Arsenic transforming abilities of groundwater bacteria and the combined use of *Aliihoeflea* sp. strain 2WW and goethite in metalloid removal

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Abstract

Several technologies have been developed for lowering arsenic in drinking waters below the World Health Organization limit of 10 µg/L. When in the presence of the reduced form of inorganic arsenic, i.e. arsenite, one options is pre-oxidation of arsenite to arsenate and adsorption on iron-based materials. Microbial oxidation of arsenite is considered a sustainable alternative to the chemical oxidants. In this contest, the present study investigates arsenic redox transformation abilities of bacterial strains in reductive groundwater from Lombardia (Italy), where arsenite was the main arsenic species. Twenty isolates were able to reduce 75 mg/L arsenate to arsenite, and they were affiliated to the genera *Pseudomonas*, *Achromobacter* and *Rhodococcus* and genes of the *ars* operon were detected. Three arsenite oxidizing strains were isolated: they belonged to *Rhodococcus* sp., *Achromobacter* sp. and *Aliihoeflea* sp., and *aioA* genes for arsenite oxidase were detected in *Aliihoeflea* sp. strain

2WW and in *Achromobacter* sp. strain 1L. Uninduced resting cells of strain 2WW were used in combination with goethite for arsenic removal in a model system, in order to test the feasibility of an arsenic removal process. In the presence of 200 µg/L arsenite, the combined 2WW-goethite system removed 95% of arsenic, thus lowering it to 8 µg/L. These results indicate that arsenite oxidation by strain 2WW combined to goethite adsorption is a promising approach for arsenic removal from contaminated groundwater.

Keywords: *Aliihoeflea*; arsenate; arsenite; bio-oxidation; goethite; groundwater.

1. INTRODUCTION

Arsenic (As) is present in high concentrations in waters due to natural and anthropogenic processes and creates serious environmental concerns throughout the world. In groundwater, As can be released from solid phases through biotic and abiotic processes. The oxidation state in which As occurs in groundwater affects its mobility and the efficiency with which it can be removed in remediation processes. Arsenic is primarily present as arsenite [As(III)], which is found under anaerobic conditions, or as arsenate [As(V)], which typically occurs under aerobic conditions [1]. As(V) has greater affinity for adsorption to oxyhydroxides and clay minerals, thus resulting less mobile than As(III) [2].

Bacteria play an important role in geochemical cycling of As by different oxidation/reduction reactions, affecting its speciation and mobility [3]. As(V) reduction and As(III) oxidation are both detoxification [4] and energy-generating mechanisms [5]. While As(V) reducing metabolism promotes As mobilization due to formation of As(III), As(III) oxidation, on the contrary, is considered to reduce it by producing As(V). Moreover, in different anaerobic environments, the release of As to aquifers has been demonstrated to be carried out by dissimilatory metal-reducing bacteria in many part of the world [6-8]. Recent literature describes different assemblages of the culturable bacterial communities within As-rich groundwaters around the world and the presence of both As(V) reducers and As(III) oxidizing strains have been recorded. In West Bengal (India), strains affiliated to *Ochrobactrum*, *Achromobacter* and *Rhizobiaceae* were able to reduce As(V) via an *ars* system and they were deduced to be responsible of the release of As(III) in groundwater [9]. In Taiwan, out of 11

As-resistant strains, 10 As(V) reducing strains of *Pseudomonas*, *Psychrobacter*, *Bacillus*, *Vibrio*, *Citrobacter* and *Enterobacter* and 1 As(III) oxidizing strain of *Bosea* were isolated [10]. In China, As(III) oxidizing strains of α- and β-proteobacteria were present in the upper layer of groundwater sediments, whereas As(V) reducing gamma-proteobacteria were from anoxic sediments [11]. Knowledge of arsenic-resistant bacterial communities in Italian groundwater is still scarce. In a recent survey γ-proteobacteria were isolated from As contaminated groundwaters sampled in the Central part of Italy [12]. The strains contained an *arsB* gene, but their As resistance levels were not phenotypically demonstrated.

Human exposure to As typically occurs through drinking water and the World Health Organisation (WHO) in 2001 has revised the threshold limit for As in drinking water to 10 µg/L [13]. In the last years, efforts have been made in order to develop solutions for an efficient As removal. Various treatment methods have been developed for the removal of As from water streams, such as adsorption, anion exchange, activated alumina, reverse osmosis, modified coagulation/filtration, modified lime softening and oxidation/filtration [for a review see [14]). Any effective treatment of As contaminated water has to remove both As(III) and As(V) forms, but sometimes classical technologies are not efficient enough for the removal of As(III), due to the positively charged surfaces of adsorbents. Thus, As(III) oxidation to As(V) is a prerequisite for achieving As concentrations below the WHO threshold.

Biological As(III) oxidation is considered a sustainable alternative to the use of chemical oxidants. Many heterotrophic bacteria oxidize As(III) to detoxify their immediate environment; chemoautotrophic bacteria oxidize As(III) via: (i) aerobic oxidation [15], (ii) anaerobic nitrate- and selenate-dependent respiration [16-18], or (iii) phototrophy [19]. Biological As(III) oxidation was tested as a feasible pre-treatment in different operational conditions by using either planktonic cells [20], biofilms [21], and immobilized bacteria [22-24].

After the biological As(III) oxidation, it is necessary to remove the produced As(V) by using sorbents. Commonly used sorbents or surface-coated sorbents are based on iron compounds and they are considered highly efficient in binding As (>95%) [25,26]. Goethite is a common soil iron oxide (α-FeOOH), known to adsorb As(III) and As(V) species [27]. The formation of various inner-sphere complexes has been suggested as the primary mechanism for the sorption of As(V) on iron oxides [28]. However, both inner-sphere complexes and outer-sphere complexes have been found in the sorption of As(III) on different iron oxides [29].

