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Considerations for conducting incubations to study the mechanisms of As release in reducing groundwater aquifers

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ABSTRACT

Microbial Fe reduction is widely believed to be the primary mechanism of As release from aquifer sands in Bangladesh, but alternative explanations have been proposed. Long-term incubation studies using natural aquifer material are one way to address such divergent views. This study addresses two issues related to this approach: (1) the need for suitable abiotic controls and (2) the spatial variability of the composition of aquifer sands. Four sterilization techniques were examined using orange-colored Pleistocene sediment from Bangladesh and artificial groundwater over 8 months. Acetate (10 mM) was added to sacrificial vials before sterilization using either (1) 25 kGy of gamma irradiation, (2) three 1-h autoclave cycles, (3) a single addition of an antibiotic mixture at 1× or (4) 10× the typical dose, and (5) a 10 mM addition of azide. The effectiveness of sterilization was evaluated using two indicators of microbial Fe reduction, changes in diffuse spectral reflectance and leachable Fe(II)/Fe ratios, as well as changes in P-extractable As concentrations in the solid phase. A low dose of antibiotics was ineffective after 70 days, whereas autoclaving significantly altered groundwater composition. Gamma irradiation, a high dose of antibiotics, and azide were effective for the duration of the experiment.

Using gamma irradiation as an abiotic control, shallow grey sediment and groundwater from 3 closely spaced locations along a gradient of dissolved As concentrations (60–130–210 μg/L) in Bangladesh were incubated for 8 months with and without organic C addition (0.9 and 0.6 mM of acetate and lactate). Unexpectedly, levels of dissolved As (64 ± 68, 92 ± 70, 217 ± 68 μg/L) and P-extractable As (0.7 ± 0.2, 2.1 ± 0.5 and 2.0 ± 0.3 mg/kg) at each location were highly variable over the duration of the experiment and prevented the detection of the relatively small levels of As release that were anticipated. Maintenance of an adsorptive equilibrium with the P-extractable As concentrations seems to govern dissolved As variability. The sediment variability is attributed to natural patchiness in the distribution of aquifer properties rather than a sampling artifact. Sub-sampling a single batch of groundwater and aquifer solids over time can alleviate this problem to some extent, but the issue of the representativeness of particular samples remains.

1. Introduction

The mechanism of As release from aquifer sands of the Bengal Basin is extensively studied because elevated levels of As are widespread throughout the region's groundwater;

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the primary source of drinking water for over 100 million people (BGS and DPHE, 2001; Ahmed et al., 2006). Current research suggests that microbial Fe reduction in anoxic groundwater releases As, however this process remains poorly understood (BGS and DPHE, 2001; Oremland and Stolz, 2003). Extensive field studies have shown that dissolved Fe and As concentrations are poorly correlated (Nickson et al., 1998; BGS and DPHE, 2001; van Geen et al., 2008) and laboratory studies using natural aquifer solids have shown that Fe and As release from sediment can be decoupled (Islam et al., 2004; van Geen et al., 2004b; Gault et al., 2005; Radloff et al., 2007). Such incubations have provided important clues to the mechanisms of As release, but various aspects of experimental design may also have limited their relevance to natural conditions.

The motivation for the present contribution is two-fold. First, various sediment sterilization methods are compared in terms of their effectiveness and to what extent they preserve the original geochemical conditions of natural aquifer material. Second, using the most promising of these sterilization methods as an abiotic control, a series of extended incubation experiments using groundwater and aquifer solids from three locations along a dissolved As gradient in Bangladesh were conducted under anaerobic conditions.

1.1. Sterilization methods

There has been considerable research on soil sterilization techniques, but the effect of sterilization on geochemical properties in the context of As mobilization has not been evaluated. Autoclaving and gamma irradiation are standard physical methods for sterilizing samples, while additions of antibiotics and respiratory inhibitors (like azide) are commonly used chemical approaches. Physical sterilization methods are effective, but have the potential to alter important soil and sediment properties (Lotrario et al., 1995; Trevors, 1996; McNamara et al., 2003). Chemical treatments target specific biological processes and therefore often result in incomplete sterilization. On the other hand, these treatments leave soil and sediments nearly unaltered (Trevors, 1996; Herbert et al., 2005). One advantage of physical sterilization for use in incubation experiments is that model organisms can be introduced to the sterilized sediment, which is not possible after chemical treatment where the chemical residue remains.

Autoclaving is a popular sterilization method due to its low cost and ease of use, even though high temperatures (>110 °C) can decrease soil surface area, damage soil structure, and release nutrients (Wolf et al., 1989; Lotrario et al., 1995; Trevors, 1996). Multiple autoclaving treatments and pre-incubation of soil and water have been shown to increase the effectiveness of sterilization (Wolf et al., 1989; Lotrario et al., 1995).

Gamma irradiation has been used as a sterilization technique for over 50 years and has proved to be very successful in sterilizing soils (McNamara et al., 2003). One clear advantage, over autoclaving, is that irradiation induces smaller temperature changes during sterilization; soil temperatures rise by 35 and 60 °C for irradiation levels of 25 and 70 kGy, respectively (McNamara et al., 2003). Some changes in soil properties, including the release of nutrients, damage to soil organic matter, as well as some resilient enzymatic activity have been reported (Wolf et al., 1989; McNamara et al., 2003). A reported increase in extractable Mn following irradiation may be due interactions between solid Mn and organic matter solubilized by the procedure (McNamara et al., 2003). Changes in surface area are more severe for autoclaved samples than for irradiated ones (Wolf et al., 1989).

Antibiotics have been used to reduce the microbial population and inhibit metabolic activity. Several studies have used combinations of antibiotics to overcome multiple metabolic processes and increase their effectiveness, however antibiotic use does not result in complete sterility, particularly in studies lasting more than 30 days (Dowdle and Oremland, 1998; Wilkie and Hering, 1998; Herbert et al., 2005). Azide is an equally or more effective inhibitor than antibiotic combinations, but still does not necessarily result in complete sterilization (Wolf et al., 1989; Trevors, 1996; Dowdle and Oremland, 1998). Its application can also produce changes in ionic strength or the pH of groundwater that require the use of buffers to control (Wolf et al., 1989; Trevors, 1996). In this study, the effectiveness of all 4 sterilization methods described above – autoclaving, gamma irradiation, and additions of antibiotics and azide – have been compared by incubating natural Pleistocene sediment from Bangladesh over an extended period.

