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Arsenic biotransformation and release by bacteria indigenous to arsenic contaminated groundwater

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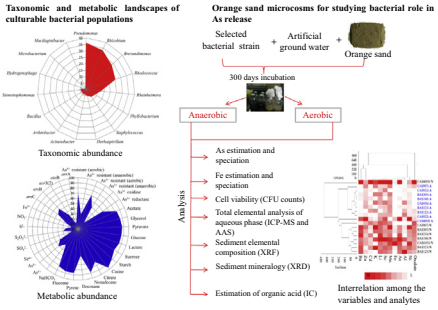
HIGHLIGHTS

- As-rich groundwater harbor bacteria with broad taxonomic and metabolic diversity.
- As- and Fe-reducing bacteria catalyze higher As release at anaerobic condition.
- As^{3+} oxidizing bacteria/oxic condition have an antagonistic role in As release.
- Fostering As^{3+} oxidizing bacteria/oxic state would control geogenic As contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

Arsenic (As) biotransformation and release by indigenous bacteria from As rich groundwater was investigated. Metabolic landscape of 173 bacterial isolates indicated broad catabolic repertoire including abundance of As^{5+} reductase activity and abilities in utilizing wide ranges of organic and inorganic respiratory substrates. Abundance of As homeostasis genes and utilization of hydrocarbon as carbon/electron donor and As^{5+} as electron acceptor were noted within the isolates. Sediment microcosm study (for 300 days) showed a pivotal role of metal reducing facultative anaerobic bacteria in toxic As^{3+} release in aqueous phase. Inhabitant bacteria catalyze As transformation and facilitate its release through a cascade of reactions including mineral bioweathering and As^{5+} and/or Fe^{3+} reduction activities. Compared to anaerobic incubation with As^{5+} reducing strains, oxic state and/or incubation with As^{3+} oxidizing bacteria resulted in reduced As release, thus indicating a strong role of such condition or biocatalytic mechanism in controlling *in situ* As contamination.

1. Introduction

Geogenic arsenic (As) contamination in groundwater of alluvial aquifers has been creating severe health problems to millions of people worldwide (Oremland and Stolz, 2005). Bengal Delta Plain (BDP) spreading large areas of Bangladesh and West Bengal (India) is considered the worst affected where more than 40 million people have been known to be exposed to drinking water containing >10 μg L^{-1} As (WHO, 2001). Understanding the processes that regulate biogeochemical cycling of this metalloid in subsurface environment including the mechanisms underlying its release into groundwater from the host minerals is a subject of high interest for developing sustainable strategies for drinking water supply in the affected regions. A number of plausible mechanisms including reductive dissolution of iron oxyhydroxide, replacement of As^{5+}

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with PO_4^{3-}, mineral weathering and oxidation of pyrites have been proposed to explain the subsurface release of As. Each of these mechanisms contain both abiotic and biotic components; but in all, role of microbially mediated processes are established beyond doubt (Islam et al., 2004; Mailloux et al., 2009). Inhabiting bacteria in contaminated groundwater have been shown to regulate the toxicity and solubility of As, as well as their release from host minerals by catalyzing redox transformations and other reactions and thereby could play critical roles in controlling the subsurface contamination and/or developing any remediation system (Gault et al., 2005; Hery et al., 2010; Islam et al., 2004; Lloyd and Oremland, 2006).

In contaminated groundwater soluble As is mostly represented by its two forms; arsenate (As^{5+}) and arsenite (As^{3+}). Abundance and distribution of these species are mainly dependent on redox condition, pH and microbial activity. In oxic condition As^{5+} is the predominant form that tends to adsorbed strongly onto the solid mineral phases (i.e., ferrioxyhydroxide, ferrihydrite, apatite, alumina, etc.). Conversely, As^{3+} which is adsorbed poorly onto such minerals and is more toxic (and more mobile) is prevalent under anoxic environment (Oremland and Stolz, 2005; Yang et al., 2014). In recent years, although considerable effort has been given to elucidate the microbial role in understanding their metabolic potential but in controlling the level of toxic metalloids in BDP remained less studied. It is imperative to know the exact role of inhabitant bacteria in controlling the release of As and its transformation to develop any mitigation strategies including long term application in bioremediation. Taxonomic identity and metabolic properties of bacteria indigenous to As contaminated groundwater and sediment have been documented (Ghosh and Sar, 2013; Liao et al., 2011; Sarkar et al., 2013). Some bacteria can reduce As^{5+} to As^{3+} during their anaerobic respiration or as a means of As detoxification, while others oxidize As^{3+} to As^{5+} during their chemolithoautotropic/heterotrophic metabolism. Recent geomicrobiological studies within As rich groundwater have revealed simultaneous presence of both As-oxidizing and -reducing abilities in the resident bacteria (Sarkar et al., 2014; Yang et al., 2014). Presence and abundance of As^{3+} oxidase and As^{5+} reductases enzymes and corresponding genes are found to be ubiquitous within the bacterial populations obtained from various As rich aquifer samples (Escudero et al., 2013). With respect to the microbial role in As mobilization, interaction of autochthonous bacteria with As, As bearing minerals and other elements present in the aquifer environment have been noted. Bacteria mobilize As either by reducing labile Fe^{3+}/As^{5+} of sediment minerals using them as alternative electron acceptors under anoxic and reducing environment or by releasing non labile Fe^{3+}/As^{5+} during nutrient acquisition or other metabolic processes and reduce the resultant As^{5+} (Hery et al., 2010; Islam et al., 2004; Zhang et al., 2012).

The present study describes the broad metabolic repertoire of indigenous groundwater bacterial community, and their involvement in regulation the level of toxic As species in the groundwater. Overall 170 pure culture bacterial isolated from different groundwater were used to ascertain their metabolic landscape of the groundwater community. Selected bacterial strains (nine) were used in sediment based microcosm studies for getting better insight into the underlying mechanism of As -transformation and -release in aquifer system.

2. Methods

2.1. Isolation, identification and metabolic characterization of bacterial isolates

Six arsenic contaminated groundwater samples were collected from existing household tubewells of different sites of Barasat and Chakdaha of West Bengal, India. Range of the As level in groundwater is 15–1364 ppb. From each of the groundwater samples, cultivable bacterial populations were isolated. The samples were serially diluted in normal sterile saline (0.9%) and 100 μL of suspension from each dilution was plated on R2A (Reasoner and Geldreich, 1985) and minimal salt medium (MSM) (Kazy et al., 1999) agar plates in triplicate and incubated at 30 °C for 7 days. Morphologically distinct colonies were selected and purified by repeated subculturing in R2A and MSM medium, respectively. Each purified bacterial strain was stored at −80 °C with 15% glycerol. Identification and taxonomic characterization of the isolated bacteria were done by 16S rRNA gene sequencing (detailed methods are given in Supplementary information). All the strains were characterized with respect to their ability to resist As under aerobic and anaerobic conditions, As^{3+} oxidase and As^{5+} reductase activities, utilization of different carbon and inorganic electron acceptors (during anaerobic growth) and presence of As resistant and transformation genes (*ars*C, *ars*B, *acr*3(2), *aio*B and *arr*A) (detailed methods are given in Supplementary information).