Goethite is reported to be more effective in As(V) than in As(III) removal, except at circumneutral pH values and higher [27,30].

Combination of different bacterial metabolisms and sorbing materials have been validated for the removal of As from groundwater (for a review see [31]). In particular, biological As(III)-oxidation combined with As(V) adsorption onto sorbing materials is considered an efficient method of removing As from polluted water. In model systems, the use of bacterial consortia in association with activated alumina [32] or of a single strain in association with chabazite and kutnahorite [33] has been shown to be effective in the removal of high initial As concentrations (98% removal of 75 mg/L; 90% removal of 100 mg/L, respectively). To the best of our knowledge, the combined use of biological As(III) oxidation and goethite has never been used for the treatment of As contaminated groundwater.

The aim of this work was to investigate the presence of As redox transformation abilities in indigenous bacterial strains and of the related genetic markers in As-rich groundwater from Lombardia (Italy), not studied so far. The study selected a suitable strain able to perform As(III) oxidation to As(V) in the condition of uninduced resting cells. This capability was then tested in the presence of goethite as sorbent and the effectiveness of the combined system in the removal of As was evaluated in model system.

2. MATERIALS AND METHODS

2.1. Sampling of Groundwaters

Water samples were collected from ten sites located in the province of Cremona (Lombardia, Italy), chosen from the dataset of the Regional Agency for Health Prevention and Environmental Protection (ARPA) of Lombardia on the basis of different levels of As in the groundwaters. The samples comes from six public-supply wells and four monitoring wells. Additionally, water sample WW was collected from an aerobic biofilter within a water treatment plant located at site B.

Water samples were purged under controlled flow before sampling, until stabilization of temperature, dissolved oxygen and redox potential (Eh).

2.2. Chemical Characterization of Water Samples

Eh and dissolved oxygen were measured *in situ* with a mV-meter PCE-228 (PCE Deutschland GmbH, Germany) and a portable dissolved oxygen-meter-HI 9146 (Hanna Instrument US Inc., Woonsocket, USA). Samples were collected into 1L sterile polyethylene bottles and brought to the laboratory in cooler bags in the dark. pH was measured on refrigerated samples within a few hours after collection, using a combination glass electrode pH-meter PCE-228 (PCE Deutschland GmbH, Meschede, Germany) Total dissolved carbon was determined with potassium dichromate standard method [34]. Sulphate was analysed by the gravimetric method using barium chloride and phosphate with the colorimetric method by Murphy and Riley.[35]. Soluble NH_4–N and NO_3–N were determined by flow injection analysis and spectrometric detection (FIAstar 5000 Analyzer, Foss Tecator, Denmark). For iron (Fe), manganese (Mn) and As determinations, samples were immediately filtered (∅ 0.22 µm) and acidified with HNO_3 to achieve a final concentration of 2% (v/v). Fe, Mn and As content were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies, USA). Standard for concentrations ranging from 0 to 1 mg/L were prepared from Multi-standard solution (Agilent Technologies, USA).

2.3. Enrichment and Isolation of Arsenic Resistant Bacteria

Mineral medium added of a carbon source was used in order to enrich AsIII and As(V) resistant bacteria from water samples. The growth medium used, hereafter referred as BBWM, consisted as follows: Solution A (g/L): KH_2PO_4 0.04; K_2HPO_4 0.04; NaCl 1.0; $(NH_4)_2SO_4$ 0.4; trace element solution 2 mL. The pH of solution A was adjusted to 6.5. Solution B (g/L): $CaCl_2$ 0.2; $MgSO_4$ 0.2. Solutions A and B were sterilized separately by autoclaving. Solution C: 0.95 M $NaHCO_3$. 490 mL of solution A and 490 mL of solution B were mixed after cooling, then supplemented with 10 mL of solution C, and with 10 mL of a vitamin solution previously sterilized by filtration over a 0.22 µm filter (Millipore, MA, USA). Sodium lactate (0.4 mol/L), previously sterilized, was added to BBWM as C source, hereafter referred as BBWM-L. The medium was then supplemented with sodium arsenite $NaAsO_2$ (0.13 g/L) or sodium arsenate $Na_2HAsO_4{\cdot}7H_2O$ (1.13 g/L) in order to select, respectively, for As(III) or As(V) resistant bacteria. The enrichment cultures were prepared by mixing 100 mL of BBWM-L (at double concentration) with 100 mL of groundwater sample

into flasks and incubated at 28°C shaking at 150 *rpm*. Four subcultures for each sample were made. After the fourth subculture, the resulting enriched cultures were serially diluted and plated onto 1.5% (w/v) agar plates made with BBWM-L separately added of As(III) (0.13 g/L) or As(V) (1.13 g/L). After 10 days of incubation at 28°C under aerobic conditions in the dark, colonies were randomly isolated from plates. Single colonies were streaked to purity by sub culturing on the same medium by three times. As(III) and As(V) resistance of strains was determined in BBWM-L liquid medium amended with As(III) 0.13 g/L or As(V) 1.13 g/L. Isolates were identified by means of 16S rRNA nucleotide sequence analysis. Strains were maintained in glycerol stocks at 70°C.

