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Microbial arsenic cycling in Italian rice paddies:

An ecological perspective

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Abstract

Arsenic (As) contamination of rice is an issue of global concern. Italy, although representing the European leader of rice production, is one of the countries mostly affected by As contamination of rice grain. Rice is mainly cultivated under continuous flooding, with the rapid depletion of oxygen in the soil. At the consequent highly reduced redox potentials, As is released into the porewater by the dissolution of iron-arsenic (Fe-As) minerals, and by the reduction of arsenate [As(V)] to arsenite [As(III)], a soluble compound that is rapidly taken up by the plants. In the presence of sulfide, As(III) co-precipitate with the formation of As_nS_n minerals. Microorganisms are known to actively oxidize and reduce As, as well as to convert inorganic to organic As via methylation. Furthermore, microorganisms that use Fe or sulfur for their metabolic activities indirectly influence As biogeochemistry in the environment.

In this study, the role of two different practices, suggested to reduce As contamination in rice fields, in shaping rice rhizospheric microbial communities were investigated. Specifically, changes in the water management and use of sulfate (SO_4^{2-}) as fertilizer were tested.

To analyze the influence of the water regime in rice rhizosphere microbiota, a semi-field experiment was set up. Plants were grown in rice field soil from Pavia (containing 18 mg kg^{-1} of As) in box plots managed with three water regimes: continuous flooding, continuous flooding with 2 weeks of drainage before flowering, and watering after complete soil drying ("aerobic rice"). In rhizosphere soil and in rhizoplane, *aioA*, *arsC*, *arsM* and *arrA* genes, encoding for different types of As transformation, as well as 16S rRNA genes belonging to dissimilatory Fe-reducing bacteria (DFeRB) and Fe-oxidizing bacteria (FeOB), were amplified and quantified with Real Time quantitative PCR (RT-qPCR). To analyze the whole active bacterial community, RNA was reverse-transcribed and 16S rRNA was amplified and sequenced by 454-pyrosequencing. The presence of DFeRB and FeOB was also highlighted in rhizoplane samples from plants at flowering stage with Fluorescence *In Situ* Hybridization (FISH). Furthermore, enrichment cultures of FeOB from roots cultivated under continuous flooding and from aerobic rice were set up on Fe(II) gradient tubes and exposed to either As(V) or As(III). Bacterial growth and related Fe(III) oxides were analyzed with Scanning Electron Microscopy (SEM) combined with Energy Dispersive X-ray Spectrometry (EDS), and used for 16S rRNA gene clone library preparation.

To test the effect of SO_4^{2-} amendment on As dissolution into the porewater, a greenhouse experiment was set up with rice plants grown in single pots on rice field soil from Carpiano (MI) (containing 30 mg kg^{-1} of As). Different pots with and without plants and with and without 0.13 % (w/w) calcium sulfate ($CaSO_4$) amendment were installed. Microbial As genes were quantified with RT-qPCR in

bulk and rhizosphere soil. In a similar experiment performed using rice field soil from Vercelli, the genome belonging to a new putative SO_4^{2-}-reducing species of the *Nitrospirae* phylum was isolated from a metagenomic library by differential genome binning. The phylogenetic affiliation of this species as well as its metabolic features were characterized by the analysis of specific marker genes and expressed proteins.

In continuous flooding, active DFeRB, As(V)-reducing and sulfur-oxidizing bacteria were stimulated, potentially contributing to the release of As into the porewater. The RT-qPCR quantification confirmed that DFeRB belonging to the genus *Geobacter* significantly increased when rice was cultivated under continuous flooding, in concomitance with a significant increase of As in the porewater over time. This supported the hypothesis that *Geobacter*, by dissolving Fe(III) minerals, promoted As solubilization. In aerobic rice, genera able to oxidize Fe(II) and/or As(III) were selected. Quantification with RT-PCR confirmed that *aioA* genes, encoding for As(III)-oxidase, were among the most abundant As genes, increasing when drainage was applied before flowering and in aerobic rice. In Fe(II) gradient tubes, As(V) promoted the enrichment of the nitrate-reducing FeOB genus *Azospira* from roots developed under continuous flooding, whereas As(III) addition inhibited the growth of FeOB. The SEM-EDS analysis revealed the presence of microorganisms covered by putative Fe encrustation as well as As-Fe oxides crystals. FISH analysis on rice rhizoplane confirmed the presence of FeRB belonging to the family *Geobacteraceae* and of both microaerophilic and nitrate-reducing FeOB, respectively belonging to the family *Gallonellaceae* and to the genus *Thiobacillus.*

The addition of SO_4^{2-} to rice field soil led, on the one hand, to a lower As release into the porewater, on the other hand, to a lower translocation of the metalloid in the plants. The bulk and rhizosphere soil bacterial community was enhanced by the addition of SO_4^{2-}, but the abundance of genes involved in As transformation did not change significantly. The analysis of a genome retrieved in a metagenomic library prepared on rice bulk and rhizosphere soil from a similar SO_4^{2-}-addition experiment, revealed the presence of a novel species belonging to the *Nitrospirae* phylum in Vercelli rice field soils. These microorganisms carry the whole genetic background for dissimilatory reduction of SO_4^{2-} and nitrate. Through the Wood-Ljungdahl pathway, they likely use acetate as electron donor. Amendment of SO_4^{2-} in the soil promoted the expression of SO_4^{2-} respiration, whereas in the control treatments genes for nitrate respiration were expressed.

These outcomes confirm and elucidate the role of the microbial community living in the rhizosphere of rice plants in decreasing As solubility when changes on the water regime are applied. Future research should be focused the possible role of endophytic bacteria on the decrease of As translocation when is rice plants are fertilized with SO_4^{2-}.

Riassunto

La contaminazione del riso da parte dell'arsenico (As) è un problema di importanza globale. L'Italia, nonostante sia il leader europeo nella produzione di riso, è tra i paesi più colpiti dalla contaminazione di As. Il riso è coltivato prevalentemente in condizioni di sommersione continua, con il rapido esaurimento di ossigeno nel suolo. Ai conseguenti altamente ridotti potenziali redox, l'As è rilasciato nella soluzione circolante dalla dissoluzione di minerali di ferro e arsenico (Fe-As) e dalla riduzione di arsenato [As(V)] ad arsenito [As(III)], un composto solubile che è rapidamente assorbito dalle piante. In presenza di sulfuro, l'As(III) co-precipitata con la formazione di minerali As_nS_n. I microrganismi possono attivamente ossidare e ridurre l'As, e convertire l'As inorganico a organico attraverso la metilazione. Inoltre, i microrganismi che usano Fe o zolfo per le loro attività metaboliche influenzano indirettamente la biochimica dell'As nell'ambiente.

In questo lavoro è stato studiato il ruolo di due diverse pratiche suggerite per ridurre la contaminazione da As nelle risaie nella costituzione delle comunità microbiche rizosferiche del riso. In particolare, sono stati testati cambiamenti del regime idrico e l'utilizzo di solfato (SO_4^{2-}) come fertilizzante.

Per analizzare l'influenza del regime idrico sulle popolazioni microbiche rizosferiche, piante di riso sono state fatte crescere in suolo di risaia di Pavia (contenente 18 mg kg^{-1} di As) in vasconi condotti secondo tre regimi idrici: sommersione continua, sommersione continua con 2 settimane di asciutta prima della fioritura e bagnature fino a completa asciugatura del suolo ("aerobic rice"). Nel suolo rizosferico e nel rizoplano sono stati amplificati e quantificati i geni *aioA*, *arsC*, *arsM* e *arrA*, codificanti per diversi tipi di trasformazione dell'As, e i geni per l'rRNA 16S appartenente a batteri Fe-respiranti (DFeRB) e Fe-ossidanti (FeOB), attraverso Real Time quantitative PCR (RT-qPCR). Per analizzare l'intera comunità batterica attiva, l'RNA è stato retro-trascritto e l'rRNA 16S amplificato e sequenziato attraverso il pirosequenziamento 454. La presenza di DFeRB e di FeOB è stata evidenziata nel rizoplano di piante allo stadio di fioritura attraverso la Fluorescence *In Situ* Hybridization (FISH). Inoltre, sono state preparate colture di arricchimento di FeOB da radici coltivate in sommersione continua e in asciutta in tubi a gradiente di Fe(II) e esposte o ad As(V) o ad As(III). La crescita batterica e gli ossidi di Fe(III) associati sono stati analizzati con la Scanning Electron Microscopy (SEM) combinati con Energy Dispersive X-ray Spectrometry (EDS), e utilizzati per la preparazione di librerie di cloni di geni per l'rRNA 16S rRNA.

Per testare l'effetto dell'aggiunta di SO_4^{2-} nel rilascio di As nella soluzione circolante, è stato effettuato un esperimento in cui piante di riso sono state coltivate in vasi singoli suolo di risaia di Carpiano (MI) (contenente 30 mg kg^{-1} of As). Sono stati previsti diversi vasi con o senza piante e con

o senza aggiunta di solfato di calcio ($CaSO_4$) allo 0.13 % (w/w). I geni microbici per la trasformazione dell'As sono stati quantificati tramite RT-qPCR nei suoli bulk e rizosferico. In una libreria di metagenomi ricavata da un esperimento simile effettuato utilizzando suolo di risaia di Vercelli, è stato isolato il genoma appartenente ad una nuova presunta specie SO_4^{2-}-respirante del phylum *Nitrospirae*. L'affiliazione filogenetica di questa specie e le sue caratteristiche metaboliche sono state caratterizzate attraverso l'analisi di specifici geni marker e proteine espresse.

La sommersione continua ha stimolato batteri DFeRB, As(V)-riduttori e zolfo-ossidanti attivi, contribuendo potenzialmente al rilascio di As nella soluzione circolante. La quantificazione RT-qPCR ha confermato un aumento significativo di DFeRB appartenenti al genere *Geobacter* quando il riso è stato coltivato in sommersione continua, in concomitanza con un aumento significativo di As nella soluzione circolante. Questo supporta l'ipotesi che *Geobacter*, solubilizzando i minerali di Fe(III), abbia promosso il rilascio di As. Nell'aerobic rice, sono stati selezionati generi in grado di ossidare Fe(II) e/o As(III). La quantificazione con RT-PCR ha confermato che i geni *aioA*, codificanti per l'As(III)-ossidasi, erano tra i più abbondanti geni per l'As, aumentando quando era stata applicata un'asciutta prima della fioritura e nell'aerobic rice. Nei tubi a gradiente di Fe(II), l'As(V) ha promosso l'arricchimento del genere FeOB nitrato-riduttore *Azospira* da radici sviluppate in sommersione continua, mentre l'As(III) ha inibito la crescita di FeOB. L'analisi SEM-EDS ha mostrato la presenza di microrganismi ricoperti da ipotetiche incrostazioni di Fe e di cristalli di ossidi di Fe-As. L'analisi FISH del rizoplano ha confermato la presenza di FeRB appartenenti alla famiglia *Geobacteraceae* e di FeOB sia microaerofili che nitrato-riduttori, rispettivamente appartenenti alla famiglia *Gallonellaceae* e al genere *Thiobacillus.*

L'aggiunta di SO_4^{2-} al suolo di risaia ha portato, da una parte, ad un ridotto rilascio di As nella soluzione circolante, dall'altra, ad una minore traslocazione del metalloide nelle piante. Le comunità batteriche dei suoli bulk e rizosferico sono state stimolate dall'aggiunta di SO_4^{2-}, ma l'abbondanza dei geni coinvolti nella trasformazione dell'As non è cambiata significativamente. L'analisi di un genoma isolato in una libreria di metagenomi costruita da suoli bulk e rizosferico in un esperimento con aggiunta di SO_4^{2-} simile, ha rivelato la presenza di una nuova specie appartenente al phylum delle *Nitrospirae* in suolo di risaia di Vercelli. Questi microrganismi possiedono l'intero corredo genetico per la riduzione dissimilativa di SO_4^{2-} e nitrato. Verosimilmente utilizzano l'acetato come donatore di elettroni attraverso la via di Wood-Ljungdahl. L'aggiunta di SO_4^{2-} ha promosso l'espressione della respirazione del SO_4^{2-}, mentre nei trattamenti di controllo venivano espressi i geni per la respirazione del nitrato.

Questi risultati hanno confermato e chiarito alcuni aspetti del ruolo delle comunità microbiche rizosferiche del riso nella diminuzione della solubilità dell'As quando viene variato il regime idrico.