2.2. Microcosm analysis

For microcosm study nine selected bacterial strains [*Acinetobacter* BAS123i (KF442760.1), *Arthrobacter* CAS4101i (KF442753.1), *Brevundimonas* CAS4005i (KF442756.1), *Pseudomonas* BAS323i (KF442756.1), *Pseudomonas* CAS907i, *Phyllobacterium* BAS224i (KF442766.1), *Rhizobium* BAS305i (KF442754.1), *Rhodococcus* CAS922i (KF442755.1) and *Staphylococcus* BAS108i (KJ493796.1)] were used. These strains were selected based on their taxonomic and metabolic representativeness. Microcosm studies were conducted by incubating As bearing orange colored Pleistocene sand (referred as orange sand) with the test bacteria under aerobic and anaerobic conditions. Orange sand was obtained from subsurface sedimentary cores recovered from drillings conducted at highly As contaminated site of Chakdaha, West Bengal (location 23°01′049.00″N/88°35′07.05″E) by Geological Survey of India (GSI), Kolkata (Pal and Mukherjee, 2009). The sand was recovered from the drill-cores under aseptic condition (inside sterilized laminar Air flow hood) using sterile spatula. Arsenic bearing "orange sand" was incubated aerobically (set A) and anaerobically (set B) in artificial groundwater with either of the nine selected strains. Prior to its use in microcosm, the orange sand was thoroughly homogenized and sterilized by autoclaving at 120 °C, 15 psi for 40 min to remove the viable cells present within it. For each set (A or B), a total of 10 microcosms, nine bioaugmented by nine individual strains and one control set devoid of any bioaugmentation was prepared. All microcosms were prepared in triplicate and incubated aerobically and anaerobically. In each set, 10 g of sterilized sand was added in serum bottle (Sigma–Aldrich, St. Louis, USA) having 20 mL artificial groundwater (composition g L^{-1}: $CaCl_2$. $2H_2O$ 0.148; NaCl 0.315; KCl 0.1067; $MgSO_4$. H_2O 0.9399; $NaNO_3$ 0.2488 and $NaHCO_3$ 0.11; to pH 7) and amended with carbon source (i.e., 10 mM glucose + 10 mM acetate). In all biotic microcosms initial bacterial cell density (added as inoculum) was maintained as 10^5–10^6 CFU mL^{-1}. For anaerobic microcosms, artificial groundwater medium was autoclaved for 45 min at 121 °C and amended with 1% (w/v) cysteine hydrochloride as a reducing agent. Bottles were capped by airtight butyl stopper, crimp sealed and bottle headspace was replaced with deoxygenated ultrapure N_2. For aerobic microcosms, except purging of N_2 and addition of reducing agent other steps remained same. Head space was filled with sterile air and bottles were closed by cotton plug. All bottles were set horizontally on a rotary shaker, gently rotating at 50 rpm at 26 °C over the entire period. Microcosms were subsampled at discrete time points with

appropriate care to maintain the same sediment:water ratio throughout the experiment. Water subsamples (3 mL) were removed periodically under sterile condition from the bottles, a part was used to determine viable cell counts and other part was used for different chemical analyses. Numbers of viable cells were enumerated by counting colony forming units using aerobically and anaerobically maintained R2A agar for set A and set B, microcosms, respectively and following the procedure as describe by Miles et al. (1938). For the set B microcosms, sample withdrawal was performed inside the anaerobic work station (Coy Laboratory Products Inc.) maintained with sterile N_2 atmosphere.

2.3. Chemical analyses

Aqueous subsamples (3 mL) obtained from all the microcosms were centrifuged at 14,000 rpm for 5 min and passed though a 0.45 μm membrane filter to remove sand particles and the filtrate was subjected to a battery of chemical tests. A part of the filtrate was immediately acidified (2% HNO_3) for inductively coupled plasma spectrometry (ICP–MS) analysis whereas the other part (remained unacidified) was used for measuring the concentration of As^{3+} and Fe^{2+}. These two elemental species were quantified spectrophotometrically using molybdenum blue method (As^{3+}) (Johnson, 1971) and ferrozine (Fe^{2+}) method (Lovley and Phillips, 1986), respectively. For the quantification of Fe^{2+} samples were completely solubilized by adding 1 mL of 0.5 N HCl to 50 μL of the desired sample and digested at 25 °C for 24 h. Concentration of Fe^{2+} was determined by adding 200 μL of sample digest to 1.5 mL of the ferrozone solution (1 g of ferrozine to 1 L of 50 mM HEPES buffer pH 7.4) and measuring absorbance at 562 nm. Total Fe and As concentrations were quantified using atomic absorbance spectrophotometer (AAnalyst™ 200, Perkin–Elmer, USA). Concentrations of Mn and other trace metals were estimated by ICP–MS (Varian 810 ICP–MS System, California). Levels of Na, K and Ca were quantified by using flame photometer (52A Flame Photometer Perkin–Elmer, USA).

Mineralogy of the orange sand and its bulk chemical analysis before and after incubation with bacteria (i.e., at zero day and 300 day, for aerobic and anaerobic sets, respectively) were monitored using X-ray diffraction (XRD) (Panalytical high resolution XRD-I, Almelo, Netherlands) and X-ray fluorescence (XRF) (PANalytical AXIOS, Almelo, Netherlands) analyses. For XRD, sand samples were oven dried at 80 °C for 24 h, ground to fine powder and analyzed with a powder diffractometer: scan range 10–100°2θ into 2°2θ/min step size using a CuKα radiation. Mineral phases were identified by matching the peaks in Joint Committee on Powder Diffraction Standards (JCPDS) database. For XRF, oven dried sediments (80 °C, 24 h) were pulverized in an agate mortar and subjected to analysis. Presence of organic acid in the aqueous phase of microcosm derived subsamples were estimated by ion chromatography (Dionex, USA) using the procedure as describe by Frey et al. (2010).

2.4. Statistical analysis

All data represented mean of three independent experiments to avoid the error in reproducibility among measurements and were expressed as mean ± SD. Association between variables were calculated by Pearson's correlation. Corelease of As and other metals (Fe, Mn, Al, K, Ba, Cd, Co, Cr, etc.) (after 300 days incubation) in both aerobic and anaerobic microcosm was analyzed by two components Principal Component Analysis (PCA). PCA was done by MVSP 3.1 software (http://www.kovcomp.com/).