2.4. Arsenic Redox Transformations Experiments with Bacterial Strains

All the isolates were characterized for their capability to oxidize As(III) and to reduce As(V). Prior to use, the strains were grown to mid-exponential phase in BBWM-L at 28°C. To test the ability of the isolates to reduce As(V) or to oxidize As(III), each strain was inoculated into three flasks each containing 20 mL of BBWM-L separately supplemented with 75 mg/L As(V) or As(III). Three flasks without As content were inoculated to compare the growth of the microorganisms in the absence of As(V) or As(III). Three flasks were also prepared without inoculum in order to check for abiotic transformation of As. All flasks were incubated at 28°C shaking at 150 *rpm* for 48 h in the dark. At each sampling time, 2 mL of cell suspensions were removed to follow cell growth (OD_{620nm}) and to determine As(V) and As(III) concentrations by spectrophotometric analysis according to the method of Dhar et al. [36].

2.5. As(III) Oxidation Experiments with Resting Cells of **Aliihoeflea** *sp. Strain 2WW*

As(III) oxidation capability of strain 2WW was tested as As(III)-induced or uninduced resting cells. Particularly, a pre-culture of 2WW cells was established in BBWM-L for 48h at 28°C in shaking condition at 150 *rpm* in the dark. Aliquots of pre-culture cell suspension were inoculated at a final cell density of 10^7 cell/mL in 100 mL of BBWM-L in the presence or in the absence of 75 mg/L As(III) in order to obtain, respectively, As(III)-induced or uninduced cells. Flasks were incubated as above described. After growth, cells were centrifuged at

10,000 *rpm*, 10°C for 30 min. Cell pellets were washed three times with Tris-HCl (5 mM, pH 7.2) buffer and resuspended in the same buffer. Resting cell suspensions (500 µL) were then introduced into 100 mL polypropylene tubes containing 50 mL of Tris-HCl (5 mM, pH 7.2) supplemented with 200 µg/L As(III) to obtain a final cell density of about 10^7 cell/mL. Resting cell experiment was carried out in triplicate for 48h in shaking condition at 150 *rpm* in the dark at 28°C and at 15°C. The two temperature were chosen in order to represent mesophilic and real groundwater conditions, respectively. At the end of the experiments, 10 mL of cell suspensions were collected, centrifuged, syringe-filtered through 0.22 µm nitrocellulose membranes. Total As, As(III) and As(V) were determined by ICP-MS analysis.

2.6. Arsenic Adsorption Experiments

A commercial goethite powder (SigmaAldrich, USA, ca. 35% wt Fe) was used as As sorbing material. Reagent-grade chemicals and Milli-Q water were used to prepare As-spiked solutions. The 1000 mg/L stock solutions of As(V) and As(III) were prepared by using their sodium salts. All adsorption experiments were carried out in triplicates at 15°C in shaking condition at 150 *rpm* in the dark in Tris-HCl buffer solution (5 mM, pH 7.2) as a model system. Batch experiments were conducted in 100 mL polypropylene tubes.
Preliminarily, the ability of goethite (4 g/L) to adsorb As(V) and As(III) was tested in experiments with increasing As(III) or As(V) concentrations (25-800 µg/L) separately added. Pre-hydration effect on goethite adsorption was also evaluated by keeping goethite in Tris-HCl for 24h before setting the experiments as above described. Pre-hydration had no effect on As adsorption to goethite.
Batch experiments combining microbial As(III) bio-oxidation and As adsorption onto goethite were carried out in the presence of goethite (4 g/L) inserted in the tubes shortly before the addition of 50 mL of As(III) 200 µg/L Tris-HCl solution. Non-induced resting cell suspension, prepared as above described, was added to obtain a final cell density of about 10^7 cell/mL. Three different control treatments were prepared in the same manner: i) As(III) Tris-HCl solution (untreated control); ii) As(III) Tris-HCl solution with goethite without resting cells (abiotic control); iii) As(III) Tris-HCl solution with resting cells without goethite (biotic control).

After 24h incubation, 20 mL suspensions were collected from each experiment, centrifuged at 10,000 *rpm*, 10°C for 30 min and syringe-filtered through 0.22 µm nitrocellulose filter membranes. Total As, As(III) and As(V) were determined by ICP-MS analysis.

2.7 Arsenic Speciation by ICP-MS analysis

Arsenic forms present in the water samples and in the experiments were determined according to Kim et al. [37]. Particularly, total As was determined in 5 mL of the filtrates previously acidified with HNO_3 to achieve a final concentration of 2% (v/v). Whereas, As(III) and As(V) contents were determined in 5 ml of samples passed through a WATERS Sep-Pak® Plus Acell Plus QMA cartridge (Waters, MA, USA). As(V) was retained in the cartridge while allowing As(III) to pass through and collected. The cartridge was then washed with 0.16M HNO_3 to extract As(V) from it. Total As, As(III), As(V) contents were determined by ICP-MS (Agilent technologies, USA). Standards of As for concentrations ranging from 0 to 1 mg/L were prepared from sodium arsenite $NaAsO_2$ (Sigma Aldrich, USA) solution.

2.8. Arsenic Resistance Gene Amplification

Primer P52f (5'-AGCCAAATGGCAGAAGC-3') and P323r (5'-GCTGGRTCRTCAAATCCCCA-3') were used for arsenate reductase *ArsC* amplification according to the protocol of Bachate et al. [38]. Primer darsB1F (5'-TGTGGAACATCGTCTGGAAYGCNAC-3') and darsB1R (5'-CAGGCCGTACACCACCAGRTACATNCC-3') were used to amplify arsenite efflux pump *ArsB* [39]. Amplification of As(III) oxidase *aioA* gene was conducted with primers aoxBM1-2F (5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3') and aoxBM3-2R (5'-TGTCGTTGCCCCAGATGADNCCYTTYTC-3-') according to the protocol of Quèmèneur et al. [40]. PCR reactions were performed in a final volume of 25 mL containing: 10 ng total DNA, 0.2mMof dNTPs, 1.75mM of $MgCl_2$, 0.4 mMof each primer, 2U of Taq polymerase, and 1x PCR buffer. All reagents were obtained from Invitrogen. PCR reactions were performed on T-Gradient Biometra apparatus. The PCR products were checked on 2% (w/v) agarose gel containing 0.01% (v/v) GelRed™ stain (Biotium, CA, USA) and visualized using the GelDoc image analyzer system (BioRad, CA, USA).