In futuro, la ricerca dovrebbe focalizzarsi sul possibile ruolo dei microrganismi endofitici nella diminuzione della traslocazione dell'As quando le piante sono fertilizzate con il SO_4^{2-}.

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Introduction

More than half of the world's populations base their nutrition on rice (http://irri.org). However, rice cropping is affected by environmental as well as health issues mainly due to the commonly used agronomic practices. Rice plants originated from aquatic plants, and most varieties are still highly sensitive to water stress (Kögel-Knabner, 2010). As a consequence, to date, rice paddies are the widest artificial wetlands. Continuous flooding leads to biogeochemical reactions in rice field soil that support, on the one hand, huge methane (CH_4) emissions into the atmosphere, on the other hand, great accumulation of arsenic (As) into rice grains (Conrad, 2009).

On January 2016, the European Community established specific limits for As content in rice, in order to avoid the consequences of long-term exposure in the European populations (Commission regulation (EU) 2015/1006). A survey of Meharg and colleagues carried out in 2009 revealed that As concentration in rice grains produced by several European countries often exceeds these limits (Meharg *et al.*, 2009). Since Italy is one of the leading European rice producers, it is extremely important to understand the mechanisms driving As contamination in rice grains, in order to find effective solutions.

Arsenic toxicity and environmental dynamics

Arsenic (As) is among the 20 most abundant elements in the Earth's crust, with average concentrations between 1 and 2 mg kg^{-1} (Lièvremont *et al.*, 2009). It can be released into food-related environments as a consequence of either natural processes or human activities, like volcanism, use of phyto-pharmaceuticals, industrial activities, acid mine drainage and wood treatment substances (Slyemi and Bonnefoy, 2012; Smedley and Kinniburg, 2002; WHO, 2001).

The World Health Organization (WHO) classified As among the ten most harmful substances for human health. Even the exposure to low concentrations of the metalloid for long periods increases the probability to develop cancer, cardiovascular and neurological disease and diabetes (Kapaj *et al.*, 2006; WHO, 2010).

According to the European Food Safety Authority (EFSA), inorganic As is generally more toxic than organic As (EFSA, 2014). However, according to *in vitro* and *in vivo* studies, the actual level of toxicity increases as follows: elemental As (As_0), arsenate [As(V)], arsenite [As(III)], dimethylarsenite [DMAs(III)], and arsine (AsH_3) (Petrick *et al.*, 2000; Mandal and Suzuki, 2002; Dopp *et al.*, 2010). Given their chemical and structural similarity, As(V) substitute phosphate (PO_4^{3-}) in selective membrane carriers as well as in several biological reactions (Csanaky and Gregus, 2001).

On the other hand, As(III) has a higher chemical reactivity and is absorbed by the cells more rapidly than As(V) through aquaglyceroporins (Rosen and Liu, 2009).

In the environment As exists in different oxidation states (-3, 0, +3, +5), often bound to iron (Fe) and sulfur (S) minerals (O'Day, 2006). Arsenic is the main constituent of more than 200 minerals like arsenopyrite (FeAsS), orpiment (As_2S_3) and realgar (As_4S_4). The most abundant inorganic As species in the environment are As(III) and As(V), whereas among the organic forms are monomethylarsinic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsinic acid (TMA). In anoxic environments, As(III) is the predominant species, more soluble, mobile and toxic with respect to As(V), the predominant species in oxic soils (Takahashi *et al*., 2004). Arsenic speciation and mobility depend on pH, redox potential and minerals present in the environment (Zhu *et al.*, 2014; Fitz and Wenzel, 2002). Arsenic in soil vary between 0.1 to 50 mg kg^{-1}, changing across different geographical regions (Mandal and Suzuki, 2002). According to the Italian law (D. Lgs. 152/2006), in soils of public green areas As concentration must be lower than 20 mg kg^{-1} dw, whereas in soils used for commercial and industrial purposes the limit is 50 mg kg^{-1} dw. Strikingly, to date no limits have been established in soils used for agricultural purposes.

Arsenic interactions with soil minerals

In soil, As and Fe biogeochemistry are strictly related (Burton *et al.*, 2008). Arsenate has a high affinity for Fe (hydr)oxides; furthermore, the same minerals oxidize As(III) to As(V), enhancing co-precipitation phenomena (Dixit and Hering, 2003). Anoxic conditions in submerged soils lead to the dissolution of Fe (hydr)oxides, increasing As mobility (Takahashi *et al.*, 2004). Some microorganisms can use Fe as either electron acceptor or electron donor for their energy metabolism. Dissimilatory Fe(III)-reducing bacteria (DFeRB) couple Fe(III) reduction with the oxidation of either organic or inorganic compounds (Lovley *et al.*, 2006). On the other hand, Fe(II) can be used by Fe(II)-oxidizing bacteria (FeOB) as electron donor, coupled with either O_2 or NO_3^- respiration (Dubinina e Sorokina, 2014). Among FeOB, neutrophilic, acidophilic, aerobes as well as anaerobes have evolved (Hedrich *et al.*, 2011). In accordance with above-mentioned consideration on As and Fe affinity, DFeRB are expected to promote As bioavailability in the environment, whereas FeOB are supposed to change As equilibrium on the reverse way (Huang, 2014).

Sulfur is involved in a complex biogeochemical cycle, with oxidation states from -2 to +6. It is one of the most abundant element on Earth, as well as an important component of biological molecules, like amino acids and coenzymes (Muyzer and Stams, 2008). Arsenite has a high affinity with sulfide (S^{2-}), with which it may precipitate to form orpiment (As_2S_3) (Rittle *et al.*, 1995; Newman *et al*., 1997; Keimowitz *et al*., 2007). Sulfur-oxidizing bacteria (SOB) oxidize different sulfur species to

gain energy for chemolithotrophic or phototrophic metabolisms, both aerobically and anaerobically (Friedrich *et al.*, 2005). On the other hand, sulfate (SO_4^{2-}) is used as final electron acceptor in organic matter degradation by dissimilatory sulfate reducing bacteria (DSRB), which concomitantly oxidize short chain fatty acids, and aromatic compounds (Muyzer and Stams, 2008). A high degree of versatility has been observed in some DSRB, which may use alternative electron acceptors like nitrate (NO_3^{2-}), nitrite (NO_2^-), Fe(III), As(V) or fumarate at specific environmental conditions (Rabus *et al.*, 2015). As a consequence of their metabolism, DSRB might promote As(III) co-precipitation with S^-, whereas SOB could potentially lead to As release by S^- mineral dissolution.

Direct microbial processing of arsenic

Microorganisms carry out a wide range of As transformations. Different strategies to avoid cell As intake as well as to resist to As accumulation inside the cells have been evolved (Slyemy and Bonnefoy, 2012). Some microorganisms change their membrane composition, for example, modulating peptidoglycan and lipopolysaccharides synthesis (Andres and Bertin, 2016). Inside the cell, As is often chelated with thiol-containing peptides or proteins (Slyemi and Bonnefoy, 2002). Intracellular sequestration into vacuoles mainly occurs in eukaryotes, like *Saccharomyces cerevisiae*, using specific vacuolar transporters (Slyemy and Bonnefoy, 2012).

The cytoplasmic reduction of As(V), coupled with As(III) extrusion is a detoxification mechanism evolved in several taxonomic groups (Yamamura and Amachi, 2014). The genes encoding for the enzyme As(V) reductase and for the As(III) efflux pump are included in the *ars* operon, which might be located either in the chromosome or in plasmids (Páez-Espino *et al*., 2009). In some microorganisms, the As(III) efflux pump is encoded by *ACR* genes (Cavalca *et al.*, 2013).

The oxidation of As(III) is a detoxification mechanism used by several autotrophic as well as heterotrophic bacterial species (Cavalca *et al.*, 2013). This reaction occurs in the periplasm and is encoded by the chromosomic *aio* operon (Andres and Bertin, 2016). The conversion of As(III) to As(V) decreases metalloid toxicity, promoting its co-precipitation with soil minerals (Dixit and Hering, 2003).

In autotrophic microorganisms, As(III) oxidation provides electrons for O_2, NO_3^- and chlorate (ClO_3^-) respiration, within a chemioautotrophic anaerobic or aerobic metabolism (Sun *et al*., 2010, Cavalca *et al.*, 2013). Previous studies demonstrated that sulfide promotes As(III) oxidation by sulfur-oxidizing microorganisms, indicating the probable co-occurrence of these two metabolism capacities in these microorganisms (Fisher *et al.*, 2008). Chemical As(III) oxidation by O_2 is very slow (Cavalca *et al.*, 2013). For this reason, microbe-mediated As(III) oxidation is more important in determining As fate in the environment.

In Bacteria and Archaea within different ecological niches, As(V) can be used as electron acceptor for respiration (Lloyd and Oremland, 2006). The electron donors usually oxidized within this metabolism can be either inorganic or organic molecules (Stolz *et al*., 2006). The respiration of As(V) is encoded by the *arr* operon (Andres and Bertin, 2016). Dissimilatory As(V)-reduction is thermodynamically favored with respect to Fe(III) and SO_4^{2-} reduction under several environmental conditions (Kocar and Fendorf, 2009).

Eventually, some microorganisms perform As(III) methylation to monomethylarsenite [MMAs(III)], dimethylarsenite [DMAs(III)] and trimethylarsenite [TMAs(III)] with the As(III) methyltransferase, encoded by the *arsM* gene (Bentley and Chasteen, 2002; Andres and Bertin, 2016). This transformation can be catalyzed by both aerobic and anaerobic bacteria (Páez-Espino *et al*., 2009). Organic As species are more volatile with respect to the inorganic ones. Therefore, As(III) methylation allows the removal of As from the system (Qin *et al.*, 2006).

Arsenic in rice paddies

Rice plants are commonly cultivated under complete flooding. However, different agronomic practices have been developed to meet environmental and water use efficiency issues. Among the less water-consuming regimes, intermittent flooding is commonly used in Chinese areas (Somenahally *et al.*, 2011b; Ma *et al.*, 2014), whereas rainfed/sprinkler irrigation has been proposed in Mediterranean areas with water limitation issues (Spanu *et al.*, 1989; Spanu *et al.*, 2009).

Complete flooding in rice paddies lead to anoxic conditions in the soil, with great effects on microbial metabolisms and As biogeochemistry (Liesack *et al.*, 2000; Lu *et al.*, 2006; Das *et al.*, 2016). In the anoxic bulk soil As(III) is massively released into the soil solution as a consequence of Fe minerals dissolution as well as by reduction of As(V) to As(III) (Yamaguchi *et al.*, 2014). On the other hands, in the close surroundings of rice rhizosphere oxygen leakage from root aerenchyma strongly impacts nutrient cycling (Kögel-Knabner, 2010). In these conditions, As(V) is the predominant species and co-precipitates with the so-called "Fe plaques" around the roots, decreasing As bioavailability (Yamaguchi *et al.*, 2014).

Microbe-mediated Fe cycle is of great relevance in rice paddies (Oremland *et al.*, 2005; Kögel-Knabner, 2010; Emerson *et al.*, 2010). Under continuous flooding, Fe-oxidizing bacteria (FeOB) contribute to Fe plaque formation on root surface (Weiss *et al.*, 2003). On the other hand, Fe (hydr)oxides in Fe plaques is used by dissimilatory Fe-reducing bacteria (DFeRB) for respiration (Hansel *et al.*, 2001). Several studies reported that Fe plaques adsorb significant amounts of As, decreasing its uptake in rice plants (Chen *et al.* 2005; Zhao *et al.*, 2009). The activity of DFeRB

might, therefore, contribute to As release into the pore water by promoting Fe (hydr)oxides dissolution (Islam *et al.*, 2004; Zhao *et al.*, 2009).

Similarly, the role of DSRB in nutrient cycling in rice paddies has emerged over the last years (Pester *et al.*, 2012). Furthermore, it has been demonstrated that high concentrations of SO_4^{2-} stimulate Fe plaque formation, with the concomitant decrease of plant As intake (Hu *et al.*, 2007). However, the mechanisms behind this phenomenon remain unclear (Jia *et al.*, 2015). In concomitance to the indirect influence of Fe and S cycling microorganisms, direct oxidative, reductive as well as methylating microbial As processes coexist in rice paddies (Zhang *et al.*, 2015; Jia *et al*., 2014; Zhu *et al.*, 2014; Huang *et al.*, 2012).

There is strong evidence that As uptake by rice plants is determined by the microbial activities occurring in the rhizosphere (Lomax *et al.*, 2012). However, the identity of the microbial species potentially responsible for As biogeochemistry in rice paddies are still poorly understood.