3. Results and discussion

3.1. Identification and metabolic characterization of bacterial isolates

In order to assess the metabolic properties of bacterial populations in As rich groundwater, more than 170 strains were isolated as pure culture from the six samples. Taxonomic identity of the strains was ascertained by 16S rRNA gene sequence analysis. Sequence similarity search (in both NCBI BLAST and RDP) as well as phylogenetic studies revealed their affiliation to phyla *Proteobacteria* (genera *Acinetobacter*, *Pseudomonas*, *Brevundimonas*, *Rhizobium* and *Phyllobacterium*), *Actinobacteria* (genera *Rhodococcus* and *Arthrobacter*) and *Firmicutes* (genus *Staphylococcus*) (Fig. 1a). Among the detected genera *Pseudomonas*, *Rhizobium*, *Brevundimonas* and *Rhodococcus* represented the major populations. Distribution pattern of these genera further indicated that except *Rhizobium*, members of the other three groups were nearly ubiquitous across the samples (Fig. S1). Genera *Rhizobium*, *Arthrobacter*, *Rheinheimera*, *Phyllobacterium* and *Herbaspirillum* were present in fewer samples but with higher abundance (10–80%) (Fig. S1). The observed taxonomic composition indicated that bacterial communities in As rich groundwater are constituted by diverse populations. Frequent abundance of most of these genera like *Pseudomonas*, *Rhizobium*, *Brevundimonas*, etc. in Ganga Bramhaputra Meghna (GBM) and other deltaic regions has been reported earlier (Ghosh and Sar, 2013; Sarkar et al., 2013; Yang et al., 2014). Previous geomicrobial studies although indicated that dissimilatory Fe/As reducing anaerobic bacteria play a major role in As release from organic C (OC) rich shallow sediment (Hery et al., 2010), but similar to the present observation, absence of bacterial groups known for dissimilatory metal reduction have been reported during earlier investigations (Ghosh and Sar, 2013; Liao et al., 2011; Sarkar et al., 2013). Metabolic potential of the bacterial communities present in As rich groundwater was evaluated by using a broad array of phenotypic and genetic tests. All the bacterial strains were tested for their As (As^{3+} and As^{5+}) resistance under aerobic and anaerobic conditions, As^{5+} reduction and As^{3+} oxidation activities, ability to utilize diverse carbon compounds (including aromatic and aliphatic hydrocarbons) as electron donor/C source and different inorganic compounds as terminal electron acceptors. As presented in Fig. 1b, ability to grow with elevated As^{5+} ($\geqslant$100 mM) or As^{3+} ($\geqslant$10 mM) under anaerobic condition was observed in 30% or 50% strains, respectively. Under aerobic condition nearly 70% strains showed their ability to withstand the same As species. Arsenate (As^{5+}) reductase activity was detected in 60% strains whereas As^{3+} oxidase activity was found to be relatively less abundant. Among the carbon sources tested, casine was most preferred, utilized by 95% of the strains followed by starch, glycerol, glucose, acetate and pyruvate. Noticeably, more than 80% strains could use long chain alkanes and poly aromatic hydrocarbons (e.g., nonadecane, pyrene, fluorine, etc.) as sole C source. With respect to utilization of different inorganic elements as terminal electron acceptors (TEAs) during anaerobic growth, 50% of the strains showed their ability to metabolize As^{5+} followed by Se^{6+}, $S_2O_3^{2-}$, and Fe^{3+}. Presence of As homeostasis genes was also observed within the strains. It was found that more than 50% strains posses cytosolic As^{5+} reductase gene *ars*C and As^{3+} transporter gene *ars*B where as nearly 10% strains contain As^{3+} oxidizing gene *aio*B. Overall the metabolic landscape of bacterial communities in As contaminated groundwater portrayed a number of very relevant properties, including ability to withstand toxic As even at anaerobic

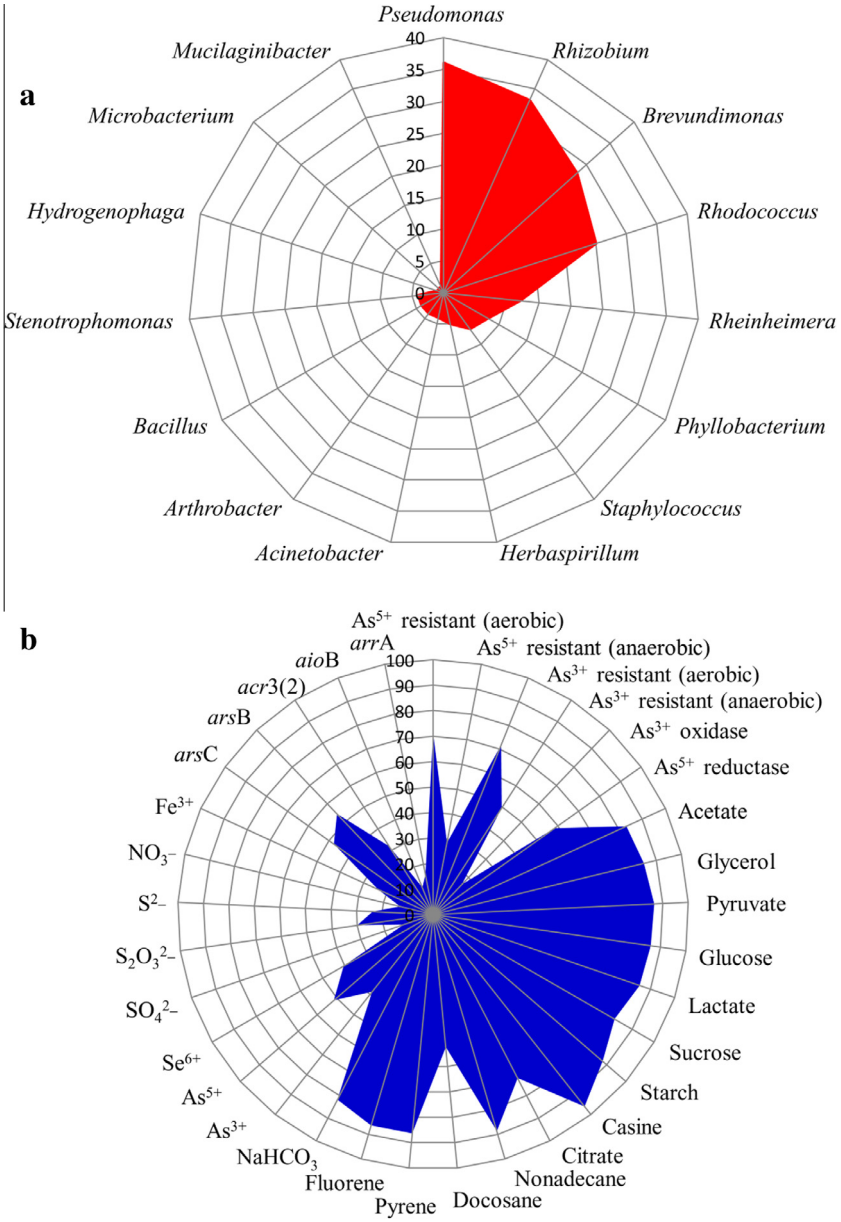

Fig. 1. Landscape of (a) microbial population and (b) metabolic activity within arsenic contaminated groundwater of West Bengal. Characters: carbon sources/e^- donors (acetate, glycerol, pyruvate, glucose, lactate, sucrose, starch, casine, citrate, docosane, pyrene, fluorine, $NaHCO_3$ and As^{3+}), terminal electron acceptors (As^{5+}, Se^{6+}, SO_4^{2-}, $S_2O_3^{2-}$, S^{2-}, NO_3^- and Fe^{3+}), As homeostasis genes (*ars*C, *ars*B, *acr*3(2), *aio*B and *arr*A).

condition, abundance of As^{5+} reductase activity and relevant genes, utilization of As^{3+} as TEA and use of multiple organic compound including complex aromatic and aliphatic hydrocarbons.

