2010), constituting a cluster of sequences distinct to those of any cultured organism (Fig. 5). Incubation with 13C-labelled substrates led to the selection of different ArrA phylotypes. After incubation with 13C-acetate, 96.7% of the ArrA sequences obtained were related to G. uraniireducens Rf4 and 3.2% to Geobacter lovleyi SZ putative ArrA sequences (δ-Proteobacteria) (Fig. 5). Incubation with 13C-lactate selected for a distinct ArrA phylotype that was not affiliated directly to any known arsenate-respiring microorganism.

Although polymerase chain reaction (PCR) amplification of arrA gene fragments from the Pleistocene sediment was not successful, arrA gene sequences were successfully amplified after incubation with lactate for 77 days. The ArrA sequences obtained from the ‘P + lactate’ treatment clustered with environmental ArrA sequences from Utah basin aquifer sediments and from Chesapeake Bay sediments (Song et al., 2009), and they were distantly related to ArrA sequences from Geobacter species (Fig. 5).

Discussion

The effect of organic substrate amendments on the phylogenetic structure of indigenous bacterial communities and their potential for arsenic mobilization were investigated in two contrasting arsenic-rich Cambodian sediments: (i) a Holocene sediment, rich in labile organic matter and with a solid phase arsenic speciation dominated by As(III), and (ii) a Pleistocene sediment, with low abundances of predominantly recalcitrant organic matter and with a solid phase arsenic speciation dominated by As(V). Detailed organic matter analysis of the two sediments is provided as Supporting Information Appendix S1.

In the microcosms that were set up with the Holocene sediment, the use of 13C-labelled substrates, DNA-SIP, gene cloning and sequencing of the heavy DNA fraction allowed the profiling of the active members of the bacterial communities, highlighting the applicability of DNA-SIP as a tool in microbial ecology studies, as previously...
Fig. 5. ArrA phylogenetic tree based on 203 AA sequences using maximum-likelihood method under Jones–Taylor–Thornton (JTT) substitution model, with a gamma distribution of rates between sites. ArrA sequences (in bold) were grouped into phylotypes based on a 98% nucleotide sequence similarity cut-off. The number of clones contained in each phylotype is indicated in brackets. Details about the different samples are shown in Table 2.
discussed (Neufeld et al., 2007a). Incubation of Holocene sediments with $^{13}$C-lactate and $^{13}$C-acetate led to the enrichment of Geobacter-related 16S rRNA sequences. However, different Geobacter-related phylotypes were enriched after amendment with $^{13}$C-lactate or $^{13}$C-acetate, which may indicate organic carbon utilization specificity at the bacterial species level. Moreover, both substrates resulted in the enrichment of sequences closely related to dissimilatory arsenate-reducing Sulfurospirillum spp. (Stolz et al., 1999; Luijten et al., 2003). Previous studies that were carried out using acetate as the organic carbon proxy also showed enrichment in sequences belonging to the Geobacter (Lear et al., 2007; Héry et al., 2010) and Sulfurospirillum genera (Lear et al., 2007), and the potentially important role of the Geobacter genus on arsenic cycling has been previously discussed in South East Asia aquifers (Lear et al., 2007; Héry et al., 2010; Lloyd et al., 2011) and more recently in a uranium-contaminated aquifer (Giloteaux et al., 2013). However, it should be noted that further work is required to confirm that enrichment of Geobacter species in these sediment systems is linked directly to arsenic cycling, and not other biogeochemical processes stimulated by the addition of organic carbon.