Despite the presence of As-precipitating reactions within rice rhizosphere, under continuous flooding As-releasing processes predominate, with the consequent accumulation of As into the grains (Meharg *et al.*, 2009). This phenomenon is exacerbated in areas highly affected by As contamination. In Bangladesh, for instance, contaminated groundwater is currently used to irrigate rice fields, transferring more than 1000 tons of As in the fields and, therefore, in rice grains (Norra *et al.*, 2005; Bachate *et al.*, 2009). In Italy, several areas designated to rice cultivation are located in the north (Lombardy, Piedmont, Veneto), where bioavailable As is often higher with respect to the southern regions (Cubadda *et al.*, 2010). In rice grains produced in these areas, also mean As content is higher, and it frequently exceeds the law limits (Sommella *et al.*, 2013).

Aim of the PhD project

The aim of this PhD project was to unravel different aspects concerning rice rhizospheric microbial populations involved in As cycle. Specifically, three aims were pursued:

- Test the effect of different water regimes on rice rhizospheric bacterial populations, to see whether changes in As content in rice grains could be attributable to specific microbial metabolic potentials (Chapters 1, 2, 3).
- Verify the decrease of As contamination induced by SO_4^{2-} fertilization and highlight possible connections with changes in As-processing microorganisms (Chapter 4).
- Reconstruct the metabolic profile of a novel putative SO_4^{2-}-reducing *Nitrospirae* species retrieved in an Italian rice field soil (Chapter 5).

1.

Rhizospheric iron and arsenic bacteria affected by water regime: implications for metalloid uptake by rice

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Abstract

Rice is characterized by high levels of arsenic accumulation, even if cultivated in non-contaminated soils. Given the limits for arsenic concentration in rice grain recently established by the European Community, it is essential to understand the mechanisms and find solutions to this issue. Arsenic bioavailability is strictly related to water management of the rice paddy as well as to iron- and arsenic-cycling bacterial populations inhabiting the rice rhizosphere.

To evaluate the effect of different agronomic conditions on the root-soil microbiota involved in arsenic mobilization, rice plants were grown in macrocosms containing non-contaminated field soil under either continuous flooding, aerobic rice regime or continuous flooding with a 14 day-period of drainage before flowering. Specific groups of iron- and arsenic-cycling bacteria were assessed by real time quantitative PCR and fluorescence *in situ* hybridization.

Continuous flooding led to the release of arsenate and iron in soil solution and produced rice grains with arsenite and organic arsenic above the recently established limits, contrary to the other agronomic conditions.

Iron-reducing bacteria affiliated to the family *Geobacteraceae* significantly increased under continuous flooding in rhizosphere soil, in concomitance to arsenate dissolution from iron minerals. The 14 day-period of drainage before flowering allowed the recycling of iron, with the increase of *Gallionella*-like iron-oxidizing bacteria. This phenomenon likely influenced the decrease of arsenic translocation in rice grains.

Regardless of the water regime, genes for arsenite oxidation (*aioA*) were the most abundant arsenic-processing genes, explaining the presence of arsenate in soil solution. The presence of arsenite and organic arsenic in rice grains produced under continuous flooding might be related to the retrieval of genes for arsenate reduction (*arsC*) and for arsenite methylation (*arsM*) in the proximity of the roots. These outcomes indicate a potential active role of rhizospheric iron- and arsenic-cycling bacteria in determining arsenic accumulation in rice grains from plants cultivated under continuous flooding, even in soil with a low arsenic content.

1.1 Introduction

Rice is one of the crops with the highest levels of arsenic (As) and one of the most important contributors to human exposure (EFSA, 2014; Hojsak *et al.*, 2015; Singh *et al.*, 2015). Noteworthy, even if rice is cultivated in soil with a low As concentration (i.e. tot As < 20 mg $kgdw^{-1}$), rice grains may accumulate inorganic As (iAs) exceeding the recently introduced limits for food quality of 100 and 200 μg kg^{-1}, respectively for baby food production and for adult consumption (Commission regulation (EU) 2015/1006). The reason for the high content of As in rice grains is that these plants are mainly cultivated under continuous flooding for the whole cropping cycle. In oxygenated soils, the most abundant form of As is arsenate [As(V)], firmly bound to iron minerals. Continuously flooded conditions of the rice paddy lead to the rapid depletion of O_2 with the consequent decrease of the reduction potential. As a consequence, the reduction of ferric iron [Fe(III)] releases As from Fe-As minerals into the porewater. Furthermore, mineral-bound As(V) is reduced by microorganisms to arsenite [As(III)], which is more mobile and toxic than As(V) (Takahashi *et al.*, 2004; Yamaguchi *et al.*, 2014). These reactions are carried out by microorganisms that either use As(V) as an electron acceptor for respiration [dissimilatory As(V) reductase, *arrA* gene] or reduce it for detoxification purposes [As(V) reductase, *arsC* gene, Zhu *et al.*, 2014]. Therefore, under continuous flooding, As bioavailability and consequent rice plant uptake increases. Several studies have documented the reduction of rice grain As content by cultivating the plants under intermittent flooding or with sprinkler irrigation (Das *et al.*, 2016; Li *et al.*, 2009; Ma *et al.*, 2014; Somenahally *et al.*, 2011b; Spanu *et al.*, 2012). The amount of As accumulated in rice grains varies among different rice varieties,

with the lowest accumulation rate being 95 μg kg^{-1}, very close to the limit for baby food production (Spanu *et al.*, 2012).

In addition to the physical and chemical factors, microorganisms affect the As cycle with a variety of direct and indirect processes. A wide range of genes are present in bacteria and archaea that encode for As-processing enzymes and transporters. With these enzymes, microorganisms can reduce As(V) to As(III), oxidize As(III) to As(V), methylate As(III) and extrude As(III) from the cell (processes reviewed by Cavalca *et al.*, 2013; Slyemi and Bonnefoy, 2012; Yamamura and Amachi, 2014). Given that Fe minerals have a higher affinity for As(V) with respect to As(III) (Liu *et al.*, 2005; Martin *et al.*, 2014; Yamaguchi *et al.*, 2014), the activity of Fe(III)-reducing bacteria (FeRB) as well as As(V)-reducing bacteria could promote the dissolution of As from soil Fe (hydr)oxides into the porewater, increasing its bioavailability (processes reviewed by Zhu *et al.*, 2014). On the other hand, in the proximity of rice roots, where oxygen is released by root aerenchyma, the activity of As(III)- and Fe(II)-oxidizing bacteria (AOB and FeOB) can both contribute to the formation and co-precipitation of As with Fe minerals, decreasing its bioavailability (Das *et al.*, 2016; Jia *et al.*, 2014).

Microorganisms also influence As speciation in rice grains. Arsenic in rice grains is mainly present as iAs and dimethylarsinic acid (DMA), with great variation between different countries of origin (Meharg *et al.*, 2009). Recent studies indicate that methylated As found in rice grains is not produced by the plant, but derives from the activity of rhizospheric microorganisms (Arao *et al.*, 2011; Jia *et al.*, 2012; Lomax *et al.*, 2012; Zhao *et al.*, 2013). Although several studies reported higher toxicity of iAs if compared to organic As, dimethylarsenite [DMA(III)] and monomethylarsenite [MMA(III)] have been demonstrated to be more genotoxic than iAs (Stýblo *et al.*, 2002; Thomas *et al.*, 2001). Therefore, understanding which microorganisms are involved in As methylation within the rice plant rhizosphere and what conditions favor their growth is of great importance.

Arsenic mobilization into rice is becoming a world-wide health issue for millions of people, yet little is known about the factors influencing microbial As solubilization in low arsenic soils of European countries. In the context of better understanding microbial As mobilization, a comprehensive study on connections between iron and arsenic-cycling bacteria in different oxic/anoxic conditions of soil in the different root compartments was carried out. The aim of the present study was to set up a detailed experiment in which iron and arsenic cycles could be deciphered by physico-chemical and biological parameters, in order to define their role in As contamination of rice grains established in different agronomic conditions.

1.2 Material and methods

1.2.1 Experimental setup and water regimes

The experiment was carried out at the Rice Research Centre (Ente Nazionale Risi) in Castello d'Agogna (Pavia, Italy) in 2013, in macrocosms set up in 0.83 m^2 plastic tanks filled with 30 cm of gravel and 25 cm of soil from a paddy field (As concentration and other selected physic and chemical characteristics are reported in Table 1). The macrocosms were located at an open-air area in front of the Rice Research Center. Water was supplied with a garden hose and capped holes at the bottom of the containers allowed water control and maintenance of aerobic conditions when required. Rice plants (*Oryza sativa* subsp. *japonica*, variety Loto) were grown under three different water regimes: continuous flooding (CF); rotational irrigations over the cropping season (aerobic rice, AR) and continuous flooding with a 14 day-period of drainage before flowering (2^{nd} internode elongation drainage, 2IED). Three replicates randomly located were set up for each water management. Dry seeding was performed on 10^{th} June in the soil fertilized with 22 g m^{-2} of urea and 40 g m^{-2} of P-K fertilizer (14-18 units respectively). The plants germinated within 10 days, and on 2^{nd} July the plants under CF and 2IED regimes were flooded and AR plants were watered. Watering of AR plants was performed only when the soil water content was below field capacity (approximately every 10 days). After nearly one month from flooding (6^{th} August), when the plants were at the 2^{nd} internode elongation stage, the 2IED macrocosms were drained for 14 days and then re-flooded until 30^{th} September and harvested on 9^{th} October. At harvesting, rice grain was separated from rice straw and then polished and ground for As extraction and speciation.

In the macrocosms soil pH and temperature were measured with SenTix® 41-3 pH electrodes directly placed in the soil. Porewater was sampled according to Cattani *et al.* (2006) through Rhizon soil moisture samplers (Rhizosphere®, Rhizosphere Research Products, Wageningen, NL) at three growing stages: tillering (28^{th} June), flowering (20^{th} August), and senescence (30^{th} September). An aliquot was immediately mixed with orthophenantroline for the measurement of Fe(II), another aliquot was acidified with 2% nitric acid (HNO_3) for As determination, while the rest was transferred into 10 ml polyethylene tubes without headspace, refrigerated and immediately transferred to the laboratory for the analysis of DOC and major dissolved anions.

1.2.2 Chemical analysis of soil and porewater

The soil samples were air-dried and all the analyses were carried out on the fine-earth fraction (< 2 mm). The particle size distribution was evaluated by the pipette method after dispersion of the sample with Na-hexametaphosphate. The pH was determined potentiometrically in a 1:2.5 soil/water

suspension; the C and N contents were determined through dry combustion (NA2100 Protein elemental analyzer, CE Instruments, Milan, Italy). The concentrations of As and Fe extractable with aqua regia (As_R, Fe_R) were determined; As was quantified with hydride generation (HG) coupled with atomic absorption spectrometry (AAS, Perkin-Elmer 4100 equipped with a FIAS 400 hydride generator; Perkin-Elmer Inc., Waltham, Massachusetts) and Fe with flame-AAS. The concentration of Fe(II) was determined with the orthophenantroline method (Loeppert and Inskeep, 1996).

The concentration of total As in soil solution was determined by Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7700x, Agilent Technologies, USA) with Octopole Reaction System (ORS system). Standard solutions of total As were prepared by dilution of a multielement standard (100 mg L^{-1}) obtained from CPI International (Amsterdam, The Netherlands). Arsenic species were measured with High Performance Liquid Chromatography (HPLC, Agilent 1100, Agilent Technologies, USA) on an anion exchange column PRP-X100 (250 x 4.6 mm, 5 μm) fitted with a pre-column. The mobile phase (1 mL min^{-1}) was made of 13.2 mM $NH_4H_2PO_4$ at pH 6. The ion intensity at m/z 75 ($^{75}As^+$) was monitored without reaction mode with carrier gas (Argon) flow rate of 0.95 L min^{-1}. Chlorine ($^{35}Cl^+$) was also monitored because chlorine matrices lead to $^{40}Ar^{35}Cl^+$ interferences. Stock solutions of As species (1000 mg L^{-1} of As) were prepared with As(III) oxide (As_2O_3), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \bullet 7H_2O$), DMA(V) from dimethylarsinic acid [$(CH_3)_2As(O)OH$] and MMA(V) from disodium methylarsenate ($Na_2CH_3AsO_3$). The standard solutions of arsenic species were obtained by diluting the corresponding stock solutions and the exact concentrations were ascertained by ICP-MS analysis. Identification of As species was confirmed by spiking real extracts with a mixture of standard solutions. The internal standard used in total and speciation analysis is ^{72}Ge. An online internal-standard addition system was used to automatically add ^{72}Ge to the instrument during analysis. Ultra-pure water was prepared by a Milli-Q system (18MΩ cm resistance, Millipore® system, Millipore, Bedford, MA). Nitric acid in analytical grade (Carlo Erba Reagents, Milano, Italy) was purified using a sub-boiling distillation system (Milestone mod. subPUR, Shelton, CT, USA).