2.9. Nucleotide Sequence Analysis

16S rRNA and arsenic resistance genes were sequenced with the respective primers using the Taq Dye-Deoxy Terminator Cycle Sequencing kit (Life Technologies Co., USA). The forward and reverse samples were run on a 310A sequence analyzer (Life Technologies Co., USA). Sequences were compared with the entire GenBank/EMBL nucleotide and amino acid databases using the BlastN and BlastX query programs (http://www.ebi.ac.uk/Tools/blastall/index.html). The EzTaxon server 2.1 was used to attribute type strain species to the newly isolated strains [41].

Sequences present in the present work were deposited to GenBank-EMBL databases.

3. RESULTS

3.1 Chemical Characterization of the Water Samples

Physical and chemical characteristics of the groundwaters along the Oglio river (in the north west of Cremona Province) are reported in Table 1. Waters from public supply wells, with the exception of that from site B-WW, were from anoxic to mildly oxic, with high hardness and contained Mn and Fe concentrations above the threshold levels for drinking waters [42]. Samples of groundwater collected in wells for the monitoring of subsurface water were from anoxic to oxic (sample from site C) and contained more sulphate and nitrates and less phosphate and ammonium than samples from public supply wells. Nevertheless, a spatial heterogeneity in the physico-chemical traits of the groundwater was revealed either for groundwater collected from monitoring wells and from public wells.

High As levels were found in eight out of ten samples: As concentration ranged from 21 to 171 µg/L. Only samples from monitoring wells of site C and H met the threshold levels of 10 µg/L. In all the contaminated samples, As(III) was the dominant As form with a ratio As(III)/As(V) ranging from 3.6 to 6.7.

Water aeration of biofilter B-WW caused a marked decrease in Fe concentration with respect to the content of the original groundwater. The As content was similar to that of the original groundwater (site B), with As(III) being totally oxidized to As(V), indicating that As was not adsorbed and immobilized by Fe(III) mineral precipitates.

Organic contaminants (*i.e*. aliphatic or aromatic hydrocarbons) and heavy metals (*i.e*. cadmium, mercury, selenium, vanadium and antimony) were absent in all the water samples.

3.2.Characterization of Arsenic Resistant Bacteria

Forty-nine bacterial strains were isolated from As(III) and As(V) amended BBWM-L plates from final step of enrichment cultures. Among these, 33 strain were able to grow aerobically in liquid mineral medium in the presence of a carbon source and of 1.13 g/L As(V) and to 0.13 g/L As(III), separately added. They were retrieved from most of the sites, and the level of As contamination did not selected for high As resistance.

Among As resistant strains, 23 isolates were able to perform redox transformation of As forms and they were grouped in 13 operational taxonomic units (OTU), according to 16S rRNA nucleotide sequence analysis (Table 2). Twenty isolates were able to completely reduce 75 mg/L As(V) to As(III) in 48h, and they belonged to ten different species. They were affiliated to different members of the genera *Pseudomonas*, *Achromobacter* and *Rhodococcus*. *ArsC* and *arsB* genes for, respectively, arsenate reductase and arsenite efflux pump present in the *ars* operon, were detected in 18 out of the 20 As(V) reducing strains, evidencing that the ability to reduce As(V) to As(III) in aerobic condition was linked to the presence of an *ars*-like detoxification system. The deduced amino acid sequence of *arsB* gene of *Pseudomonas* sp. strains 1F and 2F had 81% homology to arsenite-antimonite efflux pump of *Pseudomonas mendocina* NK-01 (Acc. Num. YP004379366). *ArsB* fragments retrieved in *Pseudomonas putida* strains 2L and 3L were highly homologous to the arsenite efflux transporter of *Pseudomonas putida* KT2440 (Acc. Num. NP744082).

As(III) oxidizing strains were more rare, as only three strains out of 23 were able to oxidize As(III) to As(V). As(III) oxidation rates were different : *Rhodococcus* sp. strain 6G oxidized 75 mg/L As(III) in 48 h, *Achromobacter* sp. strain 1L in 32 h and *Aliihoeflea* sp. strain 2WW in 24 h. The corresponding *aioA* genes for arsenite oxidase were detected in *Aliihoeflea* sp. strain 2WW and in *Achromobacter* sp. strain 1L. Deduced amino acid sequence of *aioA* gene of strain 2WW had 89% homology to large subunit of arsenite oxidase of an As(III)-oxidizing bacterium NT26 (Acc. Num. AAR05656) and 88% and 87% similarity to that of *Ochrobactrum tritici* (Acc. Num. ACK38267) and *Agrobacterium tumefaciens* 52 (Acc. Num. ABB51928), respectively. *AioA* gene of strain 1L was 100% homologous to large subunit of

arsenite oxidase of *Achromobacter* sp. 40AGIII (Acc. Num. AEL22195). *Rhodococcus ruber* strain 6G failed to give positive amplification, probably due to mismatches between the tested primers and gene sequence.

