

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

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## 21 Abstract

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

33 2WW and in *Achromobacter* sp. strain 1L. Uninduced resting cells of strain 2WW were used  
34 in combination with goethite for arsenic removal in a model system, in order to test the  
35 feasibility of an arsenic removal process. In the presence of 200 µg/L arsenite, the combined  
36 2WW-goethite system removed 95% of arsenic, thus lowering it to 8 µg/L. These results  
37 indicate that arsenite oxidation by strain 2WW combined to goethite adsorption is a  
38 promising approach for arsenic removal from contaminated groundwater.

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

## 44 1. INTRODUCTION

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

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

66 As-resistant strains, 10 As(V) reducing strains of *Pseudomonas*, *Psychrobacter*, *Bacillus*,  
67 *Vibrio*, *Citrobacter* and *Enterobacter* and 1 As(III) oxidizing strain of *Bosea* were isolated  
68 [10]. In China, As(III) oxidizing strains of  $\alpha$ - and  $\beta$ -proteobacteria were present in the upper  
69 layer of groundwater sediments, whereas As(V) reducing gamma-proteobacteria were from  
70 anoxic sediments [11]. Knowledge of arsenic-resistant bacterial communities in Italian  
71 groundwater is still scarce. In a recent survey  $\gamma$ -proteobacteria were isolated from As  
72 contaminated groundwaters sampled in the Central part of Italy [12]. The strains contained an  
73 *arsB* gene, but their As resistance levels were not phenotypically demonstrated.  
74 Human exposure to As typically occurs through drinking water and the World Health  
75 Organisation (WHO) in 2001 has revised the threshold limit for As in drinking water to 10  
76  $\mu\text{g/L}$  [13]. In the last years, efforts have been made in order to develop solutions for an  
77 efficient As removal. Various treatment methods have been developed for the removal of As  
78 from water streams, such as adsorption, anion exchange, activated alumina, reverse osmosis,  
79 modified coagulation/filtration, modified lime softening and oxidation/filtration [for a review  
80 see [14]]. Any effective treatment of As contaminated water has to remove both As(III) and  
81 As(V) forms, but sometimes classical technologies are not efficient enough for the removal of  
82 As(III), due to the positively charged surfaces of adsorbents. Thus, As(III) oxidation to As(V)  
83 is a prerequisite for achieving As concentrations below the WHO threshold.  
84 Biological As(III) oxidation is considered a sustainable alternative to the use of chemical  
85 oxidants. Many heterotrophic bacteria oxidize As(III) to detoxify their immediate  
86 environment; chemoautotrophic bacteria oxidize As(III) via: (i) aerobic oxidation [15], (ii)  
87 anaerobic nitrate- and selenate-dependent respiration [16-18], or (iii) phototrophy [19].  
88 Biological As(III) oxidation was tested as a feasible pre-treatment in different operational  
89 conditions by using either planktonic cells [20], biofilms [21], and immobilized bacteria [22-  
90 24].  
91 After the biological As(III) oxidation, it is necessary to remove the produced As(V) by using  
92 sorbents. Commonly used sorbents or surface-coated sorbents are based on iron compounds  
93 and they are considered highly efficient in binding As (>95%) [25,26]. Goethite is a common  
94 soil iron oxide ( $\alpha$ -FeOOH), known to adsorb As(III) and As(V) species [27]. The formation of  
95 various inner-sphere complexes has been suggested as the primary mechanism for the  
96 sorption of As(V) on iron oxides [28]. However, both inner-sphere complexes and outer-  
97 sphere complexes have been found in the sorption of As(III) on different iron oxides [29].

1 98 Goethite is reported to be more effective in As(V) than in As(III) removal, except at  
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3 99 circumneutral pH values and higher [27,30].  
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5 100 Combination of different bacterial metabolisms and sorbing materials have been validated for  
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7 101 the removal of As from groundwater (for a review see [31]). In particular, biological As(III)-  
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9 102 oxidation combined with As(V) adsorption onto sorbing materials is considered an efficient  
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11 103 method of removing As from polluted water. In model systems, the use of bacterial consortia  
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13 104 in association with activated alumina [32] or of a single strain in association with chabazite  
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15 105 and kutnahorite [33] has been shown to be effective in the removal of high initial As  
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17 106 concentrations (98% removal of 75 mg/L; 90% removal of 100 mg/L, respectively). To the  
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19 107 best of our knowledge, the combined use of biological As(III) oxidation and goethite has  
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21 108 never been used for the treatment of As contaminated groundwater.  
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23 109 The aim of this work was to investigate the presence of As redox transformation abilities in  
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25 110 indigenous bacterial strains and of the related genetic markers in As-rich groundwater from  
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27 111 Lombardia (Italy), not studied so far. The study selected a suitable strain able to perform  
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29 112 As(III) oxidation to As(V) in the condition of uninduced resting cells. This capability was  
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31 113 then tested in the presence of goethite as sorbent and the effectiveness of the combined  
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33 114 system in the removal of As was evaluated in model system.  
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## 34 116 **2. MATERIALS AND METHODS**

### 36 117 37 38 118 *2.1. Sampling of Groundwaters*

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42 120 Water samples were collected from ten sites located in the province of Cremona (Lombardia,  
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44 121 Italy), chosen from the dataset of the Regional Agency for Health Prevention and  
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46 122 Environmental Protection (ARPA) of Lombardia on the basis of different levels of As in the  
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48 123 groundwaters. The samples comes from six public-supply wells and four monitoring wells.  
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50 124 Additionally, water sample WW was collected from an aerobic biofilter within a water  
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52 125 treatment plant located at site B.