The observed metabolic versatility of indigenous bacterial community seems remarkable. Members of the genera *Acinetobacter*, *Brevundimonas*, *Pseudomonas* and *Rhizobium* isolated earlier from various As contaminated groundwater have been shown to utilize multiple electron donors and/or acceptors (Gihring et al., 2001; Ghosh and Sar, 2013; Sarkar et al., 2014). Along with superior As resistance, presence of As^{5+} reductase activity as detected in the test isolates corroborated earlier reports on bacterial strains isolated from As rich groundwater of BDP and elsewhere (Liao et al., 2011; Sarkar et al., 2013). Presence and abundance of As transforming ability within the indigenous bacteria of As rich groundwater is considered to be an evolutionary outcome to allow aerobic/facultative anaerobic metabolism within the alluvial aquifers (Liao et al., 2011). While the inhabitant bacteria often gain metabolic advantage through the redox transformation of As (e.g., As^{5+} reduction), eventually it facilitates mobilization of this metalloid (As^{3+} has much less affinity to remain adsorbed on host minerals). Abilities of the test isolates to utilize multiple sugar molecules, different hydrocarbon compounds as their energy and/or carbon source and use of alternate electron acceptor(s) during anaerobic condition, clearly indicate that the indigenous organisms are well equipped to survive and flourish under As rich oligotrophic condition.

3.2. Microcosm study on bacterial role in As release

For microcosm study nine bacterial strains were selected based on metabolic properties and taxonomic affiliation of the predominant populations. All these strains were endowed with As reductase activity and strains *Staphylococcus* BAS108i and *Arthrobacter* CAS4101i showed additional ability to utilize Fe^{3+} as TEA. The

detailed genetic and metabolic characteristics of these strains are presented in Table S1. Sediment microcosm study was performed to investigate the potential role of groundwater bacteria on As transformation and regulation of As contamination level in aquifer environment. Viability of augmented cells within the microcosm environment was monitored by CFU counts at distinct time intervals (Fig. S2). All the strains maintained their viability and showed growth under both aerobic and anaerobic incubations along with As rich orange sand. Among the strains, *Rhizobium* BAS305i, *Pseudomonas* CAS907i and *Rhodococcus* BAS123i showed higher growth with 2–3 orders of magnitude increase in cell number within the first 7 days of incubation (under aerobic as well as anaerobic conditions) (Fig. S2a and b). Rest of the strains also showed increase in cell number, but to a lesser extent. During subsequent incubation upto 300 days most of the strains maintained their cell viability while a few showed upto 1 order of magnitude lowering in CFU counts. The survival and growth of the test bacteria in low nutrient (mostly inorganic), As rich environment constituted by As rich orange sand in artificial groundwater medium corroborated well with their observed metabolic versatility allowing them to use the available resources within the microcosm. Increased growth and culturability of bacterial strains from As rich groundwater of BDP in low nutrient As amended medium is also previously reported (Sarkar et al., 2013).

All the bacterial strains used in the microcosm study showed release of sediment bound As in the aqueous phase, although its concentration varied according to the strain and incubation conditions. Kinetic study revealed that irrespective of the strain added and incubation condition, release of As (estimated as total As) was completed within the first 7–21 days of incubation and in all biotic sets As was mostly released as As^{3+} (Figs. 2 and 3). Aerobic microcosms bioaugmented with either of the following strains,

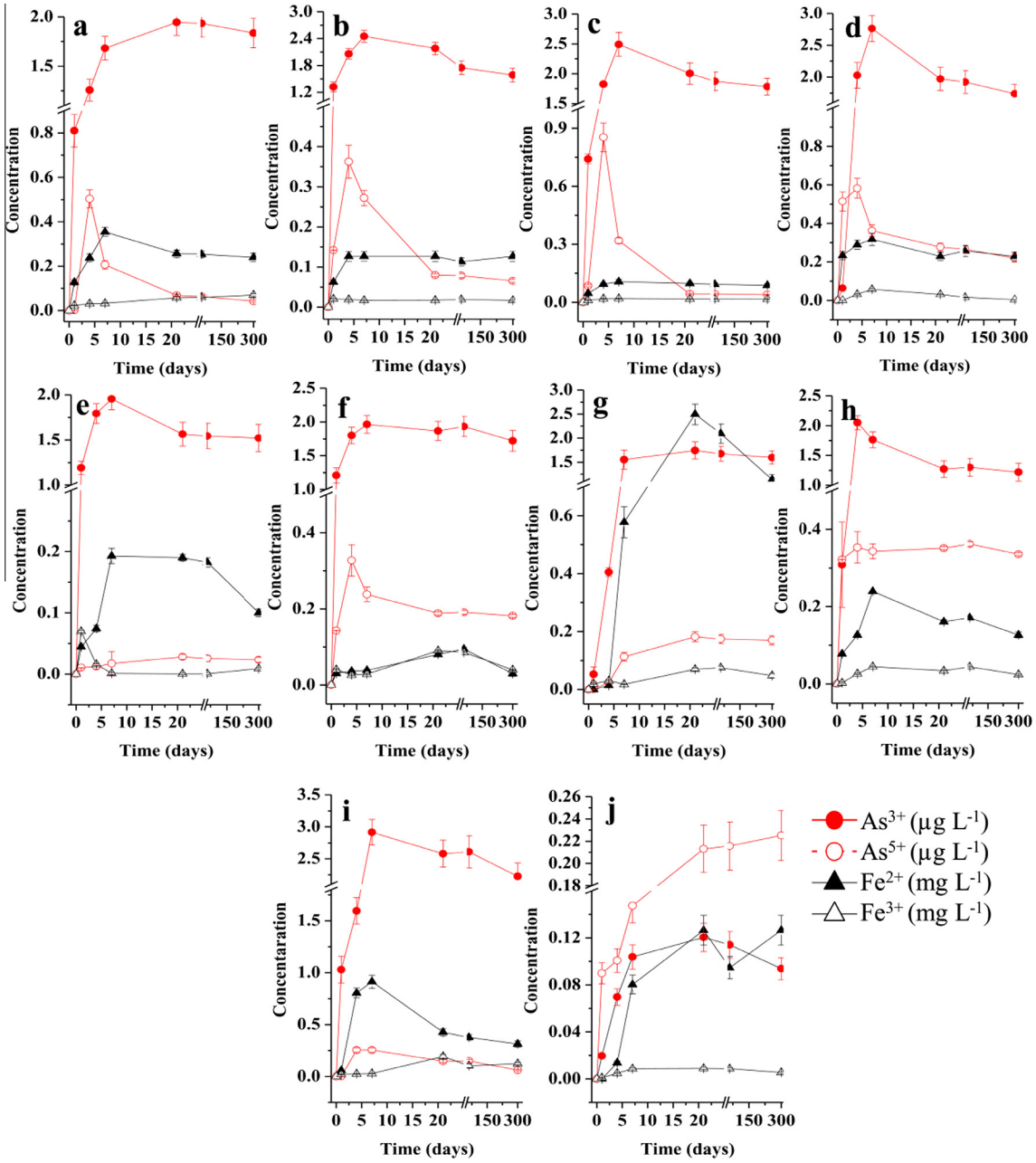

Fig. 2. Estimation of arsenic and iron release in aqueous phase of aerobic microcosm set up, over 300 days incubation. In aerobic microcosm isolates denoted as (a) BAS123i (*Acinetobacter*), (b) CAS4101i (*Arthrobacter*), (c) CAS4005i (*Brevundimonas*), (d) BAS323i (*Pseudomonas*), (e) CAS907i (*Pseudomonas*), (f) BAS224i (*Phyllobacterium*), (g) BAS305i (*Rhizobium*), (h) CAS922i (*Rhodococcus*), (i) BAS108i (*Staphylococcus*) and (j) aerobic control. Error bars indicating standard deviations ($n = 3$).