1.2. Previous incubations investigating as mobilization

Obtaining well preserved sediment and groundwater is essential for setting up incubations that are representative of *in situ* conditions. Common methods that have been used include refrigerating or freezing of sediments, flushing with N_2, and maintaining aquifer material under anoxic conditions (Islam et al., 2004; van Geen et al., 2004b; Gault et al., 2005; Swartz et al., 2005; Radloff et al., 2007). X-ray absorption near-edge spectroscopy (XANES) has shown that speciation of As in sandy sediment is particularly sensitive to exposure to even low levels of O_2; maintenance of samples under N_2 is therefore important (Rowland et al., 2005). Several studies have relied on the use of artificial groundwater, presumably due to the difficultly of obtaining, transporting, and storing anoxic groundwater (Islam et al., 2004; Gault et al., 2005). Artificial groundwater and solid phases have also been used in incubation studies to create a simplified matrix. Such studies have provided a number of valuable mechanistic insights (Benner et al., 2002; Roden, 2004; Coker et al., 2006; Herbel and Fendorf, 2006; Kocar et al., 2006). Their direct relevance to processes occurring in the natural environmental has yet to be determined, however.

Minimizing sample disturbances from natural conditions, including changing sediment-water ratios and maintaining groundwater flow conditions, is perhaps the most difficult aspect of field conditions to replicate in the laboratory. The majority of incubation experiments have been conducted in batch or sacrificial mode with lower sediment-water ratios than occur in an aquifer, with excess water to allow for monitoring over time. Such batch

experiments also do not address how groundwater flow could alter microbial dynamics (Roden et al., 2000). Some studies have attempted to replicate the effects of groundwater flow, but they have been limited so far to model systems (Roden et al., 2000; Benner et al., 2002; Herbel and Fendorf, 2006; Kocar et al., 2006). Another potential artifact that accompanies sampling, preparation of incubations (including homogenizing), and sample agitation is increased exposure of surface sites (Radloff et al., 2007).

Organic substrate additions along with sample sterilization have been important tools for characterizing and distinguishing microbially-mediated from abiotic processes. Common practice is to add organic substrate to levels (10 mM or more of acetate or equivalent) that are significantly greater than natural conditions (Islam et al., 2004; Roden, 2004; van Geen et al., 2004b; Gault et al., 2005; Coker et al., 2006; Herbel and Fendorf, 2006). Significant changes in microbial population response to such additions have been documented (Islam et al., 2004) and suggest that this practice may not necessarily induce transformations that dominate in aquifers where metabolism is C-limited. Several recent studies have amended samples with less organic substrate and these additions were still sufficient to induce responses different from the unamended samples, providing stronger evidence of the importance of microbial processes (Benner et al., 2002; Polizzotto et al., 2005; Kocar et al., 2006; Radloff et al., 2007). Sterilized controls are still needed to determine to what extent abiotic mechanism(s) could be responsible for As release from sediment in Bangladesh, however (Polizzotto et al., 2005).

Another difficulty in identifying key processes leading to As mobilization has been the limited duration of most incubation experiments, typically less than 3 months (Islam et al., 2004; van Geen et al., 2004b; Gault et al., 2005; Polizzotto et al., 2005). The build-up of As levels in groundwater over time in Bangladesh appears to occur at a rather slow and relatively constant rate (Radloff et al., 2007; Stute et al., 2007), therefore longer duration studies may be necessary to detect the relatively small changes expected. The experiments presented in this paper were all intended to address some of the potential artifacts reviewed in this section, including using natural sediments and groundwater preserved in a N_2 atmosphere from sample collection to experiment completion, using sacrificial vials to maintain a higher sediment-water ratio in order to more closely resemble aquifer conditions, and using abiotic and organic amendments to elucidate As mobilization processes over the 8 month incubation period.

2. Methods

2.1. Sediment collection

Several sterilization methods were tested using orange-colored Pleistocene sediment collected in January 2001 with a split-spoon sampler at 45 m depth in Araihazar, Bangladesh (90.60° E, 23.79° N; (Horneman et al., 2004; Zheng et al., 2005). The sections were sealed with wax and shipped at ambient temperature and have been stored at 4 °C since May 2001. The cores were opened in the laboratory in an anaerobic chamber (Coy Laboratories), although it cannot be excluded that they could have been exposed to air during storage due to diffusion through the core liner or wax seal. The Pleistocene aquifer in this region is characterized by low dissolved As concentrations (<5 μg/L) and orange-colored sediment with leachable Fe(II)/Fe ratios <0.5.

Sediment and groundwater were collected in December 2006 for incubation experiments from another area within Araihazar (Site K: 90.63° E, 23.79° N). Additional samples were collected to further characterize this new site in May 2007. The needle-sampler (van Geen et al., 2004a) was used at five locations to simultaneously collect samples of sediment and groundwater for aquifer characterization. The locations are labeled by referring to their distance (in meters) from the nearby stream – K10, K60, K150, K240 and K280, respectively. The elevation of the ground surface increases gradually by 2 m along this transect away from the stream. Sample depths are listed relative to ground level near the stream.

Material for the incubations was collected using several methods from 3 drill holes ~90 m apart to cover a range of dissolved As concentrations. Sediment at K240 was collected at 8.5 m depth using one core from a piston device analogous to a Shelby tube, while groundwater was collected at 9.7 m using a modified drive-point groundwater sampler. At K150 sediment and groundwater were collected using the same methods: sediment from 3 cores collected between 6 and 6.7 m depth and groundwater from 5.9 m. At K60, sediment and groundwater were obtained by combining needle-sampler slurries from 5 depths between 6 and 8.4 m.

2.2. Sterilization experiments

In 10 mL glass vials, 2.5 g of preserved Pleistocene sediment was mixed with 6 mL of artificial groundwater. The composition of the artificial groundwater is based on that of actual groundwater at this location and consists of 243.6 mg/L $NaHCO_3$, 24 mg/L $CaCO_3$, 6.1 mg/L $MgCl_2$, 2.5 mg/L $MgSO_4$, 0.6 g/L NaH_2PO_4 and 0.8 mg/L Na_2HAsO_4 (Zheng et al., 2005). The vials were capped with blue butyl rubber stoppers (Bellco Glass), purged with N_2, and gently agitated in the dark on a rocking shaker for the duration of the experiment.