1.2.3 Total arsenic and speciation in rice grain

The white rice grain samples were milled with a blender under controlled temperature according to Huang *et al.* (2010). Total As was determined by the open-vessel procedure using a modification of the method proposed by Pillai *et al.* (2010). Grain samples (about 0.5 g) were digested with 6 mL of 67% HNO_3 and 1 mL of H_2O_2 in a heating block system (DIGIPREP, SCP Science) in 50 ml polypropylene tubes (digiTUBES, SCP Science) at 95°C for 2 hours. The digested grain solutions were filtered with 0.45 μm filters (digiFILTER, SCP Science) after appropriate dilution with Milli-Q

water. For determination of different As species, approximately 1.5 g of pulverized rice grains were mineralized using 0.28 M HNO_3 at 95°C for 90 min in a heating block system (DIGIPREP) in 50 ml polypropylene tubes (digiTUBES, SCP Science). In each analytical batch a reagent blank and Certified Reference Materials (CRM) were used (NIST 1568a rice flour, NMIJ-CRM-7503 and BCR 610 groundwater). The total As certified values of NIST 1568a rice flour were 290 ± 30 μg kg^{-1}. The total As, As(III), As(V) and DMA(V) certified values of NMIJ-CRM-7503 were respectively 98 ± 7 μg kg^{-1}, 71 ± 3 μg kg^{-1}, 13 ± 1 μg kg^{-1} and 13 ± 1 μg kg^{-1}. The As certified value of BCR 610 groundwater was 10.8 ± 0.4 μg kg^{-1}. The concentration of total As in rice grain was determined by ICP-MS with ORS system. The separation and quantification of As species were performed by HPLC immediately after extraction.

1.2.4 Rhizosphere soil and rhizoplane collection for microbiological analyses

Sampling of rhizosphere soil and rhizoplane fractions for microbiological analyses was performed at tillering, flowering and senescence. Three plants were picked from each replication of the water regimes and pooled in one composite sample per treatment, in accordance with Somenahally *et al.* (2011a). The roots and the attached soil were used for rhizoplane and rhizosphere soil separation, performed according to Cavalca *et al.* (2010). Briefly, the epigeal parts of the plants and the bulk soil around the roots were removed. The rhizosphere soil/roots block was submerged in tetrasodium pyrophosphate buffer (0.2 %, pH 8.0) for 1 h at 30°C under 180 rpm shaking. The resulting suspension was centrifuged at 10000 g for 10 min at 4°C, providing the rhizosphere soil fraction. The roots were then washed thoroughly with sterile distilled water and submerged in 1x phosphate-buffered saline (PBS) solution in ratio 1:2 (w/v). To obtain the rhizoplane fraction, 3 cycles of sonication for 30 s each were applied to the roots and the suspension was centrifuged as previously mentioned.

1.2.5 Microscopy analysis

Rhizosphere soil and rhizoplane fractions collected at tillering, flowering and senescence were processed for 4,6-diamine-2-phenylindole (DAPI) staining and fluorescence *in situ* hybridization (FISH), using 16S rRNA probes listed in Table A1. To fix the microbial cells, approximately 0.25 g of each sample were incubated at 4°C with 3% paraformaldehyde (PFA) for 3 h. The samples were then washed with 1x PBS and resuspended in 2 mL of 5 mM Na_2EDTA. To increase the quality of the assay, 5 mg of polyvinylpolypyrrolidone were added and the samples were shaken at 300 rpm for 30 min. After 5 min of incubation, 1 mL of each sample was transferred into 1 mL of Nycodenz solution (1.3 g mL^{-1}) and centrifuged at 18°C for 30 min at 16400 g. For the following steps 1.8 mL of supernatant were used. The total microbial counts were performed by DAPI staining 18 μL of each

sample. The samples diluted in NaCl solution (9 g L^{-1}) were mixed with DAPI solution to a final concentration of 5 µg mL^{-1} and incubated at room temperature for 15 min in the dark. Samples were then immobilized on black 0.2 µm Isopore™ GTBP membrane filters (Millipore). For FISH analysis, different concentrations of the samples were eluted in 1x PBS and immobilized on white 0.2 µm Isopore™ GTBP membrane filters (Millipore). The filters were then dehydrated progressively in 50%, 80% and 96 % ethanol for 6 min. The hybridization solution was prepared by diluting the probes and the related competitors in the hybridization buffer (900 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS, formamide according to Table A1) to a final concentration of 2 ng μL^{-1}. For the total count of active bacteria, the three EUB probes were mixed together in the same hybridization solution with the same concentration. *In situ* hybridization was performed on filter portions placed on silicon grease coated slides with 100 µL of hybridization solution. The slides were incubated at 46°C for 5 h, then washed with washing buffer at 48°C for 10 min. The last rinse was performed in ice-cold water to block the hybridization reaction. After drying, the filters were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), in order to reduce autofluorescence of the samples, and observed with a fluorescence microscope (Zeiss Axioskop) supplied with a Mercury Short Arc HBO 50W/ACL2 OSRAM UV lamp and Zeis 1 and 15 filter sets for DAPI and FISH respectively. The cells were counted in 20 microscopic fields using a calibrated grid.

1.2.6 Nucleic acids isolation and quantitative real time PCR

Total DNA was isolated using the RNA PowerSoil® Total RNA Isolation Kit and RNA PowerSoil® DNA Elution Accessory KIT (MO BIO), according to manufacturer's instructions. The isolated RNA samples were used for analyses not described in this work.

Different targets were evaluated for the quantification of genes related to arsenic cycle in rice rhizosphere. The 16S rRNA genes of total bacteria and of three representative groups of iron bacteria and genes involved in the direct processing of arsenic were chosen (Table A2). The primers were selected from the literature and the thermal protocols were used accordingly, with the exception of primers arsMF1 and arsMR2, which amplify part of the gene for As(III)-methyltransferase (*arsM*). When this primer pair was tested according to Jia *et al.* (2013), the PCR reaction produced non-specific amplifications. Therefore, a range of annealing temperatures was tested. At 74 °C of annealing temperature PCR fragments with the expected size (ca. 350 bp) were produced. Cloning and sequencing of these fragments confirmed the amplification of the desired target (Table A2). All reactions were set up in a 20 µL mixture volume containing 1x Titan HotTaq EvaGreen® qPCR Mix (Bioatlas), primers with concentrations according to Table A2, 20 ng of template DNA and PCR-

grade water (Sigma-Aldrich). The qPCR was performed on a MJ Mini™ cycler equipped with a MiniOpticon™ system (BIO-RAD). The melting curves were calculated at the end of each run. Each sample was amplified in triplicate. To calculate the gene copy number, standard curves were created amplifying known amounts of DNA isolated from bacterial strains or cloned plasmids containing the target (Tables A2 and A3).

1.2.7 Clone library preparation and sequence analysis

To create the clone libraries, standard PCR was performed with the same thermal protocol described for qPCR, mixing 1x Taq PCR Master Mix (QIAGEN) with the primers, 20 ng of template DNA and PCR-grade water to a final volume of 25 μL. The PCR products were cloned using the TOPO® TA Cloning® Kit (Invitrogen), with TOP10 chemically competent *E. coli* cells and pCR™2.1-TOPO® vector, according to manufacturer's intructions. The positive clones were sequenced and the plasmid was extracted with UltraClean™ 6 minutes Mini Plasmid Prep Kit™ (MO BIO). In Table A3 the clones selected for real time qPCR analysis are listed.

1.2.8 Statistical analysis

The statistical analysis of the data was performed with SPSS software version 21. One-way analysis of variance (ANOVA), Tukey's b, Duncan and t test at $p < 0.05$ were used for comparisons.

1.2.9 Accession numbers

Sequences obtained in this study have been deposited to Genbank with accession numbers from KX592445 to KX592464 (Table A3).

1.3 Results

1.3.1 Soil characteristics

The soil was sandy-loam textured, acidic and with a relatively low organic C content (Table 1) and it was representative of the paddy fields in Pavia province. The As content was 11.4 mg kg^{-1}, in line with the local background. The As concentration did not vary among macrocosms and no differences were found when the soil was re-analyzed after the end of the experiment (data not shown).

1.3.2 Iron and arsenic dissolution in porewater and rice grain contamination

The concentration of As in the porewater (Fig. 1) was below the detection limit just before the flooding for all the water treatments, since anoxic conditions were not yet established in soil. When

the plants reached the flowering stage in the CF treatment the average As concentration in solution was 125 μg L^{-1}, and increased to average 190 μg L^{-1} at the end of the cropping season (30th September). In soil solutions of CF treatments As(V) increased from 1.40 μg L^{-1} to 180 μg L^{-1} in the end of the plant life cycle, whereas As(III) increased to 40 μg L^{-1}. In the 2IED treatment dissolved As was almost absent at the flowering stage, as a consequence of the aerobic conditions established during the 14 days of drainage; it increased to an average of 17 μg L^{-1} at the end of the cropping season, remaining well below the concentration of the CF treatment. Under 2IED As(V) and As(III) increased to 10 μg L^{-1}. In the AR treatment, as expected, dissolved total As as well as As species remained almost negligible in all stages. The concentrations of dissolved Fe(II) and DOC followed the same trend as As in all the water treatments, both increasing with the intensity of the flooding conditions (Fig. 2). Soil pH in all samples was acidic, with values ranging from 5.7 to 6.5 (Table 2).
Arsenic content of rice grain varied significantly among the different water treatments, in agreement with the concentration in soil solution. Total As in rice grains of CF plants was 237 μg kg^{-1}, significantly more abundant with respect to 2IED and AR plants, containing 68 and 5 μg kg^{-1} respectively (Table 3). In CF rice grains, As(III) and DMA accounted for 54.4% and 42.6% respectively, whereas As(V) represented only a minor fraction.

1.3.3 qPCR and FISH detection of iron-cycling bacteria

Bacterial populations involved in the iron cycle at different rhizosphere compartments were detected by quantitative analysis of target genes by real time qPCR and FISH analysis.
Eubacteria in rhizosphere soil were significantly influenced by the different water regimes ($p \leq 0.05$, Table A5). In the rhizoplane, this target accounted for a gene copy number gdw^{-1} in the order of 10^8 at flowering stage and of 10^{10} at senescence in CF, significantly less abundant with respect to 2IED and AR ($p \leq 0.05$). This trend was more evident in the rhizoplane with respect to rhizosphere soil, suggesting a stronger influence of root proximity under more oxic conditions. This trend was confirmed also by the FISH probe Eub338, although the data of cell number gdw^{-1} were one order of magnitude lower (Fig. 3). This might be attributable to FISH analysis detecting active bacterial populations, whereas qPCR relies on the quantification of targets from DNA of the total bacterial community.
Referring specifically to FeRB populations, *Geobacteraceae* were 2-4 orders more abundant with respect to *Shewanellaceae*, both in rhizosphere soil and in the rhizoplane of all agronomic conditions (Fig. 4a and 4b). During flowering, *Geobacteraceae* were more abundant in rhizosphere soil than in the rhizoplane. In rhizosphere soil of CF and 2IED *Geobacteraceae* 16S rRNA genes were in the order of 10^9 copies gdw^{-1}, significantly higher with respect to AR ($p \leq 0.05$). In the rhizoplane, this

target was significantly more present in 2IED plants with respect to CF and AR ($p \leq 0.05$, Fig. 4a), in accordance with FISH detection with probe Geo1423 (Fig. 3). During senescence, *Geobacteraceae* were higher in rhizosphere soil in CF and 2IED, whereas in AR they were significantly more abundant in the rhizoplane ($p \leq 0.05$, Table A5).

Shewanellaceae were more abundant in rhizosphere soil than in rhizoplane, from flowering to senescence regardless of the water regimes (Fig. 4b and Table A5). According to FISH data, *Shewanellaceae* (probe Shew227) were more active in CF and 2IED rather than in AR (Fig. 3 and Table A4).