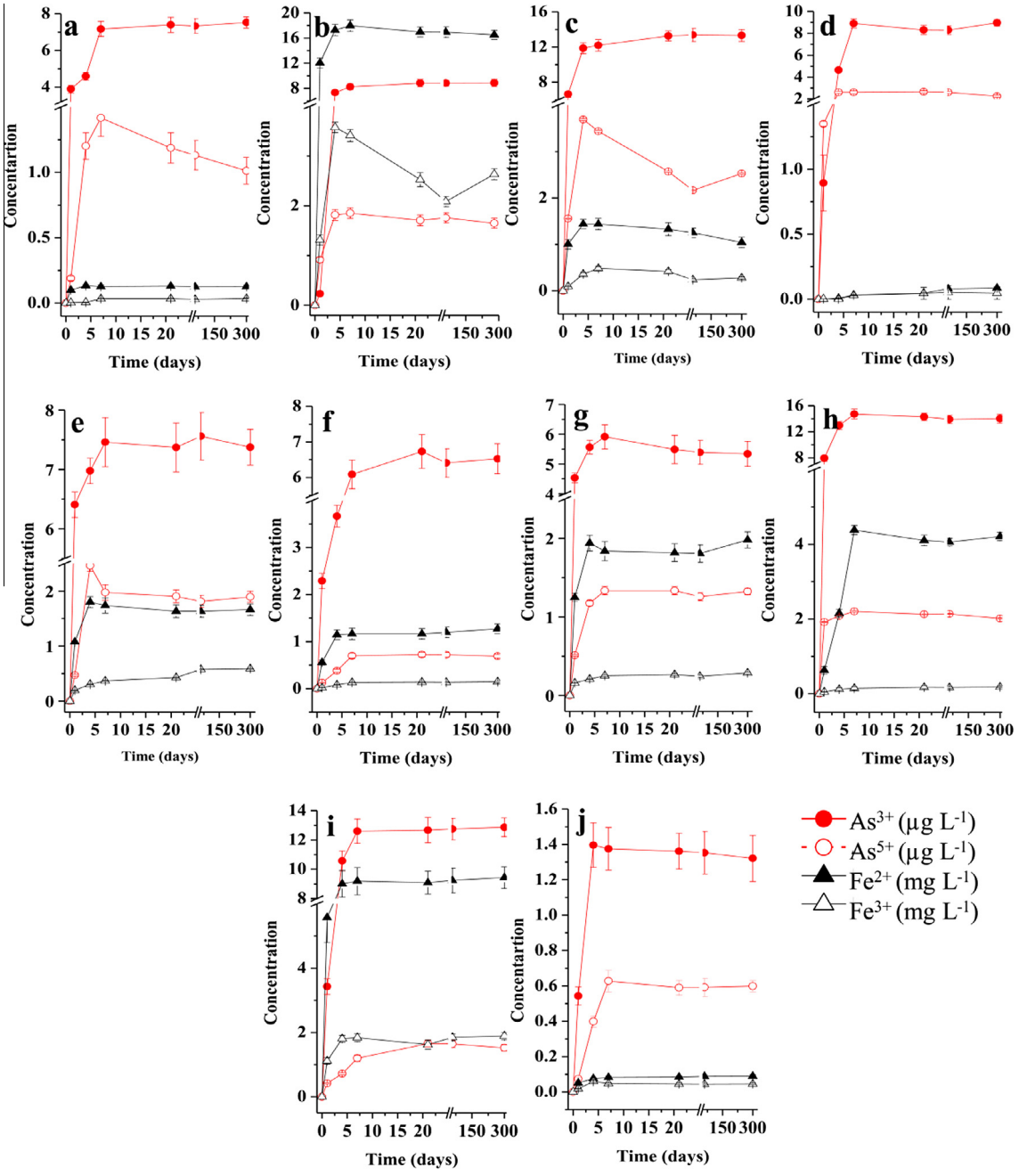

Fig. 3. Estimation of arsenic and iron release in aqueous phase of anaerobic microcosm set up, over 300 days incubation. In anaerobic microcosm isolates denoted as (a) BAS123i (*Acinetobacter*), (b) CAS4101i (*Arthrobacter*), (c) CAS4005i (*Brevundimonas*), (d) BAS323i (*Pseudomonas*), (e) CAS907i (*Pseudomonas*), (f) BAS224i (*Phyllobacterium*), (g) BAS305i (*Rhizobium*), (h) CAS922i (*Rhodococcus*), (i) BAS108i (*Staphylococcus*) and (j) anaerobic control. Error bars indicating standard deviations ($n = 3$).

viz., *Staphylococcus* BAS108i, *Pseudomonas* BAS323i, *Pseudomonas* BAS907i, *Rhodococcus* BAS922i, *Phyllobacterium* BAS224i, *Brevundimonas* CAS4005i and *Arthrobacter* CAS4101i showed 2–7.5 fold increase in aqueous As level within the first 7 days (maximum level 2.3 µg L^{-1} As with *Staphylococcus* BAS108i). For *Acinetobacter* BAS123i and *Rhizobium* BAS305i maximum rise in aqueous As occurred within the first 21 days only (Fig. 2a and g). In subsequent period, As concentration remained almost unchanged in all the sets. Compared to the aerobic set, anaerobic incubation resulted significantly higher As release (maximum upto 8 folds) in biotic microcosm and the concentration of aqueous As varied widely (6.6–16 µg L^{-1}) with respect to the bacterial strains used (Fig. 3a–j). Relatively higher As release (>10 µg L^{-1}) was noted for *Rhodococcus* CAS922i, *Brevundimonas* CAS4005i, *Staphylococcus* BAS108i and *Arthrobacter* CAS4101i (Fig. 3 h, c, i, and b). Except the microcosm amended with *Arthrobacter* CAS4101i, all other higher As releasing microcosms showed maximum release of As within 7 days. Noticeably, the strains which caused only moderate to low As release under aerobic condition, could led to relatively higher As release under anaerobic incubation; particularly with the strains *Staphylococcus* BAS108i, *Arthrobacter* CAS4101i, *Pseudomonas* CAS907i, *Rhizobium* BAS305i and *Pseudomonas* BAS323i. Overall, it could be noted that aerobic condition favored significantly less As release by all the test bacteria and the level of As (total) in each biotic microcosm remained within a close regime (1.6–2.3 µg L^{-1}).