3.3 As(III) oxidation by resting cells of **Aliihoeflea** *sp. 2WW*

Among the As(III) oxidizing bacteria, *Aliihoeflea* sp. strain 2WW was the most efficient As(III) oxidizer at the tested conditions and it was chosen for As(III) oxidation experiments as resting cell at 28 and 15°C, as As(III)-induced and uninduced resting cells (Figure 1). The two temperatures were chosen in order to compare the activity of the strain at mesophile temperature (28°C) and at environmental temperature of groundwater at the sampling time (15°C).

As(III)-induced resting cells of strain 2WW were able to oxidize completely 200 µg/L As(III) to As(V) in 8 h at 28°C and in 24 h at 15°C, respectively (Fig. 1A), while non-induced resting cells completely oxidize it in 24 h at 28°C and in 32 h at 15°C, respectively (Fig. 1B). This data indicated that, although the process was slower, at 15°C uninduced resting cells of strain 2WW were able to oxidize completely an amount of As(III) comparable to that present in the most contaminated groundwater sample A.

In order to envisage the use of a bacterial strain in the As(III) oxidation step for the As removal process, *Aliihoeflea* sp. strain 2WW was chosen as candidate in a combined bio-oxidation and adsorption experiment.

3.4 Arsenic Adsorption by Combined Bio-oxidation and Goethite System

Goethite was chosen as a model sorbent because of its high affinity for As(V) [43], its low cost and wide availability. Arsenic adsorption capability of goethite is reported in Table 3. As(V) had a higher affinity than As(III) for goethite over the studied concentration range, and it resulted almost totally adsorbed up to a concentration of 200µg/L in the tested conditions. At the same As(III) concentration, close to the highest retrieved in water samples, 75% As(III) was adsorbed, thus leaving in solution As exceeding the 10 µg/L threshold.

The combination of bio-oxidation of As(III) to As(V) and adsorption to goethite was then tested in order to improve the As removal efficiency. Similarly to the explorative trial, goethite was able to remove 85% As(III) and a negligible chemo-oxidation of As(III)

occurred in the abiotic control (Fig. 2). In biotic control *Aliihoeflea* sp. strain 2WW converted completely As(III) to As(V). In the combined bio-oxidation and adsorption system, the removal of As was higher than 95%. Total soluble As decreased to 8 µg/L, thus meeting the WHO threshold limit. At the end of the experiment, As(V) was the only As form in solution, indicating that the ability of strain 2WW to oxidize As(III) was not affected by the presence of goethite.

4. DISCUSSION

The south-east of Lombardia region has many sites with naturally occurring As levels higher than the maximum threshold indicated by the WHO for drinking water (10 µg/L); consequently, the widespread groundwater pollution affects the water of public captation wells of this area.

Arsenic groundwater contamination may occur under both reducing and oxidizing conditions, and the ratio As(III)/As(V) can vary significantly, depending on the condition of *in situ* oxidation state of water [44]. It has been reported that the depth distribution of groundwater within the aquifer varies seasonally creating a dynamic nature of the water in terms of oxic-anoxic states [45]. Moreover, after digging wells, certain oxidation condition could be established through transportation of aerobic groundwater and diffusion of oxygen through vadose zone [11]. These dynamic conditions could stimulate bacterial population with both As(V)-reducing and As(III)-oxidizing abilities. Aerobic enrichment cultures of As(V)-reducing and As(III)-oxidizing bacteria established with the groundwater under study gave evidence of the presence of bacterial populations involved in the As cycle. Bacterial strains able to convert As by redox reactions were investigated and retrieved in groundwater of Lombardia region for the first time, evidencing that As-resistant bacteria appear to be widely distributed in natural environments with different level of contamination, in accordance with previous literature [9,10,46]. The isolated bacteria belonged to the genera *Pseudomonas*, *Achromobacter* and *Rhodococcus*, broadly represented among As-resistant bacterial strains isolated from As-contaminated groundwater [9,10,46]. Interestingly, As(III) oxidising *Aliihoeflea* sp. strain 2WW was described for the first time to be part of As cycling in As contaminated environment [47]. Genome analysis of *Aliihoeflea* sp. strain 2WW confirmed these abilities [48].

Most of the isolates of the present study were positive to As genetic markers and they differed with respect to the gene they carried. There was a good agreement between the presence of the As genes and As transforming capabilities of the isolates, with exception of *Rhodococcus* species, that resulted negative for all the As genes investigated; this might be indicative of low sequence homology with the primers set used. In accordance with previous results [10,49], these data corroborate the hypothesis that the *ars* operon could be slightly different even though the strains are categorized into the same genera, supporting the hypothesis of an horizontal transfer of the As genes within bacterial populations.

The fundamental understanding of the biochemistry and metabolic pathways involved in As resistance can be translated into strategies for "engineering" bacteria for effective As remediation. Active treatments for As removal from water benefit from the knowledge of As bacterial metabolism and in general are based on natural consortia, pure cultures of As resistant bacteria or Fe- and Mn-oxidizing bacteria that can transform and/or capture As forms indirectly [31].