In this study, the enrichment of potential arsenate-respiring Geobacter and Sulfurospirillum spp. and the detection of ArrA sequences in the heavy DNA fractions was not accompanied by As(III) mobilization from As(V) naturally present in the Holocene sediment during the incubation period, in contrast with previous microcosm studies (Lear et al., 2007; Héry et al., 2010). Thus, it is not clear whether naturally occurring As(V) served as the electron acceptor during the lactate/acetate utilization. The As(V) in the solid phase comprised only 7% of the total solid phase arsenic, and a significant fraction could be non-bioavailable, for example incorporated into recalcitrant mineral phases. Because Geobacter spp. are known Fe(III)-reducing bacteria, it is more likely that substrate utilization was mainly coupled with reduction of mineral Fe(III)-oxides present in the Holocene sediments. This is supported by the increase of soluble Fe(II) concentrations observed during similar incubations, suggesting that iron reduction occurred in the Holocene microcosms (data not shown).

The phylogenetic analysis of the bacterial community in the Pleistocene sediment (P clone library; Fig. 2) showed that it shared no common OTUs with the bacterial community in the Holocene sediment. This finding indicates that contrasting bacterial communities inhabit Pleistocene and Holocene sediments, and that these communities may have different potentials for substrate utilization and As(III) mobilization. Moreover, no known Fe(III)- or As(V)-reducing bacteria were initially detected in the Pleistocene sediment, despite the relatively diverse bacterial community present, in agreement with a previous study (Sutton et al., 2009), and arrA gene PCR amplification was not successful.

However, the addition of a simple and labile organic carbon substrate (lactate) stimulated the microbially mediated release of As(III) into the aqueous phase (0.16 μM on day 77, Fig. 1C). Incubation with lactate favoured the dominance of sequences closely related to the sulphate-reducing bacterium Desulfovibrio DSM 1925 (López-Cortés et al., 2006) but not to Geobacter spp. and Sulfurospirillum spp., as found previously in the ‘H + $^{13}$C-lactate’ treatment. The dominance of a sulphate-reducing bacterium in microcosms that were not amended with sulphate could be explained by the fact that the natural sulphate content in the Pleistocene sediment prior to incubation was 40 mg l$^{-1}$, but it was depleted by the 28th day of incubation (data not shown). Moreover, another Desulfovibrio species, Desulfovibrio desulfuricans G20, has been shown to reduce 20 mM As(V) in liquid cultures with lactate as the electron donor and sulphate as the electron acceptor, using a detoxification mechanism regulated by an arsRBC operon and an arsC gene (Li and Krumholz, 2007). In the past, it had been suggested that Desulfovibrio spp. might be contributing to arsenate respiration in Mono Lake waters (Hoeft et al., 2002), but no evidence of arsenate respiratory reductase genes (arrA and arrB) has been found in the D. desulfuricans G20 genome (Li and Krumholz, 2007). In our study, the translated ArrA sequences that were retrieved from the ‘P + lactate’ treatment were only distantly related to ArrA sequences from Geobacter species. Thus, it is not clear whether the low As(III) mobilization in these Pleistocene microcosms was driven by a yet uncharacterized arsenate-respiring Desulfovibrio species or by members of the microbial community present in low abundances. Although Geobacter 16S rRNA gene sequences have been detected previously in Pleistocene sediments (Al Lawati et al., 2012), their involvement in arsenic mobilization in the Cambodian Pleistocene sediments is not supported clearly by the present study.

Future experiments with $^{13}$C-labelled substrates will allow a more accurate identification of the active members of microbial communities in Pleistocene sediments, capable of dissimilatory arsenate reduction. However, our findings demonstrate clearly that Pleistocene sediments are poised ready for As reduction and mobilization, limited only by the lack of suitable organic substrates. This is consistent with Postma and colleagues (2012) who suggested that lower groundwater arsenic in similar aquifers in northern Vietnam is associated with the occurrence of older, less reactive organic matter. In the presence of an organic carbon proxy such as lactate, As(III) mobilization mediated by indigenous microorganisms can occur in Pleistocene aquifers under anoxic conditions. This
finding, in contradiction with what was previously observed for Pleistocene sediments from Bangladesh (Sutton et al., 2009; Dhar et al., 2011; Al Lawati et al., 2012), has major implications for future strategies that aim to reduce the exposure to arsenic by switching to consumption of groundwater from Pleistocene aquifers, particularly where there is significant advective transport of labile surface-derived organic carbon electron donors (Harvey et al., 2002; van Geen et al., 2013; Lawson et al., 2013; Mailloux et al., 2013).