During this study it was clearly observed that all the bacterial isolates used were not only able to survive and grow actively

during their incubation with orange sand but more importantly facilitated mobilization of As from the constituting host minerals as well. Higher release of As within the anaerobic microcosms perhaps indicated the strong role of anaerobic metabolism in mobilization of this metalloid. Although except *Pseudomonas* and *Rhizobium*, members of the other genera used have not been reported to posses dissimilatory metal reducing activity, yet, the level of mobilized As and its predominant species (i.e., As^{3+}) within the aqueous phase corroborated very well with previous findings on sediment microcosm incubation with Fe reducing *Shewanella* sp. (Dhar et al., 2011). Within the orange sand As is present mainly as As^{5+}, which remained bound with various Fe/Mn/Al minerals or mineral phases. Presence of As^{3+} as the major aqueous As species within the biotic microcosms from the early phase of incubations possibly indicated the specific role of bacteria in mobilization of As from its bound state and its subsequent transformation. With respect to the geochemical properties of the sediment and the observed microbial phenomena it may be inferred that upon release from its bound state, As^{5+} is reduced by bacterial reductases. Reduction of As^{5+} could either be for detoxification or energy generation or both. All the test bacterial strains were capable of As^{5+} reduction and showed As^{5+} reductase activity. Presence of higher aqueous As mostly (as As^{3+}) within the anaerobic microcosms further indicated that compared to only detoxification based As^{5+} transformation, possible involvement of respiratory reductase that facilitates use of As^{5+} as TEA under anaerobic condition could be responsible for As mobilization.

3.3. Release of Fe, other elements and characterization of As bearing sediment

Coupled with As, release of Fe in the aqueous phase of all biotic microcosms was evidenced (Figs. 2 and 3). Concentration of aqueous Fe varied considerably with respect to the bacterial strain augmented and incubation conditions. Under the aerobic condition, maximum Fe release was detected with *Rhodococcus* CAS922i (0.3 mg L^{-1}) followed by *Phyllobacterium* BAS224i and *Staphylococcus* BAS108i (Fig. 2h, f and i). Strain *Arthrobacter* CAS4101i which did not show any significant Fe release under aerobic condition released highest concentration of Fe (19 mg L^{-1}) (Fig. 3b) followed by *Staphylococcus* BAS108i and others in anaerobic incubation. In all these sets aqueous Fe reached its maximum in 7 days, and was mostly represented by the reduced species (Fe^{2+}). Microcosm with the *Rhizobium* BAS305i, showed a relatively slower pace of Fe release and attained the maximum concentration after 21 days. Within the aerobic microcosms, relatively higher concentration (up to 33 fold) of Fe was released compared to that of As (Fig. S3c). During 300 days anaerobic incubation, concentration of total Fe increased up to 2–7 fold within the initial 7 days and it remained nearly invariable in the subsequent period (Fig. S3d). Noticeably, both the Fe reducing strains *Staphylococcus* BAS108i and *Arthrobacter* CAS4101i showed significantly higher release of Fe^{2+} as well as Fe^{3+} compared to other strains under anaerobic incubation (Fig. 3b and i). Additionally, it was noted that in case of *Arthrobacter* CAS4101i, release of Fe^{2+} preceded that of As^{3+}. A nearly similar trend was also observed with strain *Staphylococcus* BAS108i as well. In contrast to non Fe reducing strains wherein concomitant increase of As (mostly As^{3+}) and Fe (Fe^{2+}) was observed, this observation with Fe reducing strains remained intriguing.

Together with the release of As and Fe, possible mobilization of other elements from the host minerals was further monitored. Elemental analysis of aqueous phase samples recovered from the microcosms was performed through ICP–MS spectroscopy. It was observed that several elements like Al, Mn and K were co-released in the aqueous phase of both aerobic and anaerobic biotic microcosms (Fig. 4). Detail quantification of elements detected in the aqueous phase is presented in Table S2. Compared to the respective abiotic controls, increased mobilization of these elements in all the biotic sets indicated microbial dissolution of host minerals of orange sand. Release of Al (1.6–23 μg L^{-1}), K (1–1.7 mg L^{-1}) and Mn (2.2–3.4 mg L^{-1}) was most conspicuous within the aerobic

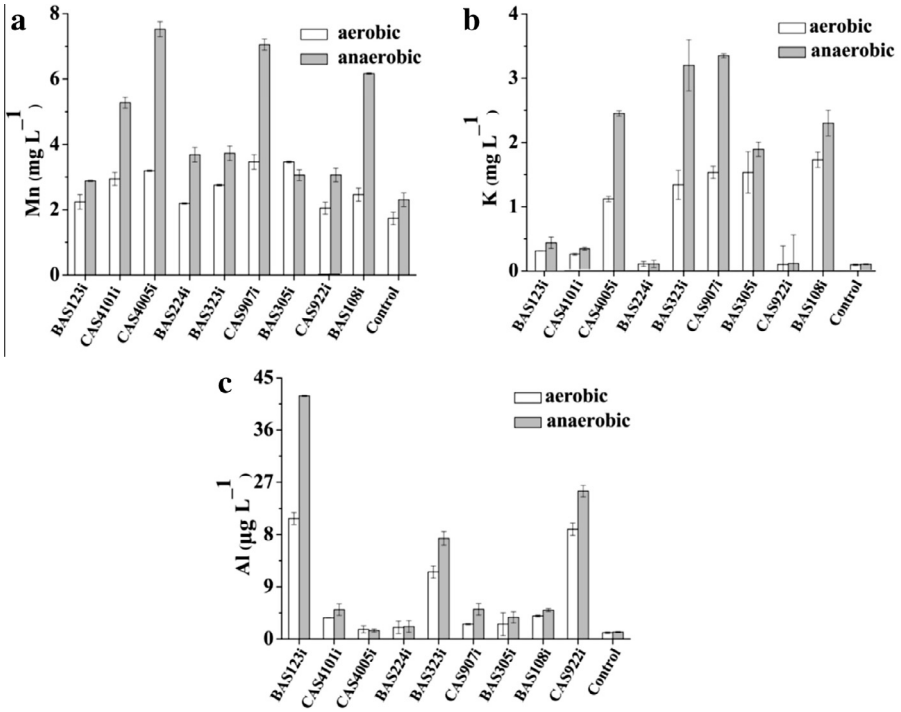

Fig. 4. Concentrations of Mn, K and Al within the aqueous phase of microcosms (after 300 days). Error bars indicating standard deviations ($n = 3$). The bacterial strains represented in X axis are as follows: BAS123i (*Acinetobacter*), CAS4101i (*Arthrobacter*), CAS4005i (*Brevundimonas*), BAS323i (*Pseudomonas*), CAS907i (*Pseudomonas*), BAS224i (*Phyllobacterium*), BAS305i (*Rhizobium*), CAS922i (*Rhodococcus*) and BAS108i (*Staphylococcus*).

microcosms bioaugmented with *Staphylococcus* BAS108i, *Acinetobacter* BAS123i and *Rhodococcus* CAS922i (Fig. 4). Noticeably, incubation under anaerobic condition favored release of much higher concentration of all these metals. Additionally, presence of a few other elements like Li, Sc, Cr, Ni, etc. was also noted within the anaerobic set (Table S2). Particularly, microcosms with *Acinetobacter* BAS123i, *Rhodococcus* CAS922i or *Pseudomonas* BAS323i showed higher release of Al. *Brevundimonas* CAS4005i, *Pseudomonas* CAS907i, *Staphylococcus* BAS108i and *Arthrobacter* CAS4101i led to higher release of Mn. In order to ascertain the inter relationship among the observed chemical variables, viz., As, Fe, Mn, Na, K, etc. as measured within the all biotic microcosms, two component PCA analyses were conducted (Fig. 5). Two completely different groups could be distinguished in both the PCA plots. In the first PCA, group I consisted of all the elements except Al was located on the left side of the score-plot; while group II included only Al. In the second PCA, except Cr and Ni all other elements remained closely associated to each other. The statistical analysis indicated that co-release of all other test elements, except Al or Ni and Cr in anaerobic or aerobic condition, respectively, has significant correlation with that of As. Overall observations implicated a strong role of bacterial activities in solubilizing and thus releasing the elements from the solid phase sediment.