53 126 Water samples were purged under controlled flow before sampling, until stabilization of  
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55 127 temperature, dissolved oxygen and redox potential (Eh).  
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### 57 58 129 *2.2. Chemical Characterization of Water Samples*

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3 131 Eh and dissolved oxygen were measured *in situ* with a mV-meter PCE-228 (PCE Deutschland  
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5 132 GmbH, Germany) and a portable dissolved oxygen-meter-HI 9146 (Hanna Instrument US  
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7 133 Inc., Woonsocket, USA). Samples were collected into 1L sterile polyethylene bottles and  
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9 134 brought to the laboratory in cooler bags in the dark. pH was measured on refrigerated samples  
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11 135 within a few hours after collection, using a combination glass electrode pH-meter PCE-228  
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13 136 (PCE Deutschland GmbH, Meschede, Germany) Total dissolved carbon was determined with  
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15 137 potassium dichromate standard method [34]. Sulphate was analysed by the gravimetric  
16  
17 138 method using barium chloride and phosphate with the colorimetric method by Murphy and  
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19 139 Riley.[35]. Soluble  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  were determined by flow injection analysis and  
20  
21 140 spectrometric detection (FIAstar 5000 Analyzer, Foss Tecator, Denmark). For iron (Fe),  
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23 141 manganese (Mn) and As determinations, samples were immediately filtered ( $\varnothing$  0.22  $\mu\text{m}$ ) and  
24  
25 142 acidified with  $\text{HNO}_3$  to achieve a final concentration of 2% (v/v). Fe, Mn and As content were  
26  
27 143 determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent  
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29 144 Technologies, USA). Standard for concentrations ranging from 0 to 1 mg/L were prepared  
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31 145 from Multi-standard solution (Agilent Technologies, USA).  
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### 33 147 **2.3. Enrichment and Isolation of Arsenic Resistant Bacteria**

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35  
36 149 **Mineral medium** added of a carbon source was used in order to enrich As(III) and As(V)  
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38 150 resistant bacteria from water samples. The growth medium used, hereafter referred as  
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40 151 BBWM, consisted as follows: Solution A (g/L):  $\text{KH}_2\text{PO}_4$  0.04;  $\text{K}_2\text{HPO}_4$  0.04; NaCl 1.0;  
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42 152  $(\text{NH}_4)_2\text{SO}_4$  0.4; trace element solution 2 mL. The pH of solution A was adjusted to 6.5.  
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44 153 Solution B (g/L):  $\text{CaCl}_2$  0.2;  $\text{MgSO}_4$  0.2. Solutions A and B were sterilized separately by  
45  
46 154 autoclaving. Solution C: 0.95 M  $\text{NaHCO}_3$ . 490 mL of solution A and 490 mL of solution B  
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48 155 were mixed after cooling, then supplemented with 10 mL of solution C, and with 10 mL of a  
49  
50 156 vitamin solution previously sterilized by filtration over a 0.22  $\mu\text{m}$  filter (Millipore, MA,  
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52 157 USA). Sodium lactate (0.4 mol/L), previously sterilized, was added to BBWM as C source,  
53  
54 158 hereafter referred as BBWM-L. The medium was then supplemented with sodium arsenite  
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56 159  $\text{NaAsO}_2$  (0.13 g/L) or sodium arsenate  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  (1.13 g/L) in order to select,  
57  
58 160 respectively, for As(III) or As(V) resistant bacteria. The enrichment cultures were prepared by  
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60 161 mixing 100 mL of **BBWM-L** (at double concentration) with 100 mL of groundwater sample  
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1 162 into flasks and incubated at 28°C shaking at 150 rpm. Four subcultures for each sample were  
2 163 made. After the fourth subculture, the resulting enriched cultures were serially diluted and  
3 164 plated onto 1.5% (w/v) agar plates made with BBWM-L separately added of As(III) (0.13  
4 165 g/L) or As(V) (1.13 g/L). After 10 days of incubation at 28°C under aerobic conditions in the  
5 166 dark, colonies were randomly isolated from plates. Single colonies were streaked to purity by  
6 167 sub culturing on the same medium by three times. As(III) and As(V) resistance of strains was  
7 168 determined in BBWM-L liquid medium amended with As(III) 0.13 g/L or As(V) 1.13 g/L.  
8 169 Isolates were identified by means of 16S rRNA nucleotide sequence analysis. Strains were  
9 170 maintained in glycerol stocks at 70°C.