Experimental procedures

Site location and sediment collection

The two sampling sites were chosen according to data available from previous studies in order to collect sediment samples that correspond to Holocene and Pleistocene aquifers. The sites near the Mekong River in Kandal Province, Cambodia, had been identified previously as a region of high arsenic groundwater hazard, with concentrations ranging between 2.8 and 12 mg kg\(^{-1}\) (Polizzotto and colleagues, 2008). Arsenic contents in the crustal abundance of arsenic is 1.5 mg kg\(^{-1}\) whereas the average crustal abundance of arsenic is 1.5 mg kg\(^{-1}\). The Holocene sediments typically comprise clays, peats and silts overlying fine grey sands (Tamura et al., 2007; 2009). According to Polizzotto and colleagues (2008), arsenic contents in the sediments in this area range between 2.8 and 12 mg kg\(^{-1}\), whereas the average crustal abundance of arsenic is 1.5 mg kg\(^{-1}\). The Holocene sediments typically comprise clays, peats and silts overlying fine grey sands (Tamura et al., 2007; 2009). Tamura and colleagues (2009) characterized older Pleistocene deposits as orange sands and silts occasionally underlying the Holocene sediments. Two distinct sediments were selected for this study based on sampling location and information inferred about their geological history, as well as from visual observations made on the retrieved cores: one Holocene sediment (H) and one deeper sediment assumed to be ‘Pleistocene’ (P) on the basis of its lithological characteristics, which were consistent with Pleistocene sediments previously described in the same area by Tamura and colleagues (2009), and its occurrence below sediments of inferred Holocene age. The Holocene sediments were collected at Rotaing (Cambodia) on 11 July 2008 [Global Positioning System (GPS) positions were 48P 0503670, 1271762]. The well used for drilling fluid (water) was 63 m deep. Two cores were collected between 11 and 11.5 m depth, and the sediments were composed of a mix of clay and fine-grained grey sand. The Pleistocene sediments were collected at Sre Ampil, Chheu Teal, Kean Svay District (Cambodia) on 22 July 2008 (GPS positions were 0509998, 1266782). The well used for drilling was 31 m deep. Four cores were collected between 12 and 13.5 m depth, and the sediments were composed of fine to medium orange sand.

All sediment samples were collected using a ‘core catcher’ going down inside driller pipes, thereby removing the possibility of contamination from borehole sides. When the cores were collected sequentially, the borehole was flushed with groundwater between collection for 1 min (i.e. no drilling between sample collection and quick flush with groundwater).

As mobilization in contrasting Cambodian sediments

The drilling fluid (water) was always sourced from nearby anoxic groundwater well (> 20 m). All the samples were sealed into O2-impermeable bags (with at least one O2 stripper included) using a larger glove bag under N\(_2\) and refrigerated within 6 h of collection. They were maintained refrigerated during transport by courier to Manchester (UK), where they were handled using strictly anoxic conditions, stored at 10°C to minimize oxidation (Rowland et al., 2005) and used in microcosm studies within 2 weeks.

Sediment characteristics

Solid-phase organic matter characterization, including TOC and TN determinations and lipid biomarker characterization, was performed as previously described in Héry and colleagues (2010). TC and TN values were determined using a EuroVector EA3000 CHN elemental analyser (EuroVector, Milan, Italy). The inorganic carbon content was determined using a modified Strohlein Coulomat 702 analyser (Markus de Vries, Schwabach, Germany), and TOC was calculated by subtracting inorganic carbon from TC values. Experimental procedures related to organic matter analyses are provided as Supporting Information Appendix S1.