Five sterilization methods were tested and compared against two non-sterilized sample sets. Before sterilization, 10 mM acetate was added to each of the samples to be sterilized as well as to one of the non-sterile sample sets. Sterilization methods carried out on the next day included (1) gamma irradiation at 1.3 kGy/h for 19.5 h for a total dose of 25 kGy, (2) autoclaving at 121 °C for 1 h on 3 consecutive days, (3) Guillard's reagent, an antibiotic mixture at it's typical strength, or low dose, and (4) at a 10 times higher concentration, or high dose, and (5) 10 mM of azide. The gamma ray source was provided by a Gammacell 220 (MDS Nordion International). Guillard's reagent consisted of 9.8 mg (16250U) penicillin, 2 mg chloramphenizol, and 5 mg streptomycin per 12.5 mL distilled water (Wilkie and Hering, 1998). This concentrated solution was added to the incubations at a ratio of 5 mL per L of groundwater.

2.3. Site K incubation experiments

Incubation experiment samples were prepared in the field within a few hours of collection. Close to anaerobic conditions (less than 1% O_2) were maintained using a portable glove box (Bel-Art), which was continuously flushed with ultra high purity N_2 gas and monitored with an O_2 indicator strip (Becton Dickinson). The sediment samples (~150 g) from each location were combined in a bowl and mixed with a spatula before distributing into individual vials. Approximately 5 g of wet sediment and 5 mL of groundwater were added to 10 mL glass vials, which were then capped with blue butyl rubber stoppers and sealed in the chamber. Twenty-five days after collection, a subset of vials were amended with an organic C addition; 0.9 mM acetate and 0.6 mM lactate were added to supply enough electron donors to potentially reduce approximately 10% of the Fe present in the sediment, i.e. more than an order of magnitude lower concentration of substrate per gram sediment than used in the sterilization experiments. This organic combination was chosen to stimulate a wider range of organisms than acetate alone (Madigan, 2006). Abiotic controls were exposed to 25 kGy of gamma irradiation, as in the sterilization experiment, the day following organic amendment. The vials were gently agitated in the dark with a rocking shaker over the duration of the experiment.

2.4. Groundwater and sediment analysis

The diffuse spectral reflectance of the sediment was measured throughout both experiments to monitor the extent of Fe reduction (Horneman et al., 2004). The slope of reflectance between 530 and 520 nm, reported in units of % reflectance per 10 nm (%/10 nm) and referred to here as ΔR without units, is used as an indication of Fe speciation in the solid phase. Grey, reduced sediment has a flatter reflectance spectrum and therefore has a smaller slope as a function of wavelength than orange–brown material containing an elevated proportion of Fe(III) oxyhydroxides. The diffuse spectral reflectance of the sediment was measured periodically through all glass vials that had not yet been sacrificed for analysis. The standard error is therefore based on a decreasing number of measurements over time.

At 4 time points during both the sterilization and incubation experiments, replicate vials were sacrificed to analyze groundwater and sediment properties. Groundwater samples were filtered through 0.45 μm syringe filters, acidified to 1% HCl (Optima) and analyzed by High-Resolution Inductively Coupled Plasma Mass Spectrometry (HR ICP-MS) for As, Fe, Mn, S, P, Na, K, Mg and Ca (Cheng et al., 2004). Sediment samples were analyzed to determine the speciation and availability of As and Fe. A 1 M phosphate solution was used to estimate the amount of As adsorbed in the solid phase and available for mobilization (Keon et al., 2001; Jung and Zheng, 2006) and was analyzed by HR ICP-MS following a protocol similar to the one used for groundwater. Speciation of As in the groundwater and phosphate extractions from the incubation experiments were determined using differential pulse cathodic stripping voltammetry (DPCSV) using an Eco Chemie Autolab voltammeter and a Metrohm 663 VA electrode stand (Brinkmann Instruments) (He et al., 2004; Jung and Zheng, 2006). Iron speciation of the sediment was determined by extraction with a hot 1.2 M HCl solution and measured using ferrozine (Horneman et al., 2004). It has been shown previously that, to a first approximation, ΔR decreases linearly from 1 to 0 as leachable Fe(II)/Fe increase from 0 to 1 (Horneman et al., 2004; van Geen et al., 2006).

3. Results

3.1. Sterilization of pleistocene sediment

The sterilized vials and their controls were monitored over the course of 8 months for changes in the dissolved and solid phase. The effectiveness of sterilization was gauged by monitoring potential changes in the acetate-amended sediment expected from microbial Fe reduction, specifically changes in the diffuse reflectance spectrum and leachable Fe(II)/Fe ratios as well as changes in the concentration of P-extractable As in the solid phase (Zheng et al., 2005). Dissolved As concentrations measured over the course of these experiments are not discussed in detail because levels remained low throughout, regardless of the treatment (<40 μg/L). The one exception is the autoclaved series, which resulted in dissolved As concentrations <2 μg/L for the duration of the experiments (Table 1).

The sediment from the core initially contained a P-extractable As concentration of 0.10 ± 0.03 mg As per kg sediment (Table 1). After only 1 day of incubation with artificial groundwater (and acetate in all but one of the ser-

Table 1
Variation in dissolved and solid phase concentrations at the start and end time points of the sterilization experiment

Day	P-ext As (mg/kg)		Fe ratio		As (μg/L)		Fe (mg/L)		Mn (mg/L)	
	Core	170	Core	170	1	170	1	170	1	170
Non-sterile w/o Acetate		0.20		0.22		15		0.02		0.10
Non-sterile		0.46		0.66		24		1.23		0.55
Radiation	0.10	0.30	0.15	0.10	20	15	0.08	0.04	0.12	0.21
Autoclave	±	0.13	±	0.12	±	1	±	0.01	±	0.13
Antibiotic – standard	0.03	0.51	0.05	0.31	13	24	0.12	0.81	0.08	0.52
Antibiotic – high		0.22		0.16		5		0.01		0.17
Azide		0.24		0.12		10		0.02		0.37

The initial time point of the dissolved concentrations came one day after the incubations began, while the initial sediment properties are from the core sediment (pre-incubation).

ies) and immediately after sterilization, P-extractable As concentrations increased in all samples and displayed considerable variability between treatment types (Fig. 1A). By day 14, however, P-extractable concentrations in all but two treatments converged to ~0.25 mg/kg and remained essentially constant for the next 7 months. In contrast, P-extractable As concentrations systematically increased in the non-sterile incubations with acetate (to 0.70 ± 0.03 mg/kg), as well as in the incubations sterilized with the low dose of antibiotics (to 0.60 ± 0.03 mg/kg), during the first two months and remained elevated thereafter. The autoclaved samples had lower P-extractable As concentrations than the non-sterile without acetate samples and the other sterilizing amendments. However, the difference was not greater than the inter-sample variability.