#### 171 172 **2.4. Arsenic Redox Transformations Experiments with Bacterial Strains**

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174 All the isolates were characterized for their capability to oxidize As(III) and to reduce As(V).  
175 Prior to use, the strains were grown to mid-exponential phase in BBWM-L at 28°C. To test  
176 the ability of the isolates to reduce As(V) or to oxidize As(III), each strain was inoculated into  
177 three flasks each containing 20 mL of BBWM-L separately supplemented with 75 mg/L  
178 As(V) or As(III). Three flasks without As content were inoculated to compare the growth of  
179 the microorganisms in the absence of As(V) or As(III). Three flasks were also prepared  
180 without inoculum in order to check for abiotic transformation of As. All flasks were incubated  
181 at 28°C shaking at 150 rpm for 48 h in the dark. At each sampling time, 2 mL of cell  
182 suspensions were removed to follow cell growth (OD<sub>620nm</sub>) and to determine As(V) and  
183 As(III) concentrations by spectrophotometric analysis according to the method of Dhar et al.  
184 [36].

#### 185 186 **2.5. As(III) Oxidation Experiments with Resting Cells of Aliihoeflea sp. Strain 2WW**

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

1 194 10,000 rpm, 10°C for 30 min. Cell pellets were washed three times with Tris-HCl (5 mM, pH  
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3 195 7.2) buffer and resuspended in the same buffer. Resting cell suspensions (500 µL) were then  
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5 196 introduced into 100 mL polypropylene tubes containing 50 mL of Tris-HCl (5 mM, pH 7.2)  
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7 197 supplemented with 200 µg/L As(III) to obtain a final cell density of about 10<sup>7</sup> cell/mL.  
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9 198 Resting cell experiment was carried out in triplicate for 48h in shaking condition at 150 rpm  
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11 199 in the dark at 28°C and at 15°C. The two temperature were chosen in order to represent  
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13 200 mesophilic and real groundwater conditions, respectively. At the end of the experiments, 10  
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15 201 mL of cell suspensions were collected, centrifuged, syringe-filtered through 0.22 µm  
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17 202 nitrocellulose membranes. Total As, As(III) and As(V) were determined by ICP-MS analysis.  
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## 20 204 ***2.6. Arsenic Adsorption Experiments***

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23 206 A commercial goethite powder (SigmaAldrich, USA, ca. 35% wt Fe) was used as As sorbing  
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25 207 material. Reagent-grade chemicals and Milli-Q water were used to prepare As-spiked  
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27 208 solutions. The 1000 mg/L stock solutions of As(V) and As(III) were prepared by using their  
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29 209 sodium salts. All adsorption experiments were carried out in triplicates at 15°C in shaking  
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31 210 condition at 150 rpm in the dark in Tris-HCl buffer solution (5 mM, pH 7.2) as a model  
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33 211 system. Batch experiments were conducted in 100 mL polypropylene tubes.

34 212 Preliminarily, the ability of goethite (4 g/L) to adsorb As(V) and As(III) was tested in  
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36 213 experiments with increasing As(III) or As(V) concentrations (25-800 µg/L) separately added.  
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38 214 Pre-hydration effect on goethite adsorption was also evaluated by keeping goethite in Tris-  
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40 215 HCl for 24h before setting the experiments as above described. Pre-hydration had no effect on  
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42 216 As adsorption to goethite.

43 217 Batch experiments combining microbial As(III) bio-oxidation and As adsorption onto goethite  
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45 218 were carried out in the presence of goethite (4 g/L) inserted in the tubes shortly before the  
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47 219 addition of 50 mL of As(III) 200 µg/L Tris-HCl solution. Non-induced resting cell  
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49 220 suspension, prepared as above described, was added to obtain a final cell density of about 10<sup>7</sup>  
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51 221 cell/mL. Three different control treatments were prepared in the same manner: i) As(III) Tris-  
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53 222 HCl solution (untreated control); ii) As(III) Tris-HCl solution with goethite without resting  
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55 223 cells (abiotic control); iii) As(III) Tris-HCl solution with resting cells without goethite (biotic  
56  
57 224 control).



1 225 After 24h incubation, 20 mL suspensions were collected from each experiment, centrifuged at  
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3 226 10,000 rpm, 10°C for 30 min and syringe-filtered through 0.22 µm nitrocellulose filter  
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5 227 membranes. Total As, As(III) and As(V) were determined by ICP-MS analysis.  
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## 8 229 **2.7 Arsenic Speciation by ICP-MS analysis**

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12 231 Arsenic forms present in the water samples and in the experiments were determined according  
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14 232 to Kim et al. [37]. Particularly, total As was determined in 5 mL of the filtrates previously  
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16 233 acidified with HNO<sub>3</sub> to achieve a final concentration of 2% (v/v). Whereas, As(III) and As(V)  
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18 234 contents were determined in 5 ml of samples passed through a WATERS Sep-Pak® Plus  
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20 235 Acell Plus QMA cartridge (Waters, MA, USA). As(V) was retained in the cartridge while  
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22 236 allowing As(III) to pass through and collected. The cartridge was then washed with 0.16M  
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24 237 HNO<sub>3</sub> to extract As(V) from it. Total As, As(III), As(V) contents were determined by ICP-  
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26 238 MS (Agilent technologies, USA). Standards of As for concentrations ranging from 0 to 1  
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28 239 mg/L were prepared from sodium arsenite NaAsO<sub>2</sub> (Sigma Aldrich, USA) solution.  
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## 31 241 **2.8. Arsenic Resistance Gene Amplification**