Microcosm set up

Microcosms were set up as described in Héry and colleagues (2010); 10 g of anoxic sediments were placed into sterile acid washed 100 ml glass serum vials and mixed with 20 ml of sterile synthetic groundwater (Rowland et al., 2007). The microcosms were set up under an N\(_2\) atmosphere, sealed with rubber butyl stoppers, then flushed with a mixture of CO\(_2\) : N\(_2\) (ratio 20:80) for 10 min, and incubated at 20°C. With the Holocene sediment, two treatments were set up (Table 2), as microcosms were amended with 10 mM 13C-lactate or 10 mM 13C-acetate. The two treatments, including additional abiotic controls (sterilized by autoclaving), were set up in triplicate. To validate the further separation of 13C- and 12C-DNA, controls were conducted by incubating bacterial pure cultures with either 13C- or 12C-acetatem and the substrate consumption was checked over time (data not shown). With the Pleistocene sediment, the microcosms were amended with (i) 10 mM unlabelled lactate or (ii) 10 mM unlabelled acetate. Because no acetate utilization was observed during 100 days of incubation, only results from the 10 mM lactate microcosms (P + lactate) are presented (Table 2).

Organic acid quantification and arsenic speciation

Subsamples were collected at different incubation times for analyses (Fig. 1), and the solid phase was separated from aqueous phase as described previously (Héry et al., 2010). The concentrations of lactate and acetate (13C-labelled or unlabelled) in the microcosm supernatants and in the control cultures were determined using a Dionex DX120 ion chromatograph, fitted with a Dionex ICE AS1 ion-exclusion column (Dionex, Sunnyvale, CA, USA). When most of the lactate or acetate was consumed in the amended microcosms (Table 2; Fig. 1) and in the pure cultures, the incubations were stopped and the corresponding samples were stored at -80°C for further analyses.

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Arsenic speciation in the aqueous phase was determined by Ion Chromatography Inductively Coupled Plasma Mass Spectrometry, as described previously (Héry et al., 2010). Arsenic speciation in the sediments was determined using XANES spectra collected on the I18 beamline at the DIAMOND synchrotron (Didcot, UK) using linear combination analysis on the underivatized normalized spectra, in comparison with model compounds, sodium arsenite (As(III)-O) and arsenopyrite (As-S).

**DNA isolation and stable isotope probing**

DNA was extracted from all the Holocene and Pleistocene sediment samples as well as from the control cultures using the PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA). After DNA extraction from the Holocene microcosms amended with 13C-lactate and 13C-acetate (Table 2), and from the control cultures, heavy and light DNA fractions were separated on a CsCl gradient according to the protocol by Neufeld and colleagues (2007b), adapted for 3.9 ml Quick-Seal®, Polyallomer tubes (Beckman Coulter, Brea, CA, USA). The 12C- and 13C-control DNA were run in parallel to Holocene DNA samples to confirm that the DNA recovered in the heavy fraction corresponds to organisms that had actively metabolized the substrate. Following centrifugation with an Optima TL-100 ultra centrifuge (Beckman Coulter) at 178 693 g for 47 h at 20°C (maximum acceleration, no deceleration), 11 fractions of approximately 330 µl were collected from each sample, using a low-flow rate peristaltic pump, and their density was determined using an analytical balance (for each sample and the control tube). The DNA in the collected fractions was precipitated as described previously by Neufeld and colleagues (2007b), resuspended in 30 µl of Tris-EDTA buffer and quantified using a NanoDrop spectrophotometer (Nanodrop, Wilmington, DE, USA). The recovered DNA fractions were used as templates for ribosomal intergenic spacer analysis, using primers ITSReub (Cardinale et al., 2004), in order to verify that they contained distinct bacterial populations corresponding to successful 12C- and 13C-DNA separation along the density gradient (data not shown). In addition, 16S rRNA-based DGGE was performed on a selection of DNA fractions from the H + 13C-lactate and H + 13C-acetate treatments in order to demonstrate the distinct DGGE patterns among the (i) heavy DNA fraction, (ii) light DNA fraction and the (iii) total DNA (before separation on the CsCl density gradient). PCR amplifications were carried out using GC-341F and 518R (Muyzer et al., 1993) and touchdown PCR protocol (Cunliffe and Kertesz, 2006). 16S rRNA gene PCR products were purified through a QiAquick PCR purification column (QIAGEN, Valencia, CA, USA) and quantified with a Nano-drop spectrophotometer (NanoDrop, Wilmington, DE, USA). DGGE was carried out in a DCode electrophoresis chamber (Bio-Rad, Hercules, CA, USA) as described previously (Cunliffe and Kertesz, 2006). Gels were stained with SYBR Gold nucleic acid stain (Invitrogen, Carlsbad, CA, USA) and viewed under an UVItec trans-illuminator (UVitec, Cambridge, UK). Selected DGGE bands were excised from gel using a scalpel blade, crushed in 20 µl of H2O, and incubated for 48 h at 4°C. The released DNA was then re-amplified as above but using the unclamped 341F primer. 16S rRNA gene PCR products were purified as above and used for sequencing. The sequences obtained were checked for chimeras using Pintail (Ashelford et al., 2005), and their closest phylogenetic affiliation (to cultured organism, if possible) was identified by nucleotide Blastn searches.