Gallionella, Leptothrix and *Thiobacillus* genera are considered as important biotic factors influencing Fe oxidation in the rice root system and possibly affecting As uptake by the plant. *Gallionella* sp. was the least represented FeOB in the root system of rice (Fig. 3). *Leptothrix* (probe Lepto175)*, Thiobacillus*, (probe Betthio1001) and nitrate-reducing iron-oxidizing bacteria (probes BrG1-829 and BrG2-830) were in the same order of magnitude of FeRB (Fig. 3). *Gallionella*-like FeOB quantified by real time qPCR were significantly more abundant in CF and 2IED treatments ($p \leq 0.05$), whereas in AR they were always in the order of 10^3 copies gdw^{-1}.

1.3.4 Quantification of genes involved in arsenic metabolism

AioA gene copies were the most abundant in all compartments, ranging from 1×10^6 to 1×10^9 gdw^{-1} (Fig. 5 and Table A6). During flowering, *aioA* genes did not vary significantly in rhizosphere soil, whereas in the rhizoplane they were significantly more abundant in 2IED and in AR ($p \leq 0.05$, Fig. 5a). In these water regimes, they were also significantly more abundant in the rhizoplane compared to rhizosphere soil ($p \leq 0.05$). During senescence, *aioA* genes in rhizosphere soil were significantly more abundant in CF and 2IED, whereas in the rhizoplane they accounted for 1×10^9 in AR, 1×10^8 in CF and 1×10^7 copies gdw^{-1} in 2IED ($p \leq 0.05$, Table A6).

In CF treatments, *arsM* genes were significantly more abundant in rhizosphere soil with respect to the rhizoplane ($p \leq 0.05$, Fig. 5b). However, these target genes were always significantly more abundant in the rhizoplane of AR with respect to the other treatments ($p \leq 0.05$), whereas in rhizosphere soil they did not vary significantly (Fig. 5b and Table A6).

ArsC gene copies ranged from 1×10^5 to 1×10^6 gdw^{-1} (Table A6). During flowering, these genes were significantly more abundant in rhizosphere soil with respect to the rhizoplane ($p \leq 0.05$, Fig. 5c). In rhizosphere soil during flowering, this target was significantly more abundant in AR with respect to the other treatments ($p \leq 0.05$, Fig. 5c).

The primers tested for dissimilatory arsenate reductase (*arrA*) did not amplify the target in any sample.

1.4 Discussion

1.4.1 Porewater chemistry and arsenic in rice grain are affected by water treatment

The concentration of As in porewater, as well as that of Fe, was driven by changes in oxic/anoxic conditions induced by the different water management regimes. The temporary dissolution of Fe and As under flooding conditions was expected, since the solubility of both elements in soil depends on redox processes (Borch *et al.*, 2010); however, interestingly, DOC also followed the same trend. When the soil was aerobic, organic C was dissolved in porewater in comparable amounts at all sampling dates and in all water treatments, but its concentration was significantly increased when the soil was anoxic. Indeed, recent findings (Said-Pullicino *et al.*, 2016) linked the cycling of DOC in paddy soils with that of Fe, demonstrating the role of the redox conditions in regulating the amount and quality of DOC**.** Under increasingly anoxic conditions more aromatic DOC, previously stabilized within the soil mineral matrix**,** is released into solution varying the amount and quality of organic molecules available for microbial metabolism.

The concentration of As in the rice grain depended on its bioavailability in soil solution, that largely differed among the three water treatments. The rice grain produced with the traditional agronomic practice involving continuous soil flooding contained 135 μg kg^{-1} of iAs and was not suitable for baby food production, exceeding the limit of 100 μg kg^{-1}. On the other hand, iAs concentrations in rice grains produced by plants under 2IED treatment (54.8 μg kg^{-1}) and AR (4.67 μg kg^{-1}) water treatments was below this limit. Although As(V) was the predominant form of As in the porewater under CF, higher amounts of As(III) were detected in rice grains. The speciation of As in porewater was different from other studies which measured higher amounts of As(III) with respect to As(V) (Xu *et al.*, 2008). On the contrary, the trend of As concentrations and the proportions of different As species in rice grains according to the water regime were comparable with what was measured in previous studies (Arao *et al.*, 2011; Hu *et al.*, 2013; Moreno-Jiménez *et al.*, 2014; Sun *et al.*, 2014; Xu *et al.*, 2008). Rice plants take up and translocate As(III) at higher rates with respect to As(V) and in comparison to other crops, using silicon transporters (Li *et al.*, 2009; Su *et al.*, 2010). Around the roots of CF plants, due to the formation of a sharp oxic/anoxic interface, iron plaques were developed. The higher affinity of iron-plaques for As(V) with respect to As(III) could also explain the major accumulation of the latter into rice grains (Chen *et al.*, 2005).

1.4.2 Bacterial populations involved in iron cycling

As a consequence of continuous flooding, Fe(II) was released into the porewater, confirming the establishment of anoxic conditions in this system. At the same time, members of the *Geobacteraceae*

family were highly abundant in all samples. These organisms have often been reported to be a dominant component of rice rhizosphere (Cahyani *et al.*, 2008; Das *et al.*, 2016; Li *et al.*, 2011; Li *et al.*, 2012; Lu *et al.*, 2006; Wang *et al.*, 2009b). The development of *Geobacteraceae* under continuous flooding might be related to DOC release in anoxic conditions. This evidence hints that these FeRB played an important role in the promotion of Fe(II) release from Fe(III) minerals under flooded conditions.

Shewanellaceae, on the other hand, were always present, but showed opposite patterns when measured with different techniques. According to FISH analysis, *Shewanellaceae* were significantly more abundant in CF, whereas their 16S rRNA gene copies quantified with qPCR where higher in AR. These organisms are known to be metabolically versatile, being able to use different electron acceptors like O_2, Fe(II) and Mn(IV) (Lovley, 2006). Therefore, their activity could change according to the environmental conditions. In CF, they can contribute, together with *Geobacteraceae* to the release of Fe(II) by reducing Fe(III), whereas in AR they could grow using O_2. The discrepancies observed using different techniques could be due to a different coverage of the qPCR primers and the FISH probes used, as well as a possible low specificity of some of them. Another important aspect is the different procedures used for sample preparation. Isolation of DNA for qPCR was performed using a direct method, whereas for FISH cells were extracted from the soil matrix by Nycodenz density gradient separation. This procedure could have led to the loss of a considerable fraction of the total community, as has been reported in previous studies (Delmont *et al.*, 2011a, 2011b).

FeRB of the family *Geobacteraceae* and the FeOB *Gallionellaceae* and *Leptothrix* sp. in the rhizoplane were more abundant in 2IED plants. For FeOB, this aspect could be explained by the presence of the optimal O_2 concentration required for their growth (3-10 μM, Dubinina and Sorokina, 2014). Furthermore, the application of a drainage period before flowering could have promoted the turn-over of the electron acceptors and donors required by these organisms for their growth. Their higher abundance in this compartment, corresponding to the production of Fe(III), could also explain the higher abundance of *Geobacteraceae*, which rely on this substrate. A complete Fe cycle could therefore have been established in this compartment, contributing to the decrease of As release by contrasting the dissolution of Fe(III) minerals.

Interestingly, most of the FeOB retrieved in this study were not inhibited under CF. This could be an indication that in CF rice rhizosphere microhabitats with different O_2 concentrations developed as a consequence of O_2 loss by rice roots and allowed the growth of microaerophilic FeOB. Furthermore, some FeOB like *Thiobacillus* sp. couple the anaerobic reduction of nitrate to the oxidation of Fe(II) (Beller *et al.*, 2006). In fact, members of this genus were significantly more represented in CF plants (Fig. 3).

The family *Gallionellaceae* was not represented by high numbers compared to what has been observed in the rhizosphere of rice and other wetland plants (Schmidt and Eickhorst, 2013; Wang *et al.*, 2009a). Although oxic conditions are normally displayed in AR, the presence and the activity of *Gallionella* group was possibly impaired by acidic pH of the studied soils and porewater. This is in accordance to the pH values measured in the porewater (Table 2), which reveals an acidic environment where acidophilic FeOB like *Thiobacillus* sp. are better adapted than *Gallionellaceae* (Emerson *et al.*, 2010).

1.4.3 Arsenic-processing bacterial populations

The higher abundance of total bacteria in AR rhizosphere was already reported (Somenahally *et al.*, 2011b). This could be explained by the fact that under CF less O_2 is present, with the increase of bacteria with metabolisms based on alternative electron acceptors that yield a lower and slower cell growth. Furthermore, the higher concentration of As in the porewater could have had toxic effects and selected only for the As-resistant fraction of the total community.

The prevalence of As(V) in porewater is consistent with the higher number of *aioA* gene copies measured in CF rhizosphere with respect to the other target genes (Fig. 1 and 5, Table A6). These proportions reflected the values measured in the unplanted soil (Table A6). A higher abundance of *aio*A genes with respect to *arsC* and *arsM* under CF was already reported (Huang *et al.*, 2012; Jia *et al.*, 2014; Lomax *et al.*, 2012). Furthermore, in the 2IED and AR rhizospheres *aioA* genes were more abundant, similar to what was observed in previous studies (Das *et al.*, 2016). Microorganisms carrying *aioA*, although not influenced by the presence of As(III), could have actively contributed to the high As(V) concentrations measured in the porewater of CF. In this water regime, As(III) was produced by *arsC*-carrying bacteria, whereas in 2IED and AR its concentration was negligible.

The qPCR analysis revealed the presence of *arsM* genes, which confirms the potential production of MMA and DMA by rice rhizosphere microbiota. These values are within the range previously reported in unplanted rice field soils and for rice plants grown with and without organic C amendment (Jia *et al.*, 2013; Zhao *et al.*, 2013). However, these genes did not vary significantly according to the water management regime in contrast with Ma *et al.* (2014), which found an increased *arsM* gene copy number in CF plants. The outcome of our study is in accordance with the concentration of organic As in porewater that did not vary during the experiment in the different water regimes, suggesting that As methylation might not be relevant for rhizospheric bacteria, unless in the presence of As(III) in the soil solution. The bacterial methylation activity of As in the CF water regime likely produced the organic As measured in CF rice grains.

The dissimilatory reduction of As(V) did not seem to play a role in As dissolution under this experimental setup.

1.5 Conclusions

In rice field soil with low concentrations of As, a combination of direct and indirect processes influence As availability for plant uptake and for microbial methylation. As(V) increased in porewater by the release from Fe (hydr)oxides as a consequence of chemical and microbial Fe(III)-reduction by *Geobacteraceae* and *Shewanellaceae*. A 14-day drainage before flowering led to the decrease of As in soil solution and in rice grains, probably promoting the oxidation of soluble Fe(II) by FeOB, with a consequent co-precipitation of As with Fe minerals. Once As is released in soil solution, As-processing bacteria influence its speciation thus affecting rice grain contamination.

Acknowledgements

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Table 1 Selected soil physic and chemical characteristics. Values represent the means of samples from all macrocosms ± standard deviation. Fe_R and As_R are *aqua regia* extractable Fe and As respectively.

Parameter	Value	Measure unit
Sand (2.00 – 0.05 mm)	54.4 ± 1.98	%
Silt (0.05 – 0.002 mm)	39.0 ± 1.62	%
Clay (< 0.002 mm)	6.6 ± 0.71	%
pH	5.9 ± 0.05	-
Organic C	15.3 ± 0.45	g kg^{-1}
Total N	1.2 ± 0.05	g kg^{-1}
Olsen P	36.9 ± 1.33	mg kg^{-1}
Fe_R	33.1 ± 1.04	g kg^{-1}
As_R	11.4 ± 0.74	mg kg^{-1}

Table 2 Mean values of pH and temperature at three rice growth stages as affected by water management.

Phase	Continuous flooding		2nd internode elongation drainage		Aerobic rice	
	pH	Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)
Tillering	5.7	21.6	6.1	21.6	5.8	21.6
Flowering	6.2	23.6	5.9	23.3	6.0	23.8
Senescence	6.5	17.0	6.4	16.7	6.1	16.8

Table 3 Content of total As and As species in rice grains (n=3) under different water regimes. Values are expressed as μg kg^{-1} ± standard deviation.