The reflectance data are consistent with the transformations suggested by the P-extractions. After day 12, the non-sterilized samples amended with acetate show a rapid decrease in ΔR from 0.9 to 0.6 (Fig. 1B). The reflectance slope reached a plateau of 0.5 after 50 days, whereas the non-sterilized samples without acetate showed no significant change in reflectance over the course of the experiment. The samples amended with a low dose of antibiotics began to show a reduction in ΔR from 0.8 to 0.6 after day 70 and the reflectance slope continued to decrease until the end of the experiment ($\Delta R = 0.5$ on day 240). The remaining sterilization methods all show little change in reflectance, with a combined ΔR of 0.82 ± 0.03 for the high dose of antibiotics, autoclaving and azide. Irradiation caused visible discoloration of the glass vials and therefore compromised the use of reflectance measurements ($\Delta R = 0.19 \pm 0.03$) as an indication of Fe reduction.

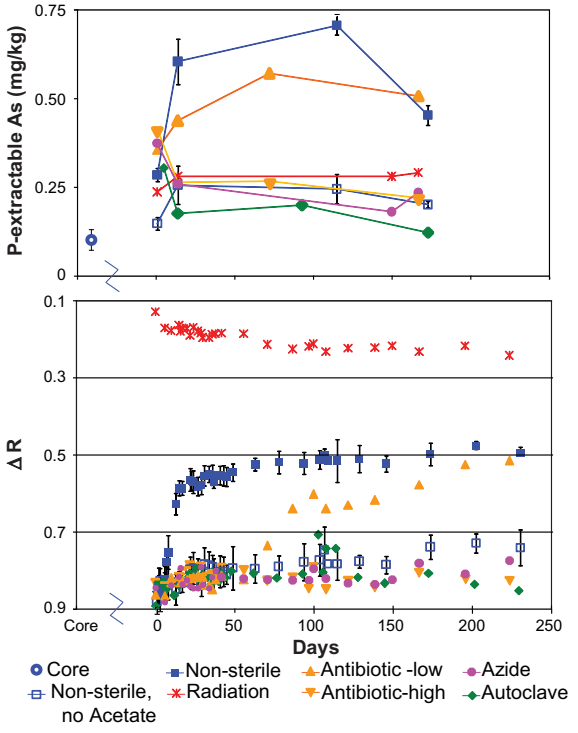

Fig. 1. (A) Phosphate-extractable As concentrations and (B) slope of diffuse spectral reflectance (ΔR) with more reduced sediments having a smaller slope in sterilized samples over the 8 month incubation. Both properties indicate Fe reduction occurring in the non-sterile and low dose antibiotic samples. Samples amended with acetate are represented with closed symbols, while open symbols show samples without acetate. The ΔR of gamma irradiated samples is offset due to the browning of glass by irradiation and should not be compared directly to the other amendments; nevertheless the ΔR of the irradiated samples remained constant for the duration of the experiment. Error bars for replicate samples of the two non-sterile amendments are shown at each time point. Error bars for other samples are similar in magnitude.

The evolution of leachable Fe(II)/Fe ratio over time determined from HCl-extractions are consistent with the trends indicated by the reflectance (Table 1). In the non-sterilized samples with acetate, the leachable Fe(II)/Fe ratio increased from 0.15 ± 0.05 in the core to a high of 0.86 ± 0.01 after day 14, and then remained above 0.65 for the remainder of the experiments. In contrast, the non-sterilized samples without acetate showed no change in Fe(II)/Fe ratio and remained <0.2 for the duration of the experiment. The samples amended with a low dose of antibiotics also had increasingly reduced sediment, with a peak Fe(II)/Fe ratio of 0.48 ± 0.02 after day 77. Leachable Fe(II)/Fe ratios fluctuated between 0.1 and 0.3 for the remaining amendments (irradiation, the high dose of antibiotics, autoclaving, and azide addition) without showing any systematic trend over time.

Dissolved Fe and Mn concentrations markedly increased in the samples that showed evidence of Fe reduction, as indicated by a decrease in ΔR and an increase in leachable Fe(II)/Fe ratios, but not in the samples that did not show evidence of reduction. Dissolved Fe concentrations increased from less than 0.1 mg/L to 1.2 mg/L in the non-sterile samples with acetate and to 0.8 mg/L in the samples amended with a low dose of antibiotics by the end of the experiment, while dissolved Fe remained less than 0.1 mg/L in the other amendments (Table 1). The concentration of dissolved Mn also increased from 0.12 mg/L to 0.5 mg/L in the non-sterile samples amended with acetate and samples amended with only a low dose of antibiotics. Dissolved Mn concentrations fluctuated between 0.1 and 0.2 mg/L for the other sterilized amendments and the non-sterilized samples without acetate and did not show any temporal trend, with the exception of the final samples amended with azide (0.37 ± 0.03 mg/L; Table 1).

3.2. Initial conditions at site K

Needle-sampling provides a two-dimensional picture of dissolved and sediment properties along a transect perpendicular to the stream at Site K where sediment and groundwater samples were collected for incubation. The 5 profiles show a gradual transition from a less reduced zone with <5 μg/L As at K280, furthest from the stream, to a highly reduced zone with >400 μg/L As at K60, near the stream (Fig. 2A). P-extractable As concentrations range from 0.4 to 3.7 mg/kg across the transect, but show no systematic spatial trend (Fig. 2B). Reflectance measurements conducted in the field indicated more reducing conditions towards the stream. However, there is considerable variability among samples separated by <1.5 m depth

(Fig. 2C). The reflectance data are consistent with leachable Fe(II)/Fe ratios increasing from ~0.5 at K280 to nearly 1.0 at K10 (Fig. 2D).