Over the last ten years, As(III)-oxidizing bacteria were used as living and resting cells for biological decontamination of synthetic and natural contaminated waters [21,23,24,33,50]. Systems based on living and resting cells offer usually high efficiency and the possibility of recovering the heavy metals, but the first requires higher amounts of maintenance and operational funding [5]. In fact, in these reactors As immobilizing/transforming microbial cultures have to be maintained under controlled conditions of nutrients and electron donors and acceptors. Non-living biomass (as resting cells) appears to present more advantages in comparison to the use of living microorganisms, since cells do not require nutrient supply and are not subjected to metal toxicity and to environmental conditions such as pH, temperature of the water to be treated [51]. In the case of a low-temperature treatment (such as groundwater with mean temperature of 10°C-15°C), the step corresponding to biological As(III) oxidation should be carried out at temperature lower than the bacterial optimal growth (i.e. 28°C). Indeed, from an economical point of view it is not possible to heat bioreactors (i.e. at 28°C) and maintain them at the optimal growth temperature of the selected bacteria. For these reasons, the use of bacteria capable to oxidize As(III) as resting cells can be advantageous to overcome these aspects. Strain 2WW possesses As(III) oxidizing capability either as living [47] and as induced and non-induced resting cells, at pH and temperature similar to those of

the groundwater under study (pH 7.2, 15°C), making the strain suitable for exploitation in the biological step of a water treatment process.

After the biological oxidation of As(III), it is necessary to remove the produced As(V) by using sorbents. Commonly used sorbents or surface coated sorbents are based on iron compounds [25] and they are considered highly efficient in binding As (>95%). Among iron based sorbents, goethite was chosen as a model because it is wide available, more effective and economic. Effectiveness of goethite in adsorbing As species was evaluated in Tris-HCl solution at different As concentrations, in order to exclude competition between oxyanions (organic or inorganic ligands such as phosphate) and As(V) for sorption sites, as postulated by Lievremont et al. [52] and to maintain stable pH conditions. Our results showed that goethite was 100-fold higher efficient in removing As(V) than As(III) at an initial As concentration of 200 µg/L, decreasing As(V) to 3 µg/L and As(III) to 43 µg/L. These findings are consistent with results from previous studies indicating higher sorption rate of As(V) than As(III) to goethite at sub-acidic pH [53]. The As(V) sorption capacity of goethite was substantially unchanged whatever the initial concentration of As, in accordance with results of a comparative study on As(V) adsorption by goethite and hematite conducted at different initial As(V) concentrations (70 µg/L and 500 µg/L) [43]. Our results evidenced that As(III) sorption capacity of goethite did not vary up to an As(III) initial concentration of 200 µg/L, whereas at concentration higher than 400 µg/L, sorption profile depended on the initial As(III) concentration. The higher affinity of goethite for As(V) than As(III) justified the exploration of the use of the As(III) oxidizing bacterium 2WW strain to improve the As removal efficiency of goethite.

The present study evidenced that the combined *Aliihoeflea* sp. strain 2WW-goethite system improved the As removal efficiency. As(III) oxidation activity of strain 2WW was not affected by the presence of goethite, and cells did not affected As(V) retention by goethite. Our findings are in accordance with Kim and co-authors who investigated the effect of different bacteria (*Enterococcus faecalis*, *Escherichia coli*, and *Bacillus subtilis*) on As removal by iron-impregnated granular activated carbon in 1 mg/L As(III)/As(V) spiked solutions [54]. The authors demonstrated that hindrance effects of bacteria on As adsorption to the surfaces of granules were minimal. Conversely, Huang et al. demonstrated the competition between *Shewanella putrefaciens* and As(V) sorbed to goethite and ferrihydrite [55]. The experiments conducted under controlled conditions of pH (7.2), temperature (15°C),

absence of competing ions (Tris-HCl solution) allowed us to conclude that As(III) bio-oxidation coupled to As adsorption of goethite was efficient in removing more than 95% of 200 µg/L As(III) from solution, suggesting that this process can be successful in lowering As levels under the threshold limit of 10 µg/L As for drinking water. These findings are in agreement with recent studies on biological As(III) oxidation combined to removal of As(V) onto various sorbents (i.e. zero-valent iron, activated alumina and goethite) in model systems [32-56]. Ike et al. showed that efficiency of activated alumina of removing 75 mg/L As(III) As from a basal salt medium was highly enhanced by microbial As(III) oxidation [32]. Similarly, Wan et al. set up reactors for biological As(III) oxidation step performed by *Thiomans arsenivorans* and subsequent As(V) adsorption onto zero-valent iron; the authors demonstrated the very high As removal capacity of the combined process from highly concentrated As solution (10 mg/L) [56].

Our findings together with previous studies suggested the potential application of the combined systems (As(III)-oxidizing bacteria and sorbents) in As removal process from water. Nevertheless, from an operational point of view, it has to be taken into account a possible decrease in As removal efficiency when the combined system 2WW-goethite would be utilized in natural groundwater due to the presence of oxyanions such as phosphate, silicate and carbonate that can compete with As(V) for sorption sites onto goethite [57-59].

5. CONCLUSIONS

Arsenic polluted groundwaters of Lombardia are reservoirs of bacterial populations able to transform As, and they may contribute to mobilization or immobilization of the metalloid in those environments. Investigating microbial As transformations under laboratory conditions may facilitate the development of desirable strategies to manage groundwater resources for supplying safe drinking water to the people of affected area. The results from this study indicate that the combination of bio-oxidation with goethite is a promising approach for arsenic removal from contaminated groundwater, although in a model system at the present stage. It would be desirable to consider different aspects. The suitable bacterial strain should be able to oxidize As(III) to As(V) in uninduced resting cell condition at the same pH and temperature naturally occurring in the groundwater. This approach would help to decrease operational costs and to avoid the addition of carbon sources and of As in the biomass production process. Moreover, arsenic concentration and forms present in water have great

impact on the adsorption efficiency of goethite. Further experiments conducted with natural groundwater samples are under way and they will help to elucidate the role of different environmental factors and of coexisting ions in affecting the performance of the system.