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34 243 Primer P52f (5'-AGCCAAATGGCAGAAGC-3') and P323r (5'-  
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36 244 GCTGGRTCRTCAAATCCCCA-3') were used for arsenate reductase *ArsC* amplification  
37  
38 245 according to the protocol of Bachate et al. [38]. Primer darsB1F (5'-  
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40 246 TGTGGAACATCGTCTGGAAYGCNAC-3') and darsB1R (5'-  
41  
42 247 CAGGCCGTACACCACCAGRTACATNCC-3') were used to amplify arsenite efflux pump  
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44 248 *ArsB* [39]. Amplification of As(III) oxidase *aioA* gene was conducted with primers aoxBM1-  
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46 249 2F (5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3') and aoxBM3-2R (5'-  
47  
48 250 TGTCGTTGCCCCAGATGADNCCYTTYTC-3') according to the protocol of Quémèneur  
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50 251 et al. [40]. PCR reactions were performed in a final volume of 25 mL containing: 10 ng total  
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52 252 DNA, 0.2mM of dNTPs, 1.75mM of MgCl<sub>2</sub>, 0.4 mM of each primer, 2U of Taq polymerase,  
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54 253 and 1x PCR buffer. All reagents were obtained from Invitrogen. PCR reactions were  
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56 254 performed on T-Gradient Biometra apparatus. The PCR products were checked on 2% (w/v)  
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58 255 agarose gel containing 0.01% (v/v) GelRed™ stain (Biotium, CA, USA) and visualized using  
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60 256 the GelDoc image analyzer system (BioRad, CA, USA).  
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## **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.

1 289 Organic contaminants (*i.e.* aliphatic or aromatic hydrocarbons) and heavy metals (*i.e.*  
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3 290 cadmium, mercury, selenium, vanadium and antimony) were absent in all the water samples.

### 4 5 291 6 292 **3.2.Characterization of Arsenic Resistant Bacteria**

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10 294 Forty-nine bacterial strains were isolated from As(III) and As(V) amended BBWM-L plates  
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12 295 from final step of enrichment cultures. Among these, 33 strain were able to grow aerobically  
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14 296 in liquid mineral medium in the presence of a carbon source and of 1.13 g/L As(V) and to  
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16 297 0.13 g/L As(III), separately added. They were retrieved from most of the sites, and the level  
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18 298 of As contamination did not selected for high As resistance.

19 299 Among As resistant strains, 23 isolates were able to perform redox transformation of As  
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21 300 forms and they were grouped in 13 operational taxonomic units (OTU), according to 16S  
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23 301 rRNA nucleotide sequence analysis (Table 2). Twenty isolates were able to completely reduce  
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25 302 75 mg/L As(V) to As(III) in 48h, and they belonged to ten different species. They were  
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27 303 affiliated to different members of the genera *Pseudomonas*, *Achromobacter* and *Rhodococcus*.  
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29 304 *ArsC* and *arsB* genes for, respectively, arsenate reductase and arsenite efflux pump present in  
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31 305 the *ars* operon, were detected in 18 out of the 20 As(V) reducing strains, evidencing that the  
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33 306 ability to reduce As(V) to As(III) in aerobic condition was linked to the presence of an *ars*-  
34  
35 307 like detoxification system. The deduced amino acid sequence of *arsB* gene of *Pseudomonas*  
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37 308 sp. strains 1F and 2F had 81% homology to arsenite-antimonite efflux pump of *Pseudomonas*  
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39 309 *mendocina* NK-01 (Acc. Num. YP004379366). *ArsB* fragments retrieved in *Pseudomonas*  
40  
41 310 *putida* strains 2L and 3L were highly homologous to the arsenite efflux transporter of  
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43 311 *Pseudomonas putida* KT2440 (Acc. Num. NP744082).

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45 312 As(III) oxidizing strains were more rare, as only three strains out of 23 were able to oxidize  
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47 313 As(III) to As(V). As(III) oxidation rates were different : *Rhodococcus* sp. strain 6G oxidized  
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49 314 75 mg/L As(III) in 48 h, *Achromobacter* sp. strain 1L in 32 h and *Aliihoeflea* sp. strain 2WW  
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51 315 in 24 h. The corresponding *aioA* genes for arsenite oxidase were detected in *Aliihoeflea* sp.  
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53 316 strain 2WW and in *Achromobacter* sp. strain 1L. Deduced amino acid sequence of *aioA* gene  
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55 317 of strain 2WW had 89% homology to large subunit of arsenite oxidase of an As(III)-oxidizing  
56  
57 318 bacterium NT26 (Acc. Num. AAR05656) and 88% and 87% similarity to that of  
58  
59 319 *Ochrobactrum tritici* (Acc. Num. ACK38267) and *Agrobacterium tumefaciens* 52 (Acc. Num.  
60  
61 320 ABB51928), respectively. *AioA* gene of strain 1L was 100% homologous to large subunit of  
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1 321 arsenite oxidase of *Achromobacter* sp. 40AGIII (Acc. Num. AEL22195). *Rhodococcus ruber*  
2 322 strain 6G failed to give positive amplification, probably due to mismatches between the tested  
3 323 primers and gene sequence.  
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5 324