Whereas the horizontal gradient towards the stream is the dominant feature of site, the vertical profile at each location reveals heterogeneity with depth as well. Sediment and groundwater for the incubations were collected from approximately the mid-point of the vertical profiles. The two locations furthest from the stream (K280 and K240) are characterized by increasing leachable Fe(II)/Fe ratios and dissolved As concentrations with depth (Fig. 2). At K240, the Fe(II)/Fe ratio increases from ~0.5

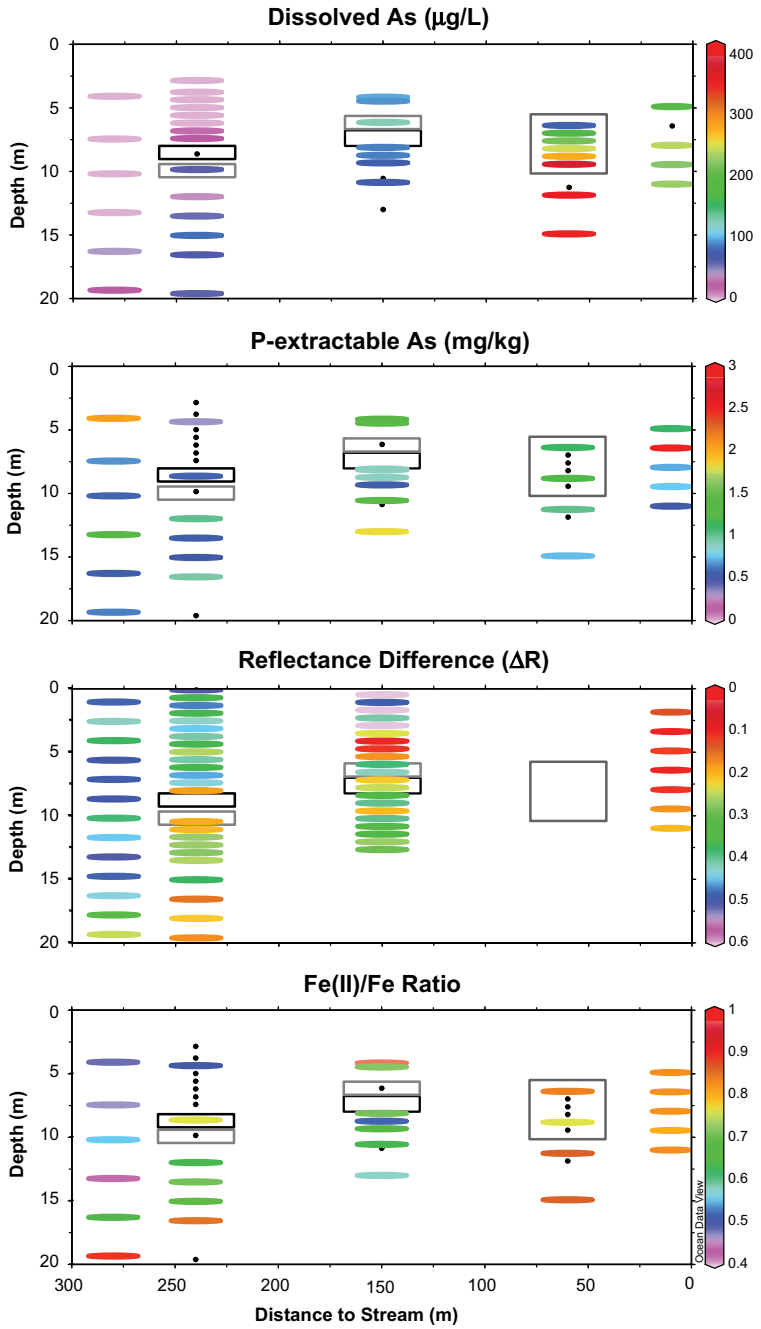

Fig. 2. Vertical profiles of groundwater and sediment properties at Site K based on needle-sampling. Five profiles are shown – K280, K240, K150, K60 and K10, indicating the distance in meters from a nearby stream. Profiles indicate more reducing sediment and increasing dissolved As concentrations towards the stream. P-extractable As concentrations are variable, but exhibit no spatial trend. There is considerable variation between closely spaced samples. Depth of sediment (black box) and groundwater (grey box) collected are indicated, at K60 they were collected simultaneously. No reflectance measurements were made at K60.

to 0.9 with depth and the dissolved As concentrations increase to a peak concentrations of 85 μg/L (Fig. 2). Incubation material from K240, collected between 8.5 and 9.7 m depth, had a Fe(II)/Fe ratio of 0.77 and 58 μg/L dissolved As.

The sediment exhibits a high level of variability within the top 10 m at K150. Reflectance measurements indicate an increase in sediment reduction from the surface to 4 m depth and then a decrease in sediment reduction from 4 to 10 m depth before increasing again below 10 m (Fig. 2). These observations are supported by leachable Fe(II)/Fe ratios that decrease with depth from ~1 to 0.5 from 4 to 9.3 m depth and then increase to 0.6 by 15 m depth. The peak dissolved As concentration of 128 μg/L occurs at 6 m which lies within the zone of lessening sediment reduction. Incubation material collected between 5.9 and 6.7 m depth had a Fe(II)/Fe ratio of 0.7 and 128 μg/L dissolved As.

At the two profiles nearest to the stream (K60 and K10), the sediment was consistently highly reduced with leachable Fe(II)/Fe ratios of 0.8 or greater (Fig. 2). Dissolved As increased from 80 to 380 μg/L from 6 to 8.4 m depth at K60. Incubation material was obtained from 5 needle samples across this depth range.

3.3. Changes in dissolved as during site K incubations

The sampling locations for the incubations were chosen to span the range of groundwater As concentrations along the transect perpendicular to the stream. Initial dissolved As levels increased from 60 to 130 to 210 μg/L in the K240, K150 and K60 incubation samples, respectively, and were accompanied by P-extractable As concentrations of 0.7 mg/kg at K240 and ~2.1 mg/kg at K150 and K60 (Fig. 3). Consistent with the difference of P-extractable As found at each site, dissolved As concentrations are low for all amendments at K240, are high and variable at K150, and are higher but less variable at K60 (Fig. 3A). Unexpectedly, dissolved As concentrations turned out to be highly variable over the course of the incubations for all amendments, especially at K150, and showed little indication of systematic changes over time (Fig. 3A).

Dissolved As concentrations in the unamended samples averaged 64 ± 68 μg/L ($n = 20$) at K240, 96 ± 70 μg/L ($n = 25$) at K150 and 217 ± 68 μg/L ($n = 27$) at K60. The unamended samples showed an initial decrease in dissolved As from when the incubation began to day 25 at K240 and K150, but concentrations returned to initial levels by the next sampling point on day 70 (Fig. 3A). In contrast, an initial increase in dissolved As occurred at K60 and concentrations remained at this higher level for remainder of the experiment. Organic amendments triggered larger variability and a higher average in dissolved As concentrations at K150, but had no apparent effect on dissolved As concentrations relative to the unamended samples at K240 or K60. Irradiation caused an initial decrease in dissolved As at all sites and was most pronounced at K60. Dissolved As concentrations recovered to pre-irradiation levels by the next sampling on day 70 at K240 and K60, but not at K150.