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Table

Table 1 Main properties of the studied water samples

Site	Water type	Depth (m)	T (°C)	pH	Redox potential (mV)	Total hardness (mg/L $CaCO_3$)	Dissolved components (µg/L)									
							Organic C	$S\text{-}SO_4$	$P\text{-}PO_4$	$N\text{-}NO_3$	$N\text{-}NH_4$	Fe	Mn	As		
														Total	As(III)	As(V)
A	public-supply	9.32	14.7	7.58	-113	282	2.11	267	165	685	2680	759	96.6	171	132	33
B	public-supply	12.33	16.3	7.62	-92	262	0.56	167	168	3.0	1231	262	70.6	24	17	4.6
B-WW	biofilter	-	16.0	7.63	+456	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	157	n.d.	22	0	24
C	monitoring-well	12.92	14.1	7.12	+161	416	1.67	27667	21	3259	69	417	435	0.7	0.3	0.4
D	public-supply	3.06	16.4	7.92	-120	n.d.	n.d.	< 0.5	131	< 0.5	1562	301	56.7	32	28	5.9
E	public-supply	3.21	16.4	8.09	-95	230	2.56	167	168	< 0.5	0.0	218	50.4	41	37	6.6
F	public-supply	0	15.4	7.98	-100	252	3.89	167	322	< 0.5	1751	260	92.6	90	71	11
G	monitoring-well	2.87	14.3	7.05	-57	507	0.11	34	79	4.0	405	4786	1191	21	18	5.0
H	monitoring-well	3.51	14.0	7.01	-100	533	3.22	40	48	< 0.5	0.0	4965	805	2.3	1.8	0.7
I	public-supply	9.93	16.1	7.73	-140	260	0.56	<0.5	112	< 0.5	1240	381	77.8	36	29	6.9
L	monitoring-well	2.20	14.0	7.17	-104	435	n.d.	4167	87	75.0	778	3198	112	53	47	7.3

n.d., not determined

Table 2 Arsenic transformation abilities and arsenic-resistance genes of bacterial strains isolated from the studied waters

site	Strain, closest relatives*	As(III) oxidation [a]	As(V) reduction [a]	Arsenic genes		
				arsC	*arsB*	*aioA*
A	3A, *Achromobacter spanius* 100% AY170848 (1)	-	+	+	-	-
WW	2WW, *Aliihoeflea* sp. 99% EF660756 (1)	+	-	-	-	+
D	1D, *Pseudomonas* sp. 100% AY690693 (1)	-	+	+	-	-
	2D, *Pseudomonas* sp. 100% AB365065 (6)	-	+	+	-	-
	4D, *Pseudomonas* sp. 100% AB633202 (2)	-	+	+	-	-
F	1F, *Pseudomonas* sp. 100% AJ551097 (5)	-	+	-	+	-
	2F, P*seudomonas stutzeri* 100% DQ211352 (1)	-	+	-	+	-
G	6G, *Rhodococcus ruber* 100% X80625 (1)	+	-	-	-	-
I	1I, *Rhodococcus* sp. 100% JN650553 (1)	-	+	-	-	-
	3I, *Rhodococcus* sp. 100% AY168591 (1)	-	+	-	-	-
L	1L, *Achromobacter* sp. 100% JN836430 (1)	+	-	-	-	+
	2L, *Pseudomonas putida* 100% HQ162489 (1)	-	+	-	+	-
	3L, *Pseudomonas putida* 100% GQ330565 (1)	-	+	-	+	-

*, numbers in brackets are referred to the abundance of isolates affiliated to the same species: [a], complete transformation of 75 mg/L arsenic in 48h

Table 3 Inorganic forms of arsenic before and after 24h contact with 4g/L goethite (mean ± standard deviation, n =3).

Soluble arsenic (µg/L)			
As(III)		As(V)	
0 h	*24 h*	*0 h*	*24 h*
0	0.3±0.3	0	0.3±0.3
25	5.1±0.9	25	0.2±0.1
50	16.1±6.6	50	0.2±0.1
100	33.6±3.4	100	0.3±0.2
200	42.6±4.6	200	3.0±1.4
400	203±26	400	15.9±4.6
800	412±15	800	75.2±20