Water regime	Tot As	As(III)	As(V)	DMA(V)	MMA(V)
CF	237 ± 38	129 ± 35	6 ± 3	101 ± 12	< 3
2IED	68 ± 4	56 ± 5	< 3	12 ± 1	< 3
AR	5 ± 3	5 ± 3	< 3	< 3	3

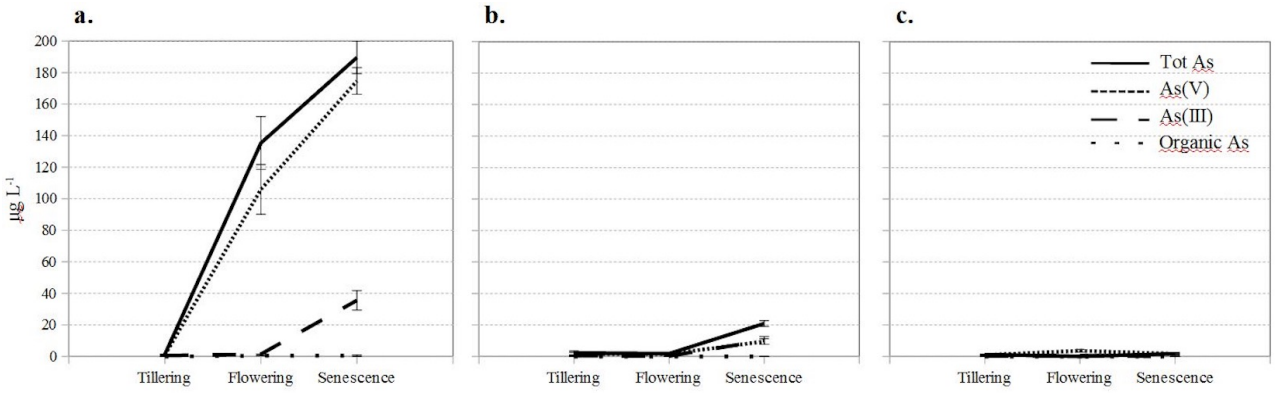

Figure 1 Concentration of total As, As(V), As(III) and organic As (µg L^{-1}) in soil solutions under continuous flooding (CF, a), 2nd internode elongation drainage (2IED, b) and in aerobic rice (AR, c) during three rice growth stages.

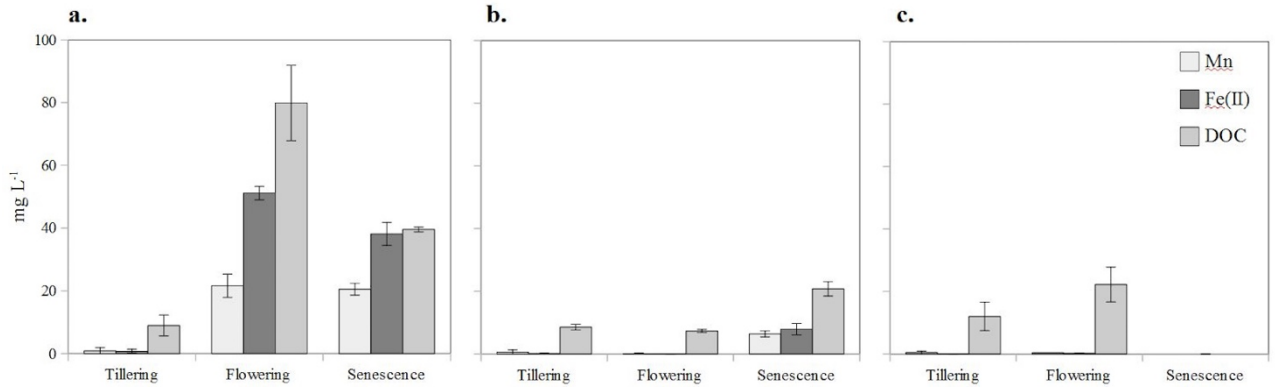

Figure 2 Concentration of soluble manganese (Mn), ferrous iron [Fe(II)] and dissolved organic carbon (DOC) under continuous flooding (CF, a), 2nd internode elongation drainage (2IED, b) and in aerobic rice (AR, c) at three rice growth stages.

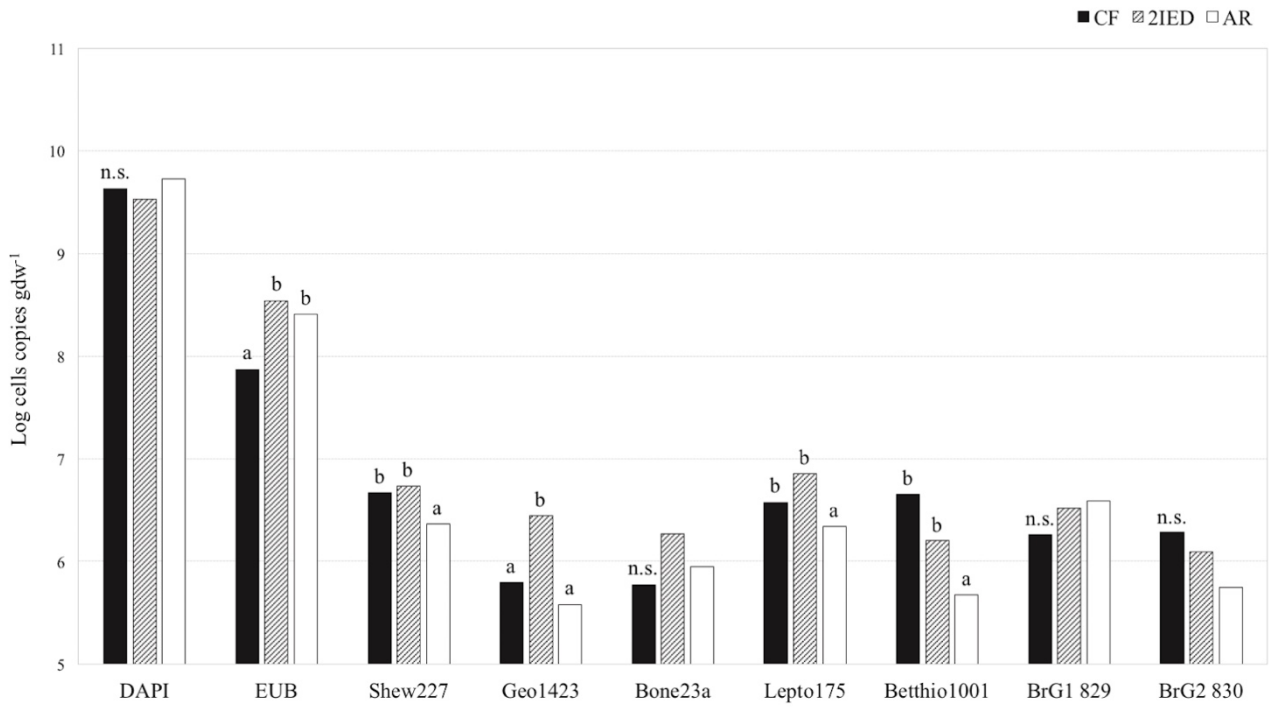

Figure 3 Cell counts obtained with DAPI staining and FISH with different probes at flowering. Letters and n.s. indicate, respectively, statistically significant groups and non-significant differences based on ANOVA (Tukey's test, $p \leq 0.05$).

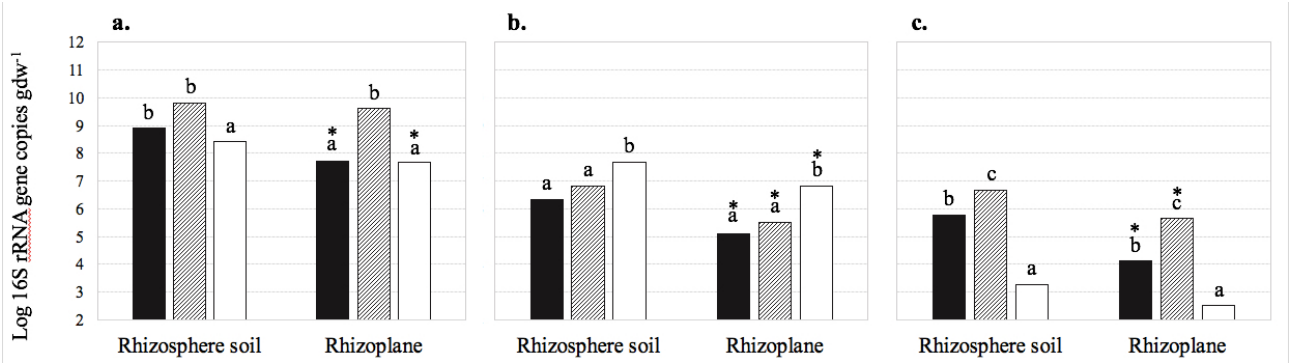

Figure 4 Gene copy abundance of 16S rRNA genes belonging to *Geobacteraceae* (a), *Shewanellaceae* (b) and *Gallionellaceae* (c) in rhizosphere soil and rhizoplane during flowering. Stars indicate values measured in the rhizoplane significantly different with respect to what measured in the rhizosphere soil (t test, $p \leq 0.05$); lowercase letters and n.s. indicate, respectively, significant differences between different water regimes within the same root-soil compartment and non-significant differences (Tukey's test, $p \leq 0.05$).

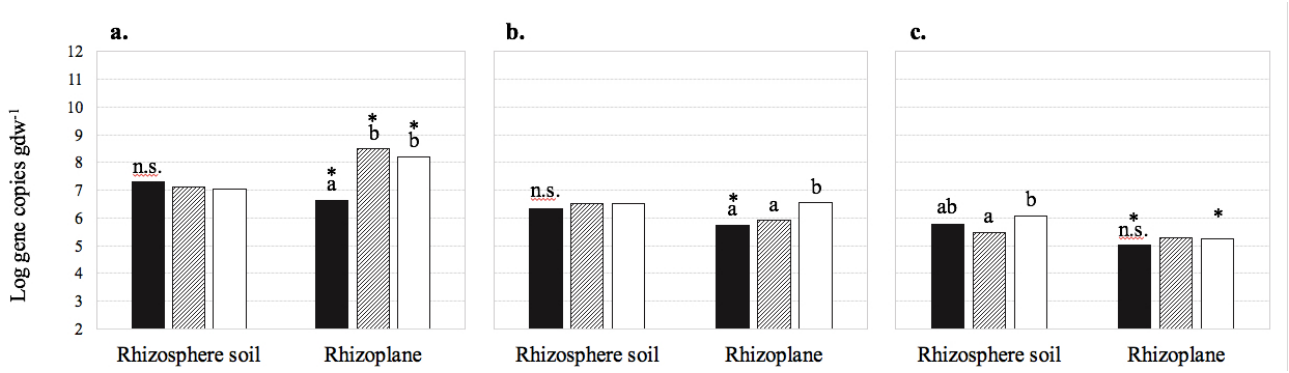

Figure 5 Gene copy abundance of *aioA* (a), *arsM* (b) and *arsC* (c) in rhizosphere soil and rhizoplane during flowering. Stars indicate values measured in the rhizoplane significantly different with respect to what measured in the rhizosphere soil (t test, $p \leq 0.05$); lowercase letters and n.s. indicate, respectively, significant differences between different water regimes within the same root-soil compartment and non-significant differences (Tukey's test, $p \leq 0.05$).

2.

Exploring the diversity of root-associated microbiota: the role of water regimes in arsenic translocation in rice

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Abstract

In recent years, the role of microorganisms inhabiting rice rhizosphere on arsenic contamination has emerged. However, little is known concerning the species and metabolic properties involved in this phenomenon. In this study, the influence of water management on the rhizosphere microbiota in relation to arsenic dissolution in soil solution was tested.

Rice plants were cultivated in macrocosms under different water regimes: continuous flooding, continuous flooding with a 2 weeks-period drainage before flowering and dry soil watered every 10 days. The active bacterial communities in rhizosphere soil and in rhizoplane were characterized by 16S rRNA pyrosequencing. An in-depth analysis of microbial taxa with direct or indirect effects on arsenic speciation was performed and related contribution were evaluated.

Continuous flooding promoted a high diversity in the rhizosphere, with the plant strongly determining species richness and evenness. On the contrary, under watering the communities were uniform, with little differences between rhizosphere soil and rhizoplane. Arsenic-releasing and arsenite-methylating bacteria were selected by continuous flooding, where they represented 8 % of the total. On the contrary, bacteria decreasing arsenic solubility were more abundant under watering, with relative abundance of 10 %. These values reflected arsenic concentrations in soil solution, respectively 135

μg L^{-1} and negligible in continuous flooding and under watering. When short-term drainage was applied before flowering, intermediate conditions were achieved.
This evidence strongly indicates an active role of the rhizosphere microbiota in driving arsenic biogeochemistry in rice paddies, influenced by water management, explaining amounts and speciation of arsenic often found in rice grains.