Speciation of dissolved As in the unamended and irradiated samples at each location indicated that dissolved As was present predominately as As(III) (Fig. 4). There was no variation in the proportion of As(III) over time or between locations. Furthermore, irradiation did not alter dissolved As speciation.

Concentrations of As in the sediment remained nearly unchanged over the course of the experiments (Fig. 3B), even for those with organic amendment. Levels of P-extractable As ranged from 0.72 ± 0.19 ($n = 20$) at K240 to 2.12 ± 0.50 ($n = 26$) at K150 to 2.02 ± 0.33 ($n = 29$) mg/kg at K60 (Fig. 2B). Irradiation showed no systematic effect at any of the locations. The speciation of P-extractable As was more variable, but adsorbed As was predominately As(III) (Fig. 4). The proportion of As(III) of the adsorbed As varied from nearly 100% at K240 to greater than 65%, in most samples, at K150 and K60. Since the complex extraction matrix can induce errors up to ±20% in the voltammetric analysis, it is reasonable to infer from these observations that the adsorbed was mostly As(III) as well. As in the case of dissolved As, there were no changes in speciation of adsorbed As over time or in response to irradiation.

3.4. Changes in other properties over time during site K incubations

Reflectance and leachable Fe(II)/Fe ratios reveal little change in sediment characteristics over the course of the incubations, regardless of the treatment (Fig. 3C and D). The ΔR of the irradiated samples are the only exception because of discoloration of the glass; this discoloration was less pronounced than in the sterilization experiments due to the difference in sediment color (a high ΔR value for the sterilization experiments vs. starting from an already low ΔR in the Site K incubations). Leachable Fe(II)/Fe ratios measured in the field indicated that K240 and K60 sediments were slightly more reducing than at K150, and they remained so throughout the experiment (Fig. 3D). Irradiation may have induced a change in Fe(II)/Fe ratio at K240 immediately after treatment, but the effect was temporary.

Dissolved Fe concentrations, and to a lesser extent Mn, also show considerable variability (Fig. 3E and F). The presence of Fe and Mn in solution for all samples does indicate that the incubation vials remained anoxic. As was observed in the case of dissolved As, there was some variation in dissolved Fe and Mn concentrations between sample collection and day 25. At K60, Fe concentrations in all amendments increased from 0.1 to 5 mg/L between collection and day 25 and remained at this level for the duration of the experiment (Fig. 3E). Needle sampling at Site K showed Fe concentrations between 6.5 and 12.75 mg/L, while the initial groundwater sample contained only 0.1 mg/L. This probably indicates precipitation due to exposure to O_2 immediately after sampling.

Evidence of sustained Fe and Mn release in the unamended samples, and not in the irradiated ones, was only observed at K150. Linear regression of the times series indicates that the releases of Fe and Mn were significant for the unamended samples (5.8 ± 1.5 mg/L-year, $r^2 = 0.90$ for Fe and 4.0 ± 1.5 mg/L-year, $r^2 = 0.82$ for Mn, $n = 8$), but not for the irradiated samples (1.5 ± 0.7 mg/L-year,