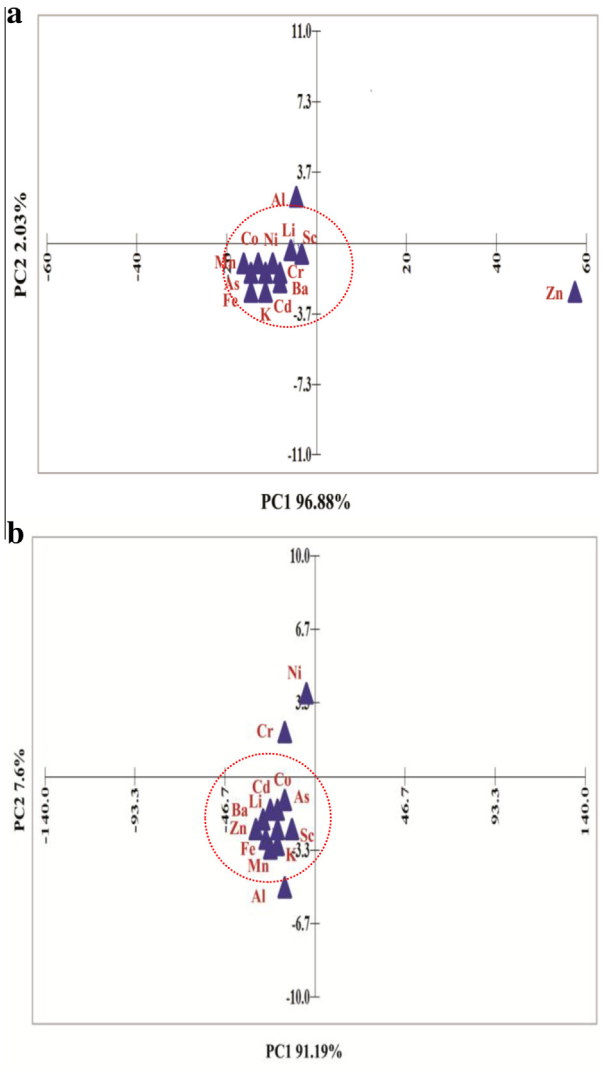

Fig. 5. Principle component analysis based on the chemical parameters of the aqueous phase of (a) anaerobic and (b) aerobic microcosms after 300 days incubation.

Following these observations on the plausible mobilization of constituting elements from As bearing orange sand, mineralogical properties of the sand were ascertained before and after incubation with bacteria. X-ray powder diffractometry (XRD) and X-ray fluorescence (XRF) analysis were performed to investigate possible changes in mineral phases present within the sand and its elemental composition. XRF analysis indicated reduced abundance of several elements including Fe, Al, K, Mn, etc. following bacterial incubation and this finding corroborated well with the elemental analysis of aqueous phase (Table S3). It was interesting to note that in all higher As releasing microcosms, elevated mobilization of one or more constituting elements of the sand occurred. XRD analysis was performed on samples from selected microcosms incubated with strains *Staphylococcus* BAS108i, *Rhodococcus* CAS922i, *Brevundimonas* BAS4005i and *Arthrobacter* CAS4101i under both aerobic and anaerobic conditions. Analyses of diffractograms confirmed that orange sand was composed of several As bearing minerals like Fe oxyhydroxide based goethite [FeO(OH)], albite ($NaAlSi_3O_8$) and glauconite ($K[(Fe,Al)_2(Si,Al)_4O_{10}(OH)_2]$) and following incubation with bacteria specific changes occurred in several of these minerals (Fig. S4). It is also noteworthy that such mineralogical changes remained more prominent in orange sand incubated under anaerobic condition than that of the aerobic one.

Within the orange sand, As is hosted mainly upon the Fe (Mn/Al)-oxides/-hydroxides minerals or mineral phases (Dhar et al., 2011; Pal and Mukherjee, 2009). Simultaneous release of Fe and other constituting elements (Al, Mn, K, etc.) of the host minerals suggests a cohesive interaction of bacterial activities with these minerals resulting into the co-mobilization of constituent elements. Release of Fe (mainly as Fe^{2+}) that occurred in most of the biotic microcosms supported specific interaction of bacterial strain with Fe rich minerals allowing its release and reduction to Fe^{2+}. Detail kinetics of As and Fe release further indicated that while elevated concentration of As (mainly as As^{3+}) appeared prior to that of Fe^{2+} (or total Fe) in microcosms bioaugmented with strains lacking Fe reducing activity, release of Fe^{2+} preceded that of As in microcosms with bacteria having Fe^{3+} reducing activity. It may be inferred that while bacterial anaerobic Fe reduction certainly lead to increased As mobilization, other metabolic activities that do not encompass Fe reduction, also facilitate As release. In case of the former, specific bacterial groups those use sediment Fe (Fe^{3+}) as TEA reduced it to soluble Fe^{2+}. Consequently, As^{5+} hosted on Fe (Fe^{3+})-minerals got dislodged to aqueous phase. Released As^{5+} is subsequently reduced by bacteria through detoxification or respiratory As^{5+} reductases or by both. In either case, (whether Fe reduction is involved or not), the sorbed As^{5+} eventually released into the aqueous medium and bacteria transform it to As^{3+}. Together with As and Fe, release of several other elements (Mn, Al, K, Na, etc.) as well as concomitant mineralogical changes clearly suggest weathering or dissolution of host minerals by bacterial activities. X-ray based studies revealed change in mineral phases particularly with respect to goethite, albite and glauconite which are composed of As, Fe, Al and K.