2.1 Introduction

Arsenic contamination of groundwater resources and soils represents an issue in many areas of the world (Singh *et al.*, 2015; Heikens, 2006). However, arsenic (As) speciation and the physicochemical characteristics of the environment determine its bioavailability more than its concentrations. In rice fields the prolonged flooding usually preferred for cultivation leads to As release from soil minerals with the consequent accumulation of the metalloid in the grains (Zhu *et al.*, 2014; Sun *et al.*, 2014; Ma *et al.*, 2014). Recent studies revealed that, on average, As content in rice from different countries exceeds the law limits established by the Commission regulation (EU) 2015/1006 (Ma *et al.*, 2014; EFSA, 2009; EFSA, 2014).
The two inorganic As species mainly present in rice field soil, arsenate [As(V)] and arsenite [As(III)], have different biogeochemical properties. Nevertheless, both As species show high affinity for iron oxides/hydroxides (Meharg, 2012).
Continuous flooding in paddy soils leads to strongly reduced conditions, with the consequent rapid dissolution of these minerals and the release of As into the porewater (Zhu *et al.*, 2014; Meharg, 2012). Furthermore, As(V) is reduced to As(III) abiotically by sulfide, ferrous iron [Fe(II)], H_2 or reduced organic acids, or by As(V)-reducing bacteria (Cavalca *et al.*, 2013; Meharg *et al.*, 2012). Several studies reported that As(III) in flooded rice fields is the predominant As species (Zhu *et al.*, 2014; Takahashi *et al.*, 2004). At highly reduced conditions, where microbial sulfate reduction is favored, sulfide produced by this activity can co-precipitate with As(III) forming a variety of minerals, such as orpiment (As_2S_3) (Zhu *et al.*, 2014; Kocar and Fendorf, 2009). If soil is aerated, for example after a drainage period, As(III) can be oxidized to As(V) by oxygen, manganese oxides and H_2O_2 as well as by microbial As(III) oxidation (Meharg, 2012).
The genetic properties and the encoded enzymatic systems that allow several groups of microorganisms to resist to high As concentrations or to use As for metabolic purposes have been recently reviewed by various authors (Andres *et al.*, 2016; Zhu *et al.*, 2014; Yamamura *et al.*, 2014; Cavalca *et al.*, 2013; Zheng *et al.*, 2012; Slyemi and Bonnefoy, 2012). Interestingly, in rice paddy soils with low As concentration a high diversity of microbial genes for As processing has been

detected (Xiao *et al.*, 2016), indicating the potential role of native communities on As transformations beyond abiotic factors. Among these processes, the microbial methylation of As(III) in rice rhizosphere is receiving great attention in the last few years. Recent studies indicated that rice roots microbiome is entirely responsible for the production of methylated As present in rice grains, which accounts for 50 % of total As content (Lomax *et al.*, 2012; Arao *et al.*, 2011; Zhao *et al.*, 2013). Furthermore, continuous flooding of rice fields has been demonstrated to increase the concentration of methylated As in rice grains (Ma *et al.*, 2014; Li *et al.*, 2009).

In addition to direct As transformations, several metabolic activities of microorganisms could indirectly influence As speciation and bioavailability in the environment. Given the above-mentioned affinity of As for iron and sulfide minerals, microorganisms involved in iron and sulfur cycles could promote either the release or the sequestration of As from the porewater of rice paddies. Dissimilatory iron-reducing bacteria (DFeRB) use ferric iron [Fe(III)] as electron acceptor for anaerobic respiration, contributing to the release of As from iron minerals (Lee, 2013). Conversely, iron-oxidizing bacteria (FeOB) are chemolithoautotrophic bacteria that couple the oxidation of Fe(II) to the reduction of a variety of electron acceptors (Emerson, 2012; Hedrich, 2011; Emerson *et al.*, 2010). With their activity, these bacteria promote the co-precipitation of As with iron minerals. As already stated, dissimilatory sulfate-reducing bacteria (DSRB) are strict anaerobes that reduce sulfate (SO_4^{2-}) to sulfide (HS^-) for their energy metabolism (Rabus *et al.*, 2015; Ramel *et al.*, 2015; Pester *et al.*, 2012; Pereira *et al.*, 2011), potentially contributing to the formation of As_2S_3 in anoxic compartments of rice fields soils. On the other hand, a variety of sulfur-oxidizing bacteria (SOB) can oxidize HS^- to SO_3^{2-}, and/or the latter to SO_4^{2-}, leading to the release of As into the porewater in rice paddies (Stubner, 1998; Friedrich *et al.*, 2005; Hamilton *et al.*, 2015; Dahl *et al.*, 2008).

The recent instructions established by the Commission regulation (EU) 2015/1006 concerning rice consumption in relation to As exposure have arisen great concern in the most important European rice producing countries like Italy. Although the scientific community has often focused the attention on As contamination of rice, especially in Asia, very little is known about the role of different rhizospheric microbial populations and the microbial metabolic processes that drive As biogeochemistry. In this study, the bacterial community inhabiting the rhizosphere of rice plants cultivated with different water regimes in an unpolluted soil has been investigated in order to identify specific populations responsible for As contamination of rice grains.

2.2 Material and methods

2.2.1 Experimental setup, sampling and chemical analyses

Rice paddy macrocosms containing 10-15 rice plants (*Oryza sativa* subsp. *japonica*, variety Loto) each were set up in tanks filled with rice field soil (sandy-loam texture; pH 6.0; 11.4 mg kg-1 of total As content and 33.1 g kg-1 of *aqua regia* extractable Fe). Three replicates for each macrocosm were managed either under continuous flooding (CF), under continuous flooding with a 2-weeks period of drainage before flowering (CF-D) or in dry soil with watering every 10 days (D). After 12 days from germination CF and CF-D macrocosms were flooded and D were watered. CF-D treatments were drained 47 days after germination for 14 days, followed by re-flooding. Then, they were re-flooded until sampling. Three plants from each macrocosm replicate were sampled at flowering, after 60 days from germination. Rhizosphere soil and rhizoplane were collected according to Cavalca *et al.* (2010). An aliquot of the unplanted soil used for the experiment was used as the time zero control. Porewater samples were obtained using Rhizon soil moisture samplers (Rhizosphere®, Rhizosphere Research Products, Wageningen, NL). The concentration of total As in the porewater samples was quantified with HG-AAS (Perkin-Elmer 4100 equipped with a FIAS 400 hydride generator; Perkin-Elmer Inc., Waltham, Massachusetts); Fe(II) was determined with the orthophenantroline method (Loeppert and Inskeep, 1996); N-nitrate, P-phosphate and S-sulfate were determined with ion chromatography [Dionex DX-500 system, AS9 analytical column, with AG9 pre-column (Dionex, Sunnyvale, CA, USA)]**.**

2.2.2 Nucleic acids isolation

Total RNA was isolated using the RNA PowerSoil® Total RNA Isolation Kit (MO BIO), according to manufacturer's instructions. To remove residual genomic DNA from isolated RNA, 1 u of DNaseI (Thermo Fisher Scientific) was added to 1 µg of RNA and each reaction was incubated according to manufacturer's instructions. The purified RNA was reverse transcribed with iScript™ cDNA Synthesis Kit (BIO-RAD) according to manufacturer's instructions.

2.2.3 Pyrosequencing of 16S rRNA

Pyrosequencing of 16S rRNA was performed from reverse-transcribed RNA isolated from the unplanted soil and from rhizosphere soil and rhizoplane sampled during the reproductive phase. Bacterial 16S rRNA was amplified with the universal bacterial primers 27F (5' - GAG AGT TTG ATC CTG GCT CAG- 3') and 1495R (5' – CTA CGG CTA CCT TGT TAC GA – 3') in triplicate in a 25 µL reaction volume containing 10 ng of cDNA, 0.3 µM primers and 1x Taq PCR Mastermix

kit (QIAGEN). The thermal incubation included a first denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 40 sec and elongation at 72 °C for 1 min and 40 sec; the final elongation was performed at 72 °C for 10 min. Replicated amplicons were pooled and purified with MinElute PCR Purification kit (QIAGEN) to a final concentration of 20 ng μL^{-1}. Pyrosequencing was performed at Molecular Research LP (MRDNA, Shallowater, TX, USA) by bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP), using the primer 27F. The sequences were processed and analyzed with the QIIME tools (Caporaso *et al.*, 2010a). Sequences with less than 200 bases of with barcodes or primer biases, homopolymers and chimeras were removed from the analysis. Operational Taxonomic Units (OTUs) were defined with a 97 % similarity cut-off. Representative sequences for each OTU were aligned to the last SILVA SSU Ref dataset (Quast *et al.*, 2013) using the PyNAST method (Caporaso *et al.*, 2010b). After taxonomic assignment, sequences belonging to chloroplasts were removed and OTU tables were generated for each sample. Phylogenetic analysis was performed using the FastTree method (Price *et al.*, 2009). To measure the bacterial diversity within the samples, the OTU tables were rarefied and different indices of alpha diversity were calculated assuming a sample size of 2000. The OTU richness and the diversity within each sample was evaluated with different alpha diversity indices (observed species, Fisher's alpha, ACE, Simpson evenness). To compare the bacterial diversity between the samples, principal coordinates analysis of the rarefied OTU tables was performed calculating unweighted and weighted unifrac distances (Hamady and Knight, 2009). To distinguish between core and rare taxa, the rank abundance was plotted. Statistically significant differences were evaluated with one-way analysis of variance (ANOVA) at $P < 0.05$ with Bonferroni's correction.

To analyze the bacterial populations potentially involved with arsenic cycle, specific genera where selected on the bases of the information available in the literature on their metabolism (Online Resource 1, Table B1). Furthermore, the genomes available for some of these genera where checked for the presence of genes related to arsenic metabolism. Genera were selected for their documented capacity to reduce As(V) as an electron acceptor [dissimilatory As(V)-reducing bacteria, DAsRB], to resist to arsenic with different mechanisms [arsenic resistant bacteria, AsRB], to oxidize As(III) [As(III)-oxidizing bacteria, AsOB] or to methylate As(III) [As(III)-methylating bacteria, AsMB]. The metabolisms that indirectly influence arsenic dynamics considered in this analysis were dissimilatory Fe(III)-reduction (DFeRB), Fe(II)-oxidation (FeOB), dissimilatory SO_4^{2-} reduction (DSRB) and sulfur-oxidation (SOB).

All the 16S rRNA sequences retrieved in this study are deposited in the NCBI Bioproject (https://www.ncbi.nlm.nih.gov/bioproject/) PRJNA353766.

2.3 Results

2.3.1 Effect of the water treatment on porewater chemistry

The dissolution of relatively high concentrations of As and Fe(II) in the porewater in the CF macrocosms was indicative of the reducing conditions induced by the continuous flooding, whereas in CF-D and D they were almost negligible at the considered sampling date (Table 1), proving that the drainage of the CF-D macrocosms had been effective in restoring an oxidative environment in soil. Nitrate and sulfate, which are reducible species, showed different patterns, being more abundant in the aerobic rice test D and in the just drained macrocosm CF-D, compared with the flooded ones. The concentration of dissolved phosphate, as expected, remained comparable in the different treatments.

2.3.2 Ecology of rice rhizosphere microbiota under different water management

Sequencing of 16S rRNA produced 230,791 reads. The average length of reads with quality score above 25 was 408 bp, therefore the sequence region beyond the nucleotide position 400 was removed in all reads. The total number of sequences that passed the quality control for each sample and the related number of OTUs are listed in Table B2.

Different indexes for alpha diversity (ACE, Simpson evenness, observed species) were calculated and compared (Fig.1). The rhizoplane samples of CF and CF-D plants showed the highest species richness (Fig. 1a). On the contrary, the rhizoplane of D was the sample with the lowest species richness. The number of expected species in all rhizosphere soil samples was similar to the unplanted soil. Simpson's evenness in all samples was below 0.5, indicating the predominance of specific groups among the whole community. In the rhizoplane of CF plants, species were more evenly distributed with respect to all the other samples. In the rhizoplane of CF-D plants, although species richness was similar to CF plants, evenness was lower. The bacterial community of the rhizosphere soil of CF plants was the most heterogeneous.

The rarefaction analysis performed on the observed species highlighted four clusters of samples according to their trend until 4000 sampled reads: the rhizoplane of CF plants with observed species > 1600, rhizosphere soil of CF plants and the rhizoplane of CF-D plants with values between 1200 and 1400, the unplanted soil, with 1000 species and both compartments of D and rhizosphere soil of CF-D plants with values between 800 and 1000 (Fig. 1b).