### 6 325 ***3.3 As(III) oxidation by resting cells of Aliihoeflea sp. 2WW***

7 326  
8 327 Among the As(III) oxidizing bacteria, *Aliihoeflea* sp. strain 2WW was the most efficient  
9 328 As(III) oxidizer at the tested conditions and it was chosen for As(III) oxidation experiments as  
10 329 resting cell at 28 and 15°C, as As(III)-induced and uninduced resting cells (Figure 1). The two  
11 330 temperatures were chosen in order to compare the activity of the strain at mesophile  
12 331 temperature (28°C) and at environmental temperature of groundwater at the sampling time  
13 332 (15°C).

14 333 As(III)-induced resting cells of strain 2WW were able to oxidize completely 200 µg/L As(III)  
15 334 to As(V) in 8 h at 28°C and in 24 h at 15°C, respectively (Fig. 1A), while non-induced resting  
16 335 cells completely oxidize it in 24 h at 28°C and in 32 h at 15°C, respectively (Fig. 1B). This  
17 336 data indicated that, although the process was slower, at 15°C uninduced resting cells of strain  
18 337 2WW were able to oxidize completely an amount of As(III) comparable to that present in the  
19 338 most contaminated groundwater sample A.

20 339 In order to envisage the use of a bacterial strain in the As(III) oxidation step for the As  
21 340 removal process, *Aliihoeflea* sp. strain 2WW was chosen as candidate in a combined bio-  
22 341 oxidation and adsorption experiment.  
23 342

### 24 343 ***3.4 Arsenic Adsorption by Combined Bio-oxidation and Goethite System***

25 344 Goethite was chosen as a model sorbent because of its high affinity for As(V) [43], its low  
26 345 cost and wide availability. Arsenic adsorption capability of goethite is reported in Table 3.  
27 346 As(V) had a higher affinity than As(III) for goethite over the studied concentration range, and  
28 347 it resulted almost totally adsorbed up to a concentration of 200µg/L in the tested conditions.  
29 348 At the same As(III) concentration, close to the highest retrieved in water samples, 75%  
30 349 As(III) was adsorbed, thus leaving in solution As exceeding the 10 µg/L threshold.  
31 350 The combination of bio-oxidation of As(III) to As(V) and adsorption to goethite was then  
32 351 tested in order to improve the As removal efficiency. Similarly to the explorative trial,  
33 352 goethite was able to remove 85% As(III) and a negligible chemo-oxidation of As(III)

1 353 occurred in the abiotic control (Fig. 2). In biotic control *Aliihoeflea* sp. strain 2WW converted  
2  
3 354 completely As(III) to As(V). In the combined bio-oxidation and adsorption system, the  
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5 355 removal of As was higher than 95%. Total soluble As decreased to 8 µg/L, thus meeting the  
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7 356 WHO threshold limit. At the end of the experiment, As(V) was the only As form in solution,  
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9 357 indicating that the ability of strain 2WW to oxidize As(III) was not affected by the presence  
10 358 of goethite.

#### 11 12 359 13 14 360 **4. DISCUSSION**

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18 362 The south-east of Lombardia region has many sites with naturally occurring As levels higher  
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20 363 than the maximum threshold indicated by the WHO for drinking water (10 µg/L);  
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22 364 consequently, the widespread groundwater pollution affects the water of public captation  
23  
24 365 wells of this area.

25 366 Arsenic groundwater contamination may occur under both reducing and oxidizing conditions,  
26  
27 367 and the ratio As(III)/As(V) can vary significantly, depending on the condition of *in situ*  
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29 368 oxidation state of water [44]. It has been reported that the depth distribution of groundwater  
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31 369 within the aquifer varies seasonally creating a dynamic nature of the water in terms of oxic-  
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33 370 anoxic states [45]. Moreover, after digging wells, certain oxidation condition could be  
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35 371 established through transportation of aerobic groundwater and diffusion of oxygen through  
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37 372 vadose zone [11]. These dynamic conditions could stimulate bacterial population with both  
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39 373 As(V)-reducing and As(III)-oxidizing abilities. Aerobic enrichment cultures of As(V)-  
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41 374 reducing and As(III)-oxidizing bacteria established with the groundwater under study gave  
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43 375 evidence of the presence of bacterial populations involved in the As cycle. Bacterial strains  
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45 376 able to convert As by redox reactions were investigated and retrieved in groundwater of  
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47 377 Lombardia region for the first time, evidencing that As-resistant bacteria appear to be widely  
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49 378 distributed in natural environments with different level of contamination, in accordance with  
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51 379 previous literature [9,10,46]. The isolated bacteria belonged to the genera *Pseudomonas*,  
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53 380 *Achromobacter* and *Rhodococcus*, broadly represented among As-resistant bacterial strains  
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55 381 isolated from As-contaminated groundwater [9,10,46]. Interestingly, As(III) oxidising  
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57 382 *Aliihoeflea* sp. strain 2WW was described for the first time to be part of As cycling in As  
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59 383 contaminated environment [47]. Genome analysis of *Aliihoeflea* sp. strain 2WW confirmed  
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61 384 these abilities [48].  
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1 385 Most of the isolates of the present study were positive to As genetic markers and they differed  
2 386 with respect to the gene they carried. There was a good agreement between the presence of  
3 387 the As genes and As transforming capabilities of the isolates, with exception of *Rhodococcus*  
4 388 species, that resulted negative for all the As genes investigated; this might be indicative of  
5 389 low sequence homology with the primers set used. In accordance with previous results  
6 390 [10,49], these data corroborate the hypothesis that the *ars* operon could be slightly different  
7 391 even though the strains are categorized into the same genera, supporting the hypothesis of an  
8 392 horizontal transfer of the As genes within bacterial populations.

