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# Impact of glucose on microbial community of a soil containing pyrite cinders: Role of bacteria in arsenic mobilization under submerged condition

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ABSTRACT

Arsenic transformation and mobilization in a pyrite cinder-polluted soil were studied under submerged conditions both in the presence and absence of glucose. The presence of the carbon source enhanced bacterial activity and a reduction in the redox potential, resulting in release of higher amounts of arsenic iron and manganese in the aqueous phase. Since arsenic solubilization was not concomitant to that of iron, desorption rather than dissolution was found to be the main mechanism controlling its release from pyrite cinders. Arsenate was reduced to arsenite whose presence increased during the time course of the experiment. Denaturing gradient gel electrophoresis analysis of 16S rRNA genes of the total bacterial community revealed that the addition of glucose stimulated uncultivable populations of *Flavobacterium* and *Paenibacillus*. The isolation technique enabled the characterisation of nineteen arsenic-resistant bacteria, mostly related to the facultative aerobic genera *Bacillus, Paenibacillus, Staphylococcus* and to *Rhodococcus* and *Micromonospora*. Most of them contained putative arsenate reductase and/or arsenite efflux pump as indicated by the presence of *ArsC* and/or *ArsB* genes. Four strains showed the ability to reduce arsenate by an intracellular detoxification mechanism, and one strain was able to oxidize arsenite, indicating that bacteria with the ability to oxidize or reduce arsenic are ubiquitous in soils. The findings confirm that bacterial activity was responsible for the arsenic reduction causing the solubilization of the metalloid from pyrite cinders to aqueous phases. Reducing conditions, such as those present in flooded soils in the presence of readily utilizable carbon sources could induce arsenic mobilization.

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## 1. Introduction

Arsenic (As) is present in high concentrations in soils due to natural- and anthropogenic processes and creates serious environmental concerns throughout the world. Arsenite As(III) and arsenate As(V) are the two inorganic forms mainly present in soils. The former is generally found under reducing environments while the latter predominates under well-oxidized conditions (Ackermann et al., 2008). However, due to slow redox transformations, both As(V) and As(III) are found in either environment. As(III) is less retained by soil colloids (Sadiq, 1997; Bissen and Frimmel, 2003), while As(V) is preferentially adsorbed on positively charged surfaces like the (hydro)oxides of Fe, Mn, and Al (Adriano, 2001; Fitz and Wenzel, 2002), the adsorption affinity being greater at low pH (Yang et al., 2002).

The bioavailability and mobility of As are governed by many physico-chemical and biological factors. Among factors affecting the equilibrium of precipitation and sorption reactions in nature, those playing determining roles are: parent mineral form and constituents of minerals, pH, redox potential, dissolved organic carbon (DOC), competing ions, interactions with Fe and Mn oxides, and chemical speciation (Adriano, 2001; Al-Abed et al., 2007; Martin et al., 2007; Islam et al., 2004, Bauer and Blodau, 2006).

However, microorganisms too play a significant role in speciation and geochemical behaviour of As (Macur et al., 2001; Routh et al., 2007; Bachate et al., 2009). Microbial reduction of As(V) can occur through a reductive intracellular mechanism of detoxification (Oremland and Stolz, 2003; Macur et al., 2004) or a dissimilatory reduction (respiration) (Oremland and Stolz, 2005). At first it acts on the *ars* operon which converts As(V) to As(III) that is pumped out of the cell, permitting the cells to survive in a highly As-contaminated environment without gaining energy. On the other hand, dissimilatory As(V) reduction usually occurs under anoxic conditions and microorganisms gain energy for growth by coupling As(V) reduction to oxidation of organic matter. Microbial oxidation

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of As(III) is carried out by heterotrophic (Gihring et al., 2001; Ehrlich, 2002) and chemoautotrophic bacteria (Santini et al., 2000; Oremland et al., 2002).

While heterotrophic As(III) oxidation is generally considered a detoxification reaction, chemoautotrophic oxidation provides energy and reducing power for $CO_2$ fixation and cell growth under aerobic (Santini et al., 2000) and nitrate-reducing (Oremland et al., 2002; Macur et al., 2004) conditions.

Whereas studies on the transport and outcome of As in terms of abiotic geochemistry are numerous (Chatain et al., 2005; Al-Abed et al., 2007; Signes-Pastor et al., 2007; Ackermann et al., 2008; Ascar et al., 2008; Noubactep et al., 2008; Phuong et al., 2008), those examining the effect of microbial processes on As behaviour in soil containing mineral processing wastes, for example of pyrite cinders, are few (Macur et al., 2001, 2004).

The research presented in this paper focuses on a soil polluted by pyrite cinders, which are a by-product of sulphuric acid manufacturing operations using pyrite ores. Because these wastes were buried under a layer of loamy sand soil, the potential release of As from the cinders poses an environmental hazard.

The purpose of this work was to study the temporal dynamics of the bacterial community and the effect of its activity on soil redox potential and on As and Fe solubilization in a submerged experimental system spiked with glucose.

The specific objectives of this study were to: (i) investigate the influence of the added carbon source on bacterial As speciation and solubilization; (ii) identify microbial populations associated with the As transformations occurring in soil microcosms using cultivation-independent 16S rDNA sequence analysis; (iii) isolate As(V)-reducing- and As(III)-oxidizing aerobic heterotrophic bacteria from soil microcosms.