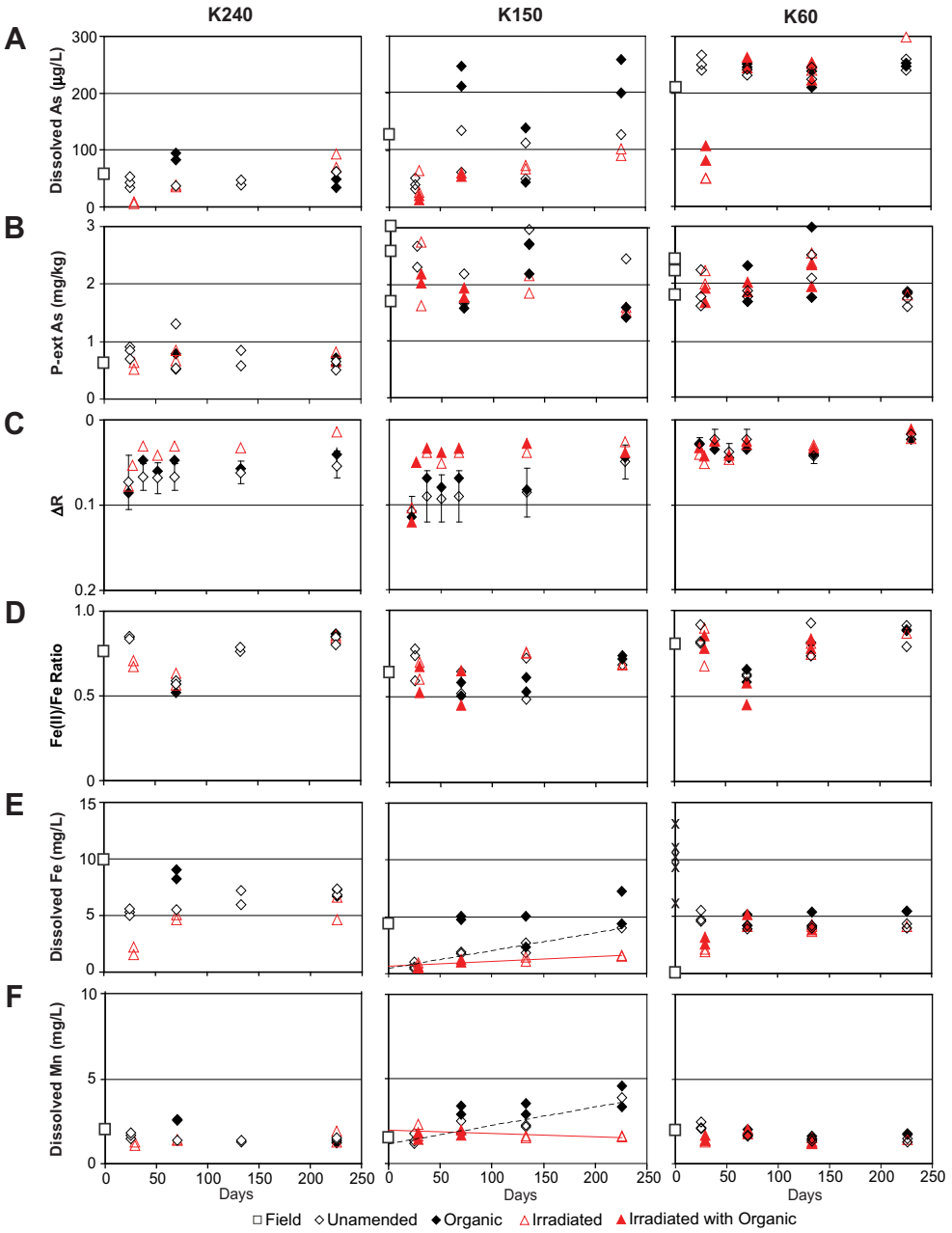

Fig. 3. Results of 8 month incubations of material from 3 locations – K240, K150 and K60 – at Site K. Multiple samples were taken at each time point and are shown here to demonstrate the inter-sample variability. Error bars in reflectance are only shown for the unamended samples, other reflectance error bars are similar in magnitude. Dissolved As, P-extractable As, difference in diffuse spectral reflectance, leachable Fe(II)/Fe ratio, dissolved Mn and Fe concentrations are shown for the duration of the experiment. The dissolved Fe concentrations of the 5 needle samples collected at K60 (shown as x marks at day 0) suggest that the initial low dissolved Fe was a sampling artifact. Linear regression lines are shown for Mn and Fe concentrations at K150 for the unamended (dashed black line) and irradiated (red line, grey in B&W in print version) samples, based on concentrations from day 25 onward. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

r^2 = 0.70 for Fe and −0.7 ± 1.5 mg/L-year, r^2 = 0.002 for Mn, n = 9). Furthermore, dissolved Fe and Mn concentrations were higher in the organic amendments after day 70 than in the unamended samples at K150. At K240 and K60, there was no sustained difference in dissolved Fe or Mn concentrations between the unamended samples, samples with organic additions, or the irradiated samples after day 25.

4. Discussion

4.1. Sterilization effectiveness

Sterilization was intended to eliminate, or at least markedly reduce, the activity of the natural microbial population in the sediment, particularly microbes responsible

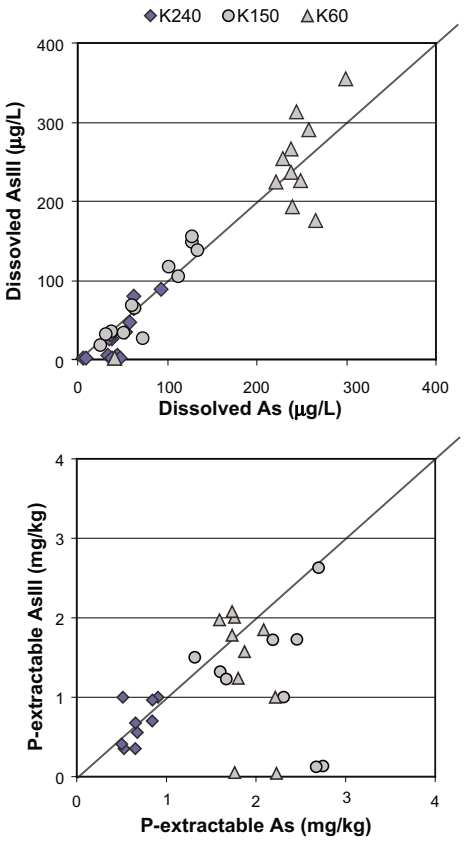

Fig. 4. Speciation of As in the dissolved and P-extractable phases in the unamended and irradiated amendment over the duration of the experiment. Grey line indicates the 1:1 line of As(III) to total As. Dissolved As is predominately As(III) at all three locations, while P-extractable As(III) varies somewhat, from over 65% to 100%.

for Fe reduction and/or As mobilization. While changes in microbial populations or activity were not directly measured, several of the treatments were successful in the sense that little change in Fe or As chemistry was observed, when compared to the extensive Fe reduction and increase in P-extractable As documented in the non-sterile incubations with acetate (Fig. 1). There was no evidence of Fe reduction or As mobilization in the irradiated, high dose antibiotics, autoclaved or azide samples for which conditions remained similar to the non-sterile without acetate samples. In contrast, the low dose of antibiotics delayed the onset of Fe reduction and As mobilization but failed to eliminate it.

Previous studies have shown that significant sediment changes may occur with autoclaving and this was confirmed here (Wolf et al., 1989; Lotrario et al., 1995; Trevors, 1996). The sediment Fe properties remained largely unchanged, while autoclaving induced slightly lower P-extractable As concentrations than measured in the non-sterilized without acetate samples. However dissolved As concentrations provided the clearest indication that autoclaving significantly alters sediment properties. Dissolved As concentrations were lower in the autoclaved samples than the other treatments (Table 1), which suggests that heat- induced crystallization of Fe oxides reduced the availability of As. Previous studies have noted the aggregation of particles and reduction of soil surface area with autoclaving (Wolf et al., 1989; Lotrario et al., 1995).

Irradiation, a high dose of antibiotics, and the addition of azide were all equally effective in inhibiting microbial activity over an extended period. The primary benefit of gamma irradiation relative to antibiotics and azide is that it preserves the option of inoculating sterilized sediment with organisms of known characteristics, which could become increasingly important for elucidating the key microbial processes that control As mobilization (Saltikov et al., 2005).

4.2. Implications of sediment heterogeneity

Sediment properties were not expected to change over the course of the Site K experiments since minor changes in sediment concentrations result in large changes in dissolved concentrations and, indeed, no systematic changes were observed (Fig. 3). The large sediment variability in a number of properties observed here has to the authors' knowledge not been reported previously. The variations in P-extractable As concentration can not be explained by As adsorption or release: a 0.5 mg/kg increase in P-extractable As would require the absorption of all the dissolved As from groundwater with an initial concentration of 500 µg/L, which did not occur. The variations in P-extractable As and the Fe(II)/Fe ratio indicate a level of heterogeneity exists in the sediment that mixing of the sediment in a bowl with a spatula under N_2 did not eliminate in the 30 subsamples collected.