9 393 The fundamental understanding of the biochemistry and metabolic pathways involved in As  
10 394 resistance can be translated into strategies for “engineering” bacteria for effective As  
11 395 remediation. Active treatments for As removal from water benefit from the knowledge of As  
12 396 bacterial metabolism and in general are based on natural consortia, pure cultures of As  
13 397 resistant bacteria or Fe- and Mn-oxidizing bacteria that can transform and/or capture As forms  
14 398 indirectly [31].

15 399 Over the last ten years, As(III)-oxidizing bacteria were used as living and resting cells for  
16 400 biological decontamination of synthetic and natural contaminated waters [21,23,24,33,50].  
17 401 Systems based on living and resting cells offer usually high efficiency and the possibility of  
18 402 recovering the heavy metals, but the first requires higher amounts of maintenance and  
19 403 operational funding [5]. In fact, in these reactors As immobilizing/transforming microbial  
20 404 cultures have to be maintained under controlled conditions of nutrients and electron donors  
21 405 and acceptors. Non-living biomass (as resting cells) appears to present more advantages in  
22 406 comparison to the use of living microorganisms, since cells do not require nutrient supply and  
23 407 are not subjected to metal toxicity and to environmental conditions such as pH, temperature of  
24 408 the water to be treated [51]. In the case of a low-temperature treatment (such as groundwater  
25 409 with mean temperature of 10°C-15°C), the step corresponding to biological As(III) oxidation  
26 410 should be carried out at temperature lower than the bacterial optimal growth (i.e. 28°C).  
27 411 Indeed, from an economical point of view it is not possible to heat bioreactors (i.e. at 28°C)  
28 412 and maintain them at the optimal growth temperature of the selected bacteria. For these  
29 413 reasons, the use of bacteria capable to oxidize As(III) as resting cells can be advantageous to  
30 414 overcome these aspects. Strain 2WW possesses As(III) oxidizing capability either as living  
31 415 [47] and as induced and non-induced resting cells, at pH and temperature similar to those of  
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1 416 the groundwater under study (pH 7.2, 15°C), making the strain suitable for exploitation in the  
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3 417 biological step of a water treatment process.

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5 418 After the biological oxidation of As(III), it is necessary to remove the produced As(V) by  
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7 419 using sorbents. Commonly used sorbents or surface coated sorbents are based on iron  
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9 420 compounds [25] and they are considered highly efficient in binding As (>95%). Among iron  
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11 421 based sorbents, goethite was chosen as a model because it is wide available, more effective  
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13 422 and economic. Effectiveness of goethite in adsorbing As species was evaluated in Tris-HCl  
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15 423 solution at different As concentrations, in order to exclude competition between oxyanions  
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17 424 (organic or inorganic ligands such as phosphate) and As(V) for sorption sites, as postulated by  
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19 425 Lievremont et al. [52] and to maintain stable pH conditions. Our results showed that goethite  
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21 426 was 100-fold higher efficient in removing As(V) than As(III) at an initial As concentration of  
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23 427 200 µg/L, decreasing As(V) to 3 µg/L and As(III) to 43 µg/L. These findings are consistent  
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25 428 with results from previous studies indicating higher sorption rate of As(V) than As(III) to  
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27 429 goethite at sub-acidic pH [53]. The As(V) sorption capacity of goethite was substantially  
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29 430 unchanged whatever the initial concentration of As, in accordance with results of a  
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31 431 comparative study on As(V) adsorption by goethite and hematite conducted at different initial  
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33 432 As(V) concentrations (70 µg/L and 500 µg/L) [43]. Our results evidenced that As(III) sorption  
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35 433 capacity of goethite did not vary up to an As(III) initial concentration of 200 µg/L, whereas at  
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37 434 concentration higher than 400 µg/L, sorption profile depended on the initial As(III)  
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39 435 concentration. The higher affinity of goethite for As(V) than As(III) justified the exploration  
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41 436 of the use of the As(III) oxidizing bacterium 2WW strain to improve the As removal  
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43 437 efficiency of goethite.