## 2. Materials and methods

### *2.1. Site description and soil sampling*

The polluted area lies in the premises of a chemical factory that produced primary base- and fine-chemicals, located in the North-East of Italy (Torviscosa, Udine) and together with the surroundings is included in the national priority list of polluted sites (Decreto Ministeriale 468/2001, 2002). The soil of the site is contaminated by pyrite cinders, a by-product of sulphuric acid manufacturing operations (800 °C roasting temperature). The cinders were deposited into the soil for about 40 years until the late 1970s. In the site, a horizon of pyrite cinders of about 1 m depth was covered by a layer of 0.2 m of carry-over, gravelly soil. In 2005, an experimental field was prepared in this area for phytoextraction experiments (Marchiol et al., 2007) by removing the covering soil of the field and mixing it with cinders in the same proportion (v/v). After three years had passed, a soil sample from the edges of the field as well as samples of the starting materials (pyrite cinders and covering soil) were collected, stored in sterile polyethylene bags, and transported to the laboratory. First, the soil was sieved with 2 mm mesh size after which one part was air-dried for chemical analysis and another part was stored at 4 °C for microbiological analysis and for set up of the microcosms.

### *2.2. Set up of microcosms*

To examine the effect of the bacterial activity on As and Fe mobilization from the soil polluted with pyrite cinders, 50 g of soil were mixed with 50 g of water in 100 ml tubes; some of these tubes were then amended with glucose (0.2%, w/w) as carbon source and the rest were left unamended. 7.83 mg of $Ca(NO_3)_2$ was added to adjust the C/N ratio of glucose-amended microcosms to 30. The same amount of $Ca(NO_3)_2$ was also added to the unamended microcosms. Control soils were prepared by adding formaldehyde (0.04%, w/v) in the sample tubes to inhibit the microbial activity (Tuominen et al., 1994). The tubes were closed with cotton plugs and incubated statically at 30 °C. Three sacrificial replicates of control, 0, and 0.2% glucose thesis were prepared for each sampling time. Time courses of pH, redox potential, total As, As(V), As(III), Fe contents in the aqueous phase were determined at successive incubation days. Soluble Mn content was checked on glucose-amended and unamended microcosms to evaluate the potential role of Mn oxides on As and Fe transformation and sorption. The counts of aerobic and anaerobic heterotrophic bacteria as well as total DNA extraction were also carried out.

### *2.3. Chemical and microbiological determinations*

Polluted soil was chemically and physically characterized in accordance with MIPAF Official Methods (2000). $pH_W$ was measured in a soil suspension with a solid:water ratio of 1:2.5 (w/v). Texture was defined with sand (50–2000 μm), silt (2–50 μm), and clay (<2 μm) fractions. Exchangeable cations were extracted with a barium chloride-triethanolamine solution at pH 8.1. To determine the total content of heavy metals and As, the samples were $HNO_3$/HCl-digested in a microwave oven (CEM, MARS5); the slowly labile fraction of heavy metals was determined by diethylene triaminepentaacetate (DTPA) extraction. Water soluble fraction of As was determined with a solid:water ratio of 1:1 (w/v). To perform As fractionation of the soil, a five-step sequential extraction procedure described by Wenzel et al. (2001) was applied. This technique graded the occurrences of As as non-specifically sorbed, specifically-sorbed, associated with amorphous and poorly-crystalline (hydro)oxides of Fe and Al, associated with well-crystallized hydrous oxides of Fe and Al, and residual phases.

Redox potential and pH in microcosms were measured in the upper aqueous phase of the tubes, retaining the electrodes until the measurements were stabilized. The tubes were successively shaken for 10 min and then centrifuged at 6000 rev $min^{-1}$ for 15 min. The supernatant was collected, filtered (0.45 μm), and stored at −20 °C until the determination was completed. As, Fe and Mn contents in the solution were determined in 10 ml of supernatant after acidification with HCl to pH 2.0. As(III) and As(V) contents were determined in 10 ml of supernatant passed through a WATERS Sep-Pak® Plus Acell Plus QMA cartridge (Waters, MA). 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 (Kim et al., 2007).

Exchangeable cations, heavy metals, Fe, Mn and As contents were determined by ICP-MS (Varian Inc.). Standards of As for concentrations ranging from 0 to 1 mg $l^{-1}$ were prepared from sodium arsenite ($NaAsO_2$) (Sigma–Aldrich) solution.

The number of heterotrophic bacteria in the polluted soil was determined using conventional plating techniques. Triplicate samples of soil (3 g) were suspended in 27 ml of sodium pyrophosphate solution (0.2%, w/v), shaken in a rotary shaker at 180 rev $min^{-1}$ for 1 h and left to rest for 10 min. Aliquots (1 ml) of the soil suspensions were serially diluted 10-fold in a physiological solution (0.9% NaCl) after which 1 ml was plated on 1:10 diluted Tryptic Soy Agar (TSA/10) in double sets of plates supplemented with sterile cycloheximide (1% w/v) to inhibit fungal growth. The incubation period was 10 days at 30 °C. Similarly, the number of As-resistant bacteria was determined on TSA/10 medium supplemented with 10 mmol $l^{-1}$ of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$, Sigma–Aldrich) or 3 mmol $l^{-1}$ $NaAsO_2$. The As-resistant heterotrophic bacteria detected were expressed as percentage of growth on TSA/10 without As addition.

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