**16S rRNA and arrA gene cloning and sequencing**

PCR amplification of isolated DNA (unlabelled or selected heavy DNA fractions, as shown in Table 2), cloning and sequencing of 16S rRNA gene and arrA gene fragments were performed as described previously (Héry et al., 2010). To account for arrA gene variability, we tested multiple sets of available primers to cover maximal arrA diversity. All arrA primers and PCR amplification protocols published to date were tested (Malasarn et al., 2004; Kulp et al., 2006; Lear et al., 2007; Fisher et al., 2008). None resulted in amplification of arrA sequences from DNA isolated from the Holocene and Pleistocene sediments. The only protocol that successfully amplify an arrA gene product was the one described previously by Héry and colleagues (2010). First, arrA was amplified using primers ArrAUF1 (TGTCAAGGHTGTA TBASNTGGGANRARGCNMT) and ArrAUR3 (GGGGTGCG CBDCHTGG) and ArrAUR3 (GCW GCCCAY TCV GGN GT) as described by (Fisher et al., 2008). A second PCR amplification was performed with primers AS2F (GTCCCNATBASNTGGGANNRARGCNMT) and AS1R (GGGGTGCG GTCYTTNARYTC) as described by Lear and colleagues (2007), using the first PCR products as templates. In this study, five 16S rRNA and four arrA gene clone libraries were prepared (Table 2); for each library, either 96 or 48 of clones were sequenced. The 16S rRNA gene sequences obtained were analysed for chimeras using Mallard (Ashelford et al., 2005).
and chimeric sequences were removed from further analysis. The phylogenetic classification of the non-chimeric 16S rRNA sequences (at a confidence threshold of 95%) was performed using the RDP Naive Bayesian rRNA Classifier Version 2.4 of the Ribosomal Database Project (Cole et al., 2009), and MOTHUR v.1.6.0 (Schloss et al., 2009) was used to cluster these sequences into OTUs at a level of similarity of 97%. The closest type strain relative to each OTU was identified using the RDP(Ribosomal Database Project)'s SeqMatch. The phylogenetic tree of the GeoBacter-related 16S rRNA sequences was drawn with MEGA5 (Tamura et al., 2011), using the neighbour-joining method and the Jukes Cantor substitution model. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. The arrA sequences obtained were submitted to BLAST search (Altschul et al., 1990), and phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura et al., 2011). The best-fitting model of molecular evolution for the dataset was calculated based on amino acid-derived sequences, and the optimal tree was performed with maximum-likelihood method under Jones–Taylor–Thornton substitution model, with a gamma distribution of rates between sites. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. All sequences of this study were deposited to GenBank, under accession numbers JQ810187-JQ810718 (16S rRNA gene sequences; Table 2) and JQ890209-JQ890225 (arrA gene sequences; Table 2).

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References


**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1.** Organic matter analysis in Holocene and Pleistocene sediments.