44  
45 438 The present study evidenced that the combined *Aliihoeflea* sp. strain 2WW-goethite system  
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47 439 improved the As removal efficiency. As(III) oxidation activity of strain 2WW was not  
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49 440 affected by the presence of goethite, and cells did not affected As(V) retention by goethite.  
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51 441 Our findings are in accordance with Kim and co-authors who investigated the effect of  
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53 442 different bacteria (*Enterococcus faecalis*, *Escherichia coli*, and *Bacillus subtilis*) on As  
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55 443 removal by iron-impregnated granular activated carbon in 1 mg/L As(III)/As(V) spiked  
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57 444 solutions [54]. The authors demonstrated that hindrance effects of bacteria on As adsorption  
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59 445 to the surfaces of granules were minimal. Conversely, Huang et al. demonstrated the  
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61 446 competition between *Shewanella putrefaciens* and As(V) sorbed to goethite and ferrihydrite  
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63 447 [55]. The experiments conducted under controlled conditions of pH (7.2), temperature (15°C),  
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1 448 absence of competing ions (Tris-HCl solution) allowed us to conclude that As(III) bio-  
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3 449 oxidation coupled to As adsorption of goethite was efficient in removing more than 95% of  
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5 450 200 µg/L As(III) from solution, suggesting that this process can be successful in lowering As  
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7 451 levels under the threshold limit of 10 µg/L As for drinking water. These findings are in  
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9 452 agreement with recent studies on biological As(III) oxidation combined to removal of As(V)  
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11 453 onto various sorbents (i.e. zero-valent iron, activated alumina and goethite) in model systems  
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13 454 [32-56]. Ike et al. showed that efficiency of activated alumina of removing 75 mg/L As(III)  
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15 455 As from a basal salt medium was highly enhanced by microbial As(III) oxidation [32].  
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17 456 Similarly, Wan et al. set up reactors for biological As(III) oxidation step performed by  
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19 457 *Thiomans arsenivorans* and subsequent As(V) adsorption onto zero-valent iron; the authors  
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21 458 demonstrated the very high As removal capacity of the combined process from highly  
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23 459 concentrated As solution (10 mg/L) [56].  
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25 460 Our findings together with previous studies suggested the potential application of the  
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27 461 combined systems (As(III)-oxidizing bacteria and sorbents) in As removal process from  
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29 462 water. Nevertheless, from an operational point of view, it has to be taken into account a  
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31 463 possible decrease in As removal efficiency when the combined system 2WW-goethite would  
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33 464 be utilized in natural groundwater due to the presence of oxyanions such as phosphate, silicate  
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35 465 and carbonate that can compete with As(V) for sorption sites onto goethite [57-59].  
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## 36 467 **5. CONCLUSIONS**

37 468 Arsenic polluted groundwaters of Lombardia are reservoirs of bacterial populations able to  
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39 469 transform As, and they may contribute to mobilization or immobilization of the metalloid in  
40  
41 470 those environments. Investigating microbial As transformations under laboratory conditions  
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43 471 may facilitate the development of desirable strategies to manage groundwater resources for  
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45 472 supplying safe drinking water to the people of affected area. The results from this study  
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47 473 indicate that the combination of bio-oxidation with goethite is a promising approach for  
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49 474 arsenic removal from contaminated groundwater, although in a model system at the present  
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51 475 stage. It would be desirable to consider different aspects. The suitable bacterial strain should  
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53 476 be able to oxidize As(III) to As(V) in uninduced resting cell condition at the same pH and  
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55 477 temperature naturally occurring in the groundwater. This approach would help to decrease  
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57 478 operational costs and to avoid the addition of carbon sources and of As in the biomass  
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59 479 production process. Moreover, arsenic concentration and forms present in water have great  
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1 480 impact on the adsorption efficiency of goethite. Further experiments conducted with natural  
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3 481 groundwater samples are under way and they will help to elucidate the role of different  
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5 482 environmental factors and of coexisting ions in affecting the performance of the system.  
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## 18 489

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**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 <sub>3</sub> )	Dissolved components (µg/L)									
							Organic C	S-SO <sub>4</sub>	P-PO <sub>4</sub>	N-NO <sub>3</sub>	N-NH <sub>4</sub>	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