3.4. Production of oxalic acid

Since mineral dissolution is often caused by microbially produced organic acids, attempt was made to detect and estimate concentrations of such acids within the aqueous phase of microcosms. Using an ion chromatography system oxalic acid, gluconic acid and other organic acids were analyzed. Among the acids analyzed, oxalic acid was detected at significantly higher concentration within the biotic microcosms (Fig. 6). Microcosms, augmented by *Brevundimonas* BAS4005i and *Pseudomonas* CAS907i showed superior production of this acid under both aerobic as well as anaerobic conditions, although under the later condition, it was considerably

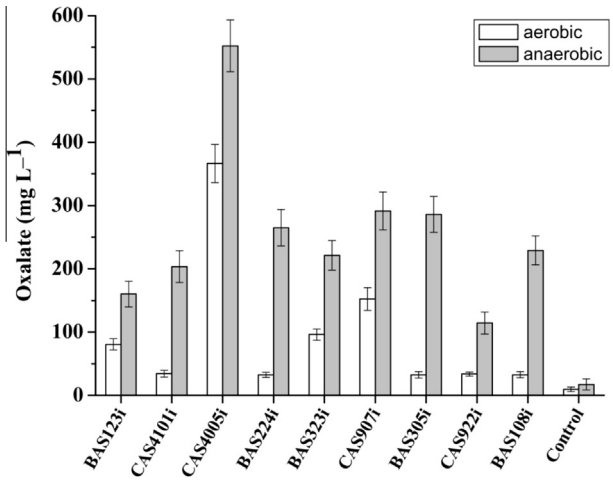

Fig. 6. Concentrations of oxalic acid present in aqueous phase of microcosm after 300 days incubation with selected bacterial strains in aerobic and anaerobic conditions (IC analysis). Error bars indicating standard deviations ($n = 3$). The bacterial strains represented in X axis are as follows: BAS123i (*Acinetobacter*), CAS4101i (*Arthrobacter*), CAS4005i (*Brevundimonas*), BAS323i (*Pseudomonas*), CAS907i (*Pseudomonas*), BAS224i (*Phyllobacterium*), BAS305i (*Rhizobium*), CAS922i (*Rhodococcus*) and BAS108i (*Staphylococcus*).

higher (2 fold). Low concentration of oxalic acid as detected in abiotic control sets probably resulted from abiotic transformation of glucose in the presence of minerals. This has already been reported by earlier investigators (Frey et al., 2010). It was observed that presence of oxalic acid was moderate to strongly correlated ($R^2 = 0.4$, $p \leqslant 0.05$) with As, and most of the other elements released in aqueous phase of microcosms incubated under both aerobic and anaerobic condition (Table 1a and b), thereby suggesting that the formation of oxalate during incubation may favor the release of most of the elements including As to some extent.

Presence of oxalic acid as the plausible bioweathering agent provides a critical insight on role of bacterial metabolites in mineral dissolution and mobilization of the elements into the aqueous phase. Microorganisms are well known to facilitate dissolution of minerals by acidification, ligand complexation, and/or by redox reactions through production of diverse metabolites e.g., organic acids, siderophores, cyanides, etc. (Frey et al., 2010; Mailloux et al., 2009; Uroz et al., 2009). Oxalate is known to act as a ligand affecting mineral dissolution by complexing metal ions at mineral surfaces and thereby enhancing their release in solution through ligand promoted dissolution. Weathering of minerals by bacteria may or may not be for energy, but certainly for supporting their growth and survival under nutrient limiting condition.

The present study demonstrates for the first time that indigenous aerobic, facultative anaerobic bacteria can transform and mobilize As from the host sediment, and delivers a most comprehensive mechanism for such phenomenon. In this microcosm study solid to water ratio was near about 12 times lower than normal aquifer condition (Dhar et al., 2011). Considering all other factors remained constant, impact of the presence of a similar dose of bacteria would increase the concentration of dissolved As concentration above WHO guideline (10 μg L^{-1}). Under anaerobic condition, *Rhodococcus* CAS922i, *Staphylococcus* BAS108i and *Brevundimonas* CAS4005i released As as high as >170 μg L^{-1} where as in aerobic condition with isolates *Staphylococcus* BAS108i and *Phyllobacterium* BAS224i it excides 20 μg L^{-1}. The study clearly showed that compared to anaerobic state, aerobic environment facilitates the indigenous bacteria to act less towards the mobilization of sediment bound As.

4. Conclusions

Indigenous bacteria isolated from groundwater posses high metabolic diversity with respect to utilization of C/e$^-$ -donors and -acceptors, As^{5+} reductase and As^{3+} oxidase activities. Sediment microcosms showed ability of all this bacteria to release As mainly as As^{3+} along with Fe, Al, Mn, *etc.* Increased level of oxalic acid indicated the role of bioweathering activity in release of As. In contrast to anaerobic incubation with As^{5+} reducing strains,

Table 1
Correlation matrix of 13 chemical parameters of the aqueous phase of (a) aerobic set and (b) anaerobic set microcosms after 300 days incubation.

	As	Mn	Fe	K	Al	Cr	Co	Ni	Zn	Cd	Ba	Na	Oxalate
(a)													
As	1												
Mn	0.298785	1											
Fe	0.308079	−0.25205	1										
K	0.419586	0.560362	−0.48456	1									
Al	0.380441	−0.47824	0.5031	0.030844	1								
Cr	−0.50276	0.040159	−0.30063	−0.14725	−0.21378	1							
Co	−0.07228	0.278833	−0.13159	−0.20866	−0.37841	−0.21348	1						
Ni	−0.3823	0.118534	−0.31646	0.041392	−0.0902	0.735227	−0.34789	1					
Zn	−0.08633	0.550424	−0.25266	−0.00387	−0.47963	0.105858	0.457349	0.446609	1				
Cd	−0.00779	0.284613	−0.06104	−0.17073	−0.32311	−0.29339	0.981062	−0.4669	0.322207	1			
Ba	−0.65182	0.205954	−0.35871	−0.16764	−0.60833	0.480585	−0.13912	0.425917	0.280824	−0.19076	1		
Na	−0.50897	−0.26862	−0.03359	−0.48886	−0.16447	0.834813	−0.20665	0.68992	0.069457	−0.28605	0.392025	1	
Oxalate	0.460635	0.391595	−0.14176	0.345064	−0.27676	−0.72078	0.25314	−0.53324	0.166374	0.303041	0.054444	−0.84878	1
(b)													
As	1												
Mn	0.17526	1											
Fe	0.39180	0.53918	1										
K	0.10516	0.61131	0.40806	1									
Al	0.05989	−0.5664	−0.2941	−0.3304	1								
Cr	0.22930	−0.4339	−0.1626	−0.13469	0.18731	1							
Co	−0.5712	0.05584	−0.4570	0.05946	−0.3222	0.41164	1						
Ni	−0.1233	−0.3060	−0.3735	−0.1850	−0.0202	0.87668	0.70309	1					
Zn	0.28927	−0.4448	−0.2055	−0.2718	0.28177	0.86566	0.15874	0.79283	1				
Cd	−0.4335	−0.13454	0.01269	−0.3511	−0.3679	−0.15532	0.20692	0.00509	−0.1773	1			
Ba	0.34610	0.49835	0.67571	0.37166	−0.4353	−0.3619	−0.4101	−0.5203	−0.35429	0.28433	1		
Na	0.31610	0.21628	0.41062	0.13331	−0.2608	−0.6599	−0.7743	−0.7445	−0.4330	−0.0351	0.51841	1	
Oxalate	0.47663	0.73357	0.73203	0.55528	−0.3848	−0.4807	−0.3583	−0.4727	−0.3889	−0.2102	0.35733	0.5410	1

incubation under oxic condition with As^{3+} oxidizing bacteria facilitates lesser As release, thereby highlighting the possible scope for controlling the aquifer contamination and/or developing any remediation system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.02.039.

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