Figure

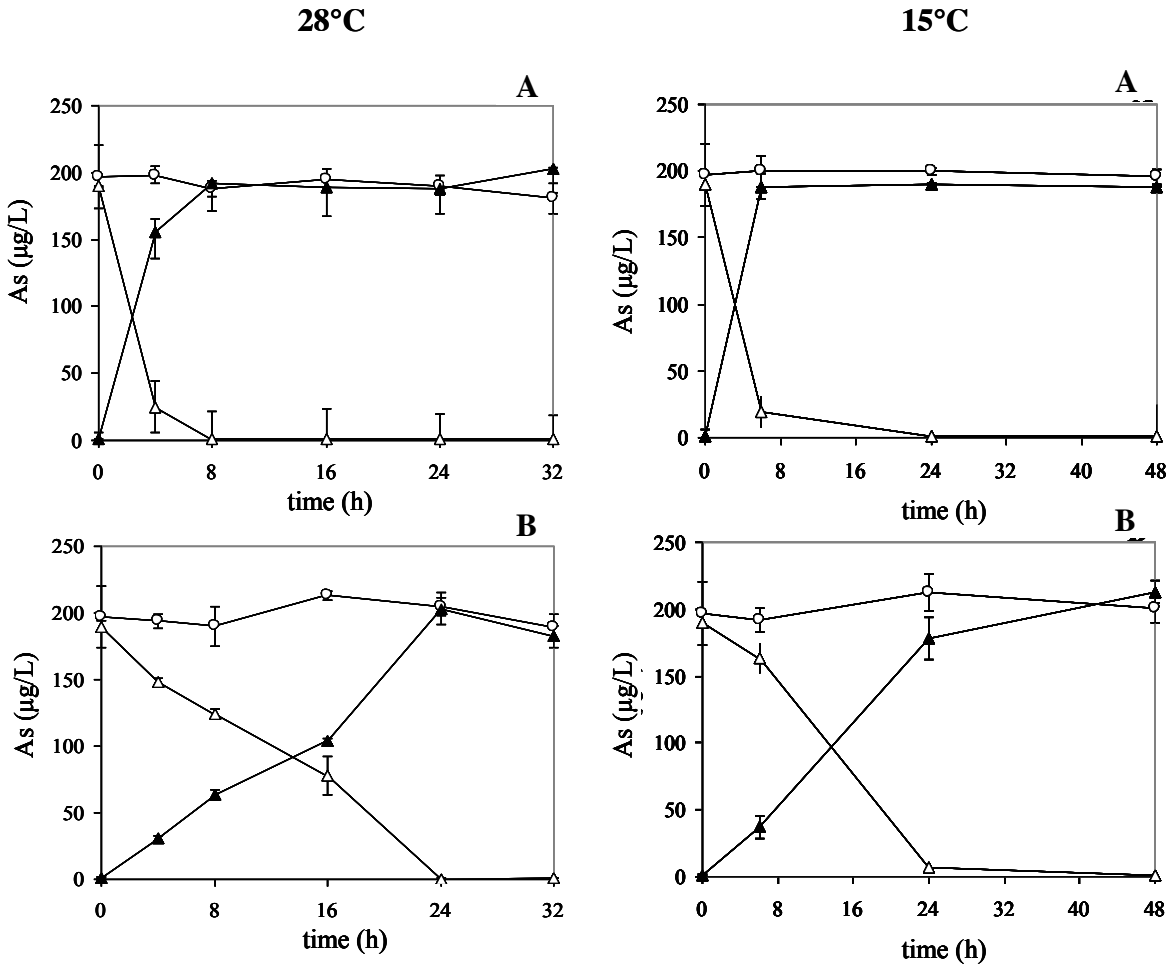

Fig. 1. Time course of As(III) oxidation by resting cells at 28°C and 15°C; A – As(III)-induced resting cells, B – non induced resting cells (mean ± standard deviation, n =3); ○, Total arsenic; △, As(III); ▲, As(V).

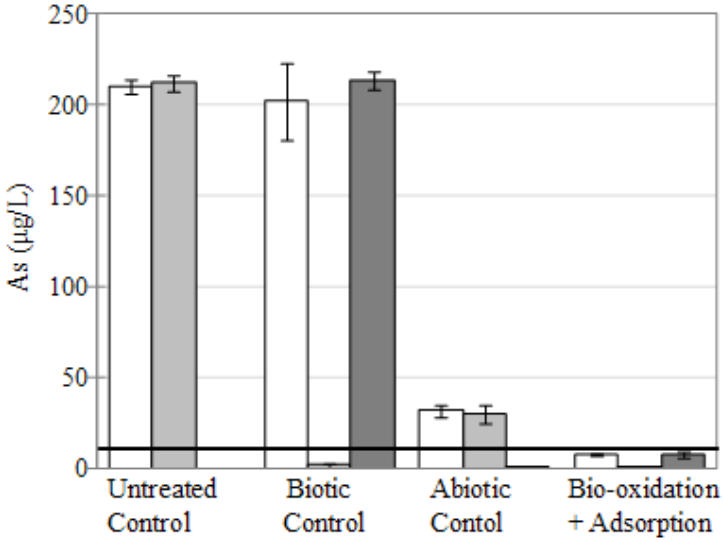

Fig. 2. Soluble arsenic forms in combined bio-oxidation and goethite system after 48h incubation in the presence of As(III) 200 µg/L. Untreated Control: As(III) Tris-HCl solution; Biotic Control: *Aliihoeflea* sp. 2WW resting cells; Abiotic Control: goethite 4g/L; Bio-oxidation + Adsorption: *Aliihoeflea* sp. 2WW resting cells and goethite 4g/L (mean ± standard deviation, n =4). □, total As; □, As(III); ■ ,As(V).

Abstract

Several technologies have been developed for lowering arsenic in drinking waters below the World Health Organization limit of 10 μg/L. When in the presence of the reduced form of inorganic arsenic, i.e. arsenite, one options is pre-oxidation of arsenite to arsenate and adsorption on iron-based materials. Microbial oxidation of arsenite is considered a sustainable alternative to the chemical oxidants. In this contest, the present study investigates arsenic redox transformation abilities of bacterial strains in reductive groundwater from Lombardia (Italy), where arsenite was the main arsenic species. Twenty isolates were able to reduce 75 mg/L arsenate to arsenite, and they were affiliated to the genera *Pseudomonas, Achromobacter* and *Rhodococcus* and genes of the *ars* operon were detected. Three arsenite oxidizing strains were isolated: they belonged to *Rhodococcus* sp., *Achromobacter* sp. and *Aliihoeflea* sp., and *aioA* genes for arsenite oxidase were detected in *Aliihoeflea* sp. strain 2WW and in *Achromobacter* sp. strain 1L. Uninduced resting cells of strain 2WW were used in combination with goethite for arsenic removal in a model system, in order to test the feasibility of an arsenic removal process. In the presence of 200 μg/L arsenite, the combined 2WW-goethite system removed 95% of arsenic, thus lowering it to 8 μg/L. These results indicate that arsenite oxidation by strain 2WW combined to goethite adsorption is a promising approach for arsenic removal from contaminated groundwater.