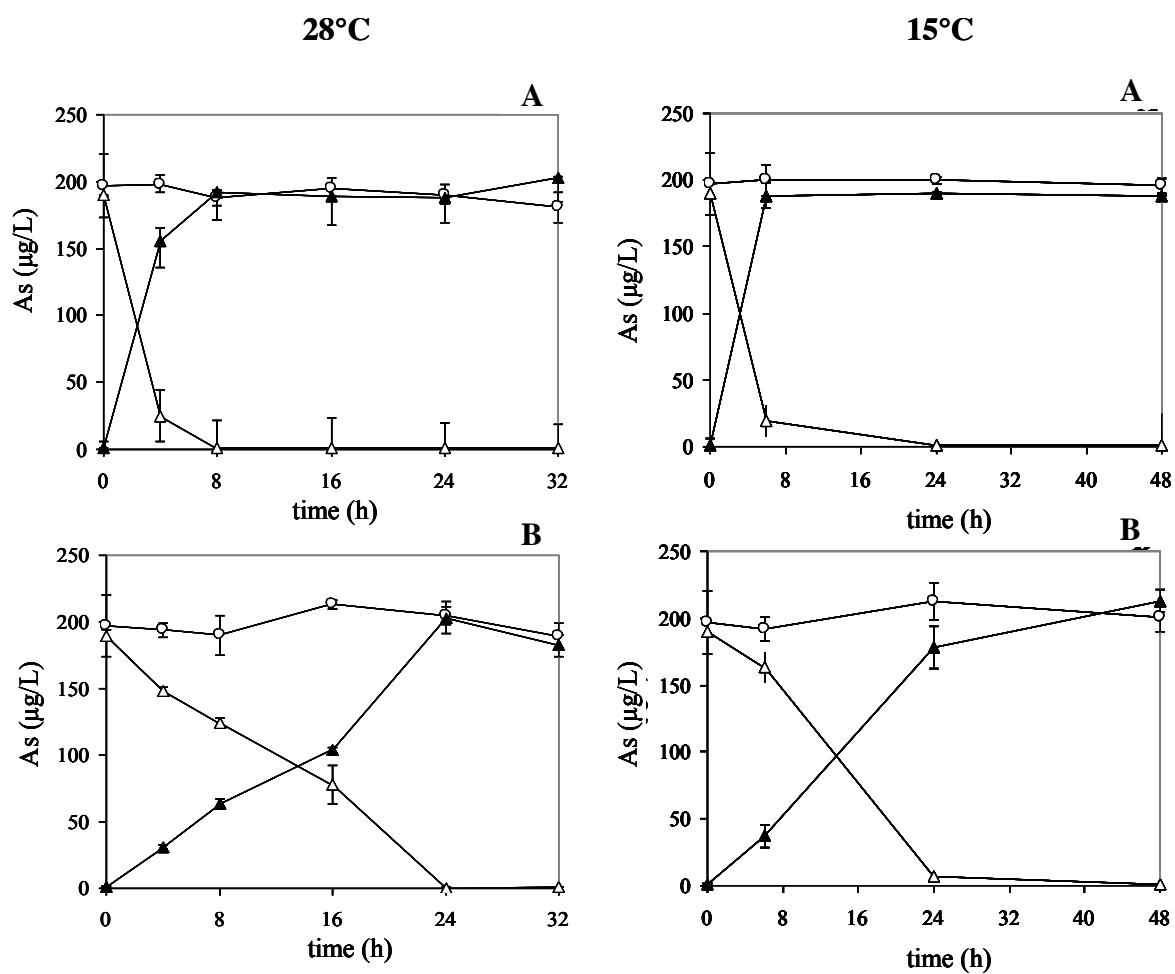
**Table 2** Arsenic transformation abilities and arsenic-resistance genes of bacterial strains isolated from the studied waters

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

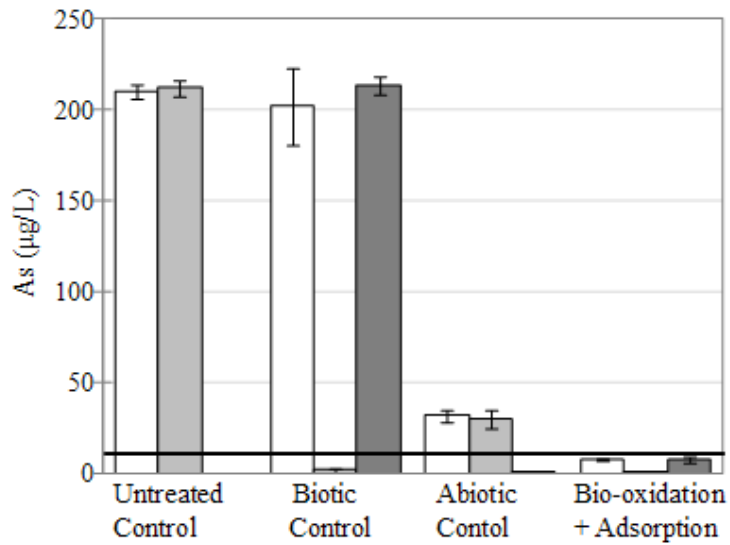
\*, numbers in brackets are referred to the abundance of isolates affiliated to the same species: <sup>a</sup>, complete transformation of 75 mg/L arsenic in 48h

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

Soluble arsenic ( $\mu\text{g/L}$ )			
As(III)		As(V)	
<i>0 h</i>	<i>24 h</i>	<i>0 h</i>	<i>24 h</i>
0	0.3 $\pm$ 0.3	0	0.3 $\pm$ 0.3
25	5.1 $\pm$ 0.9	25	0.2 $\pm$ 0.1
50	16.1 $\pm$ 6.6	50	0.2 $\pm$ 0.1
100	33.6 $\pm$ 3.4	100	0.3 $\pm$ 0.2
200	42.6 $\pm$ 4.6	200	3.0 $\pm$ 1.4
400	203 $\pm$ 26	400	15.9 $\pm$ 4.6
800	412 $\pm$ 15	800	75.2 $\pm$ 20



**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  $\pm$  standard deviation,  $n=3$ );  $\circ$ , Total arsenic;  $\triangle$ , As(III);  $\blacktriangle$ , 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.