The vertical profiles from the Site K provide some independent evidence of the extent of sediment heterogeneity. Spatial variability in the composition of groundwater, and to a lesser extent also sediment, over short distances (0.3–1 m depth) has been previously documented in Bangladesh and West Bengal, India, and does not appear to be due to sampling artifacts (Métral et al., 2008). At Site K, the greatest inter-sample variation occurred at K150 where the vertical profile indicates a large change in sediment properties from 4 to 9 m depth; with large fluctuations in the Fe(II)/Fe ratio, reflectance slope and P-extractable As (Fig. 2B–D). It is therefore not surprising that sediment collected for the incubations between 6 and 6.7 m depth was so heterogeneous. It is worth noting that solid phase heterogeneity was directly tied to variability in dissolved As during the experiment. In contrast, dissolved phase heterogeneity in the original samples did not strongly affect solid phase properties or the resulting dissolved As variability in the incubations. At K60, dissolved As concentrations increased from 80 to 380 µg/L in the 5 sediment and groundwater slurries collected over 2.4 m (Fig. 2). The average dissolved As concentrations during the incubations, however, was the least variant of the 3 locations and averaged 217 ± 68 µg/L. Over this same depth range, the Fe(II)/Fe ratio remained between 0.8 and 0.9 and P-extractable As varied between 0.7 and 1.1 mg/kg. The extensive reducing conditions at this location may have limited sediment variability.

These observations suggest that the spatial scale and magnitude of sediment variability needs to be considered when selecting samples expected to be typical of an aquifer system. This is the underlying notion of the representative elemental volume (REV), defined as the volume of an aquifer that is large enough give an "average" picture of the process being studied (Bear, 1972). Small-scale sediment and microbial heterogeneity have been reported for several aquifer systems (Brockman and Murray, 1997; Mailloux et al., 2007; Musslewhite et al., 2007), including the spatial scale of microbial Fe reduction in a coastal plain aquifer (Mailloux et al., 2007). In this last study, discrete incubations documented a wide range of Fe reduction rates while push–pull tests indicated relatively uniform rates throughout the aquifer. This was interpreted as an indication that the REV for Fe reduction in this aquifer was ~30 cm and was captured only by the push–pull tests. The spatial variability in sediment composition observed at Site K was apparently not adequately represented in the ~5 g sub-samples of sediment used for the incubations. This complication suggests future studies that capture processes at a larger scale, e.g. push–pull tests of the type conducted by Harvey et al. (2002), need to be considered.

Despite the high degree of sediment heterogeneity, dissolved As concentrations in the field and at the end of the incubations appear to be primarily driven by the P-extractable As concentrations. Such a relationship, characteristic of adsorptive equilibrium, was previously observed in a recent study of groundwater and sediment properties across Bangladesh and associated with a distribution coefficient (K_d) of ~4 cm^3/g (van Geen et al., 2008). A somewhat higher average K_d of ~13 ± 6 cm^3/g was measured at the 3 present locations and remained unchanged at the completion of the experiment (11 ± 5 cm^3/g, see Supplementary material). As observed in van Geen et al. (2008), the measured K_ds were invariant over a large concentration range (dissolved As from 10 to 300 μg/L and P-extractable As from 0.5 to 2.8 mg/kg). In addition, sample irradiation did not produce a change in K_d. The predominance of As(III) in both the dissolved and solid phase As at Site K suggests that the mechanism(s) of adsorption involve As(III) and not As(V).

4.3. Implications of changes in dissolved As

Sediment heterogeneity produced unexpected variability in dissolved As concentrations, driven by the absorptive equilibrium (Fig. 3). This induced inter-sample variability in dissolved As was greater than the previously measured increase in As concentrations for similar incubations (Radloff et al., 2007). Using the less variable samples from K240 as an example, the dissolved As concentrations at day 25 varied from 34 to 54 μg/L in the 3 unamended samples, while the previously reported release rates from batch experiments would have predicted less than a 20 μg/L increase in dissolved As over the course of the entire experiment (Radloff et al., 2007). This inter-sample variation overwhelmed the small temporal release rates anticipated and, therefore, is a severe drawback of using sacrificial vials for incubations experiments. Batch experiments appear better suited for measuring small changes, since the groundwater and sediment are continuously monitored and this type of variability is only present between replicates.

The initial decrease in dissolved As and Fe concentrations observed at K240 and K150 (and therefore increase in the As K_d) are similar to initial changes observed in previous batch incubation experiments, where up to 90% more surface absorption sites were calculated to have been exposed by sampling (Radloff et al., 2007). These observations support the notion that sample collection and preparation results in the increased exposure of surface absorption sites due to sample collection. The return to the field observed K_d by day 70 in the Site K incubation suggest that the adsorptive equilibrium may have been restored.

4.4. Validation of irradiation for abiotic controls

There was no evidence of Fe reduction and no indication of significantly altered sediment or groundwater concentrations in the irradiated samples over the duration of the Site K incubations. Dissolved Fe and Mn concentrations increased with time in the unamended and organic addition samples at K150, while no changes were observed in the irradiated samples. This suggests that irradiation prevented microbially-mediated Fe and Mn release (Fig. 3). A similar conclusion cannot be drawn for K240 or K60 because no clear release of Fe or Mn was observed in the unamended or organic addition amendments. Irradiation did induce a temporary decrease in dissolved As at K150 and K60, but this effect was less obvious with time (Fig. 3). Notably, irradiation did not induce a change in dissolved or sediment As speciation or adsorptive equilibrium. These results therefore support the use of irradiation for sterilization in long term incubations.

5. Conclusions

Four sediment sterilization methods were compared in terms of their ability to inhibit microbial Fe reduction in Pleistocene aquifer sediment from Bangladesh over a period of 8 months. Gamma irradiation, autoclaving, additions of a high dose of antibiotics and azide were all effective; only the low dose of antibiotics failed. Autoclaving caused dissolved As concentrations to decrease significantly, however. These observations combined with previous work by others rule out autoclaving as a suitable method to obtain abiotic control for incubation experiments. Whereas gamma irradiation, a high dose of antibiotics and azide all successfully prevented Fe reduction without significantly altering the composition of the sediment or groundwater, gamma irradiation has a distinct advantage for inoculation studies. Irradiation was also observed to eliminate Fe and Mn reduction in Holocene sediment from Bangladesh over a period of 8 months.

Incubations of grey sediment from the Holocene aquifer spanning a range of initial conditions revealed large sediment heterogeneity. Despite this heterogeneity, dissolved As concentrations were systematically related to P-extractable As, with a K_d of ~11 ± 5 cm^3/g. The variability in P-extractable As levels induced changes in dissolved As

during the incubations to the extent that release rates could not be measured. Sediment heterogeneity as well as evidence of disturbance even if extensive precautions are taken during collection suggest that *in situ* experiments which encompass REVs, such as push–pull tests, will be needed to identify the key processes that lead to As(III) mobilization in shallow aquifers of Bangladesh.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apgeochem.2008.07.009.

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