According to the PCoA analysis, the bacterial communities developed in both compartments of D were similar to each other, whereas CF and CF-D treatments led to a significant differentiation between rhizosphere soil and the rhizoplane (Fig. 2). Interestingly, the communities selected within

the rhizoplane of plants treated with these two water regimes were similar. The rhizosphere soil community of CF plants was more similar to the unplanted soil, whereas in CF-D plants, although the phylogenetic composition was similar to the community of rhizosphere soil under CF (CF RS) (Fig. 2a), the relative abundances where different (Fig. 2b).

2.3.3 Phylogenetic composition of the different communities

In total 40193 OTUs at genus level were found in all samples. Two opposite trends were observed concerning the number of OTUs exclusively present after each treatment in rhizosphere soil and in the rhizoplane (Online Resource 2, Fig. S1). In rhizosphere soil, 25.8 %, 30.5 % and 30.9 % of the total number of OTUs were selected by CF, CF-D and D, respectively (Online Resource 2, Fig. S1a). On the contrary, in rhizoplane the respective abundances were 33.6 %, 30.6% and 22.6 % (Online Resource 2, Fig. S1b). In rhizosphere soil, CF-D shared more OTUs with D treatments, with 4.6 % of OTUs in common with respect to 3% with CF. In the rhizoplane, CF-D shared 7.2% of the OTUs with CF treatments, compared to 1.7% in common with D.

A total number of 33 phyla was detected in the samples. The number of phyla in the unplanted soil was higher with respect to all rhizosphere compartments of rice cultivated with different water regimes (Online Resource 1, Table B2). In both compartments the number of phyla decreased from CF to CF-D to D. On the basis of the rank abundance plot, taxa with relative abundance below 0.01 % were considered as part of the rare biosphere (Online Resource 2, Fig. S2a). According to this definition, the fraction of rare phyla was higher in the UN, followed by both compartments of CF plants, both compartments of CF-D plants and both compartments of D plants (Online resource 2, Fig. S2b).

The percentage of sequences that could not be assigned to any known phylum ranged between 2.3 % and 6.9 % (Fig. 3). In the unplanted soil, the phyla *Acidobateria*, *Actinobacteria* and *Proteobacteria* were the most abundant, accounting for 9.13%, 30.69% and 36.72% of the bacterial community respectively (Fig. 3a). In CF RS, the abundance of *Proteobacteria* decreased to 25.10 %, with the concomitant increase of *Acidobacteria* and *Actinobacteria*, which accounted for respectively 12.61 % and 37.14 % of the total (Fig. 3b). In the rhizoplane under CF (CF RP), *Actinobacteria* represented only 2.52 %, whereas *Proteobacteria* and *Acidobacteria* accounted for 54.43 % and 20.83 % respectively (Fig. 3e). In CF RS *Actinobacteria* belonging to an uncultured genus of the order *Gaiellales* were significantly more abundant with respect to the other treatments, whereas in the rhizoplane the same microorganisms were more abundant in D (Fig. 3b, Table B4). The genera *Marmoricola* and *Nocardioides* also contributed with 3.8 % and 1.9 % respectively. In CF RP *Acidobacteria* of the order *DA023* and Candidatus *Chloracidobacterium* were represented by 7.81 %

and 4.21 % of the sequencing library respectively (Fig. 3e). Bacteria belonging to the class *Deltaproteobacteria* were selected by CF, accounting for 4.5 % and 6% of the total community in CF RS and in CF RP (Fig. 3a and 3e). Iron-reducing *Deltaproteobacteria* belonging to the genera *Anaeromyxobacter* and *Geobacter* were the most contributors for this class in CF RS and CF RP respectively (Online Resource 1, Table B3).

In the rhizosphere and in the rhizoplane under CF-D (CF-D RS and CF-D RP), the phylum *Proteobacteria* accounted respectively for 82.34 % and 68.10 % of all bacterial phyla (Fig. 3c and 3f). In CF-D RS, this phylum was the only one with abundance above 10 %, whereas in CF-D RP the phylum *Acidobacteria* contributed with 17.78 %. The classes *Alphaproteobacteria* and *Betaproteobacteria* were mainly responsible for the dominance of the phylum *Proteobacteria* in both compartments. In CF-D RS, members of the class *Betaproteobacteria* accounted for more than 45%, with the *Comamonadaceae* family accounting for 38% of the total bacterial community (Fig. 3c, Table B4). Within this family, *Ramlibacter*, *Piscinibacter* and other unknown genera represented 11 %, 3.3 % and 21 % of the whole community respectively. In CF-D RP, on the other hand, several members of the class *Alphaproteobacteria* made up almost 50 % of the total community (Fig. 3f). Within these members, the genus *Sphingomonas* accounted for 17 % in both compartment.

In both compartments of D (D RS and D RP) the two most abundant phyla were *Proteobacteria* and *Actinobacteria*, respectively accounting for 53.68 % and 31.31% in D RS and 60.56 % and 25.25 % in D RP (Fig. 3d and 3g)**.** Within *Proteobacteria*, the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were the most abundant. In D RS they accounted for 30.3 %, 6.8 % and 15.6 % respectively, with *Sphingomonas* responsible for 15.2 % of the total community. In D-RP the three above mentioned classes accounted for 39.2 %, 11.2 % and 9.5 %. In this compartment *Sphingomonas* and *Variovorax* represented 28 % and 6.8 % of the total community respectively. Within the phylum *Actinobacteria*, the genus *Arthrobacter* accounted for 14.3 % and 5.9 % in D RS and D-RP respectively. Different OTUs belonging to this genus were significantly more abundant in D with respect to the other water regimes (Table B4). In D-RP the genera *Marmoricola* and *Nocardioides* also contributed with 2.1 % and 2.9 % respectively.

2.3.4 Bacterial populations potentially involved in arsenic cycle

In the unplanted soil, bacteria able to resist or to process As, either by reduction, oxidation or methylation, accounted for 6.39 % of the bacterial community (Fig. B3a). Bacteria involved in iron and sulfur cycle represented 1.40 % of the total (Online Resource 2, Fig. S3b). Of the potential directly As-processing bacteria, 58.96 % were putative AsMB, 23.83 % were AsOB, 12.35 % were putatively *ars/ACR* operons-carrying species (AsRB) and 4.85 % DAsRB. Of the total indirectly As-

influencing species, 22.34% were DFeRB, 35.55 % were FeOB, 3.61 % were DSRB and 38.5 % were SOB.

During the reproductive phase, DAsRB, DSRB and DFeRB were significantly more abundant in rhizosphere soil and in the rhizoplane of plants cultivated under CF (Fig. 4). In the rhizoplane, DAsRB and DFeRB accounted for more than 2 % of the total community, whereas DSRB represented 0.1 % of the community. Bacteria able to oxidize Fe(II) or As(III) (FeOB and AsOB) in rhizosphere soil showed an opposite trend, being significantly more abundant in D plants, where they accounted for 4.34 % and 6.28 % respectively. Concerning FeOB, an opposite trend was observed for different species: *Leptothrix*, *Rhodobacter*, *Thiobacillus* and *Aquabacterium* were more abundant in CF, whereas *Thermomonas* and *Pseudomonas* were more abundant in D (Online Resource 1, Table B3). In the rhizoplane of all treatments FeOB were always below 1%. In the rhizoplane, AOB accounted for 10% of the total community in D plants, whereas in CF and CF-D plants they were always overall below 3%. Interestingly, sulfur-oxidizing bacteria (SOB) were significantly more abundant in CF plants in both compartments, doubling from 1.49 % in rhizosphere soil to 2.88% in the rhizoplane.

The abundance of AsRB in rhizosphere soil was included between 2.6 % and 3.4 %, without significant variations due to the water regimes. In the rhizoplane, on the contrary, AsRB accounted for 3.91 % of the total in CF plants, with respect to 1.14 % and 0.72 % in CF-D and D plants. Similarly, AsMB were present in rhizosphere soil with abundances between 1.93 % and 4.67 %, with no significant variations. In the rhizoplane, AsMB were significantly more abundant in CF plants (4.64 %) than in CF-D (2.66 %) and D (1.11 %) plants.

2.4 Discussion

The species richness and evenness of the samples, the PCoA analysis and the core microbiome analysis reflected the chemical characteristics determined by the different water regimes and indicated a strong influence of the presence of the plant in CF and CF-D agronomic conditions (Table 1; Fig. 1, 2, B1).

The rhizosphere of CF and CF-D plants was generally an anoxic environment, where strictly anaerobic species were selected. In the rhizoplane of these plants, the release of organic matter and oxygen by the roots likely promoted a high diversity, whereas in D rhizosphere the proximity to the plant did not lead to a sharp differentiation in the bacterial community. In rhizosphere soil of CF and CF-D treatments, populations with similar phylogenetic affiliation were selected during the vegetative phase, whereas the two-weeks drainage period led to a differentiation in the abundances but not in the phylogenetic structure of the community (Fig. 2). In rhizosphere soil of CF-D plants,

the stress induced by sharp short-term changes in the redox conditions could have selected for more versatile species in common with D. In the rhizosphere of D less species where stimulated, probably indicating a lower degree of electron acceptors restoration given by either the absence of a redox interface or by the fact that these plants do not grow under optimal conditions (i.e. continuous flooding).

The three dominant phyla found in all samples, *Proteobacteria*, *Acidobacteria* and *Actinobacteria*, reflected what commonly found in plants rhizosphere (Bulgarelli *et al.*, 2013).

Actinobacteria represented a significant part of the original community of the soil used for this experiment, contrary to what seen in previous works (Edwards *et al.*, 2015; Wörner *et al.*, 2016). These microorganisms are common soil inhabitants and plant commensals (Ventura *et al.*, 2007). Most of them, including members of the genera *Marmoricola* and *Nocardioides* and of the order *Gaiellales,* are aerobic and degrade a variety of complex polysaccharides deriving from the plant (Barka *et al.*, 2016; Kügler *et al.*, 2015; Urzì *et al.*, 2000; Lee, 2007; Dastager *et al.*, 2008; Lee and Lee, 2010; Lee *et al.*, 2011; Yoon and Park, 2006; Albuquerque *et al.*, 2011). The genus *Arthrobacter*, quite abundant in rhizosphere soil of D plants, is commonly found in soils with neutral pH. Members of this genus are versatile concerning carbon source and highly resistant to environmental stress like aridity (Jones and Keddie, 2006). The high abundance of members of the order *Gaiellales* together with Fe(III)- and SO_4^{2-}-reducing genera in the rhizosphere of CF plants indicates the presence of microhabitats with different levels of oxygen and electron acceptors.

Similarly, members of the phylum *Acidobacteria* are commonly found in soils, especially outside the roots (Bulgarelli *et al.*, 2013; Ward *et al.*, 2009). As a confirmation of these outcomes, in previous studies these organisms were found to be more abundant in bulk and rhizosphere soil with respect to the rhizoplane (Edwards *et al.*, 2015). They are usually aerobic, capable of nitrate and nitrite reduction, heterotrophs, able to degrade complex substrates and tolerant to variation of soil humidity (Ward *et al.*, 2009).

Member of the *Proteobacteria* phylum were favored by the proximity to the plant. These microorganisms have often been found more abundant in the rhizoplane and in the endosphere (Edwards *et al.*, 2015) with respect to the bulk of rice field soil. The high abundance observed in CF-D for *Alphaproteobacteria* and *Betaproteobacteria* might be due to the decrease of members of the other taxa, less resistant to the alternation of wet/dry periods. Shrestha and colleagues also observed that members belonging to these two classes were more active in oxic paddy soil (Shrestha *et al.*, 2009). The genus *Ramlibacter* sp., including aerobic heterotrophs, often isolated from soils, has been demonstrated to be resistant to dryness stress (Lee *et al.*, 2014). The genus *Sphingomonas*, strictly aerobic and facultative photoorganotroph (Yabuuchi and Kosako, 2005), found its ideal habitat in the

rhizoplane of D. *Piscinibacter* are described as chemoheterotrophs and facultative aerobic (Song and Cho, 2007; Stackebrandt *et al.*, 2009). *Deltaproteobacteria* are more frequently detected in anoxic rice paddies (Shrestha *et al.*, 2009; Pester *et al.*, 2012; Lu *et al.*, 2006). According to these observations, in this study members of this class were more abundant in CF rhizosphere compartments.

Most of the above-mentioned genera, with high relative abundance in the different treatments, are not known to have arsenic-processing capacities. This aspect probably reflects the fact that the soil used for this experiment did not contain high As concentration. Therefore, As was not the main factor shaping the bacterial communities in this environment.

In this study, we observed that DSRB were more abundant in rhizosphere soil of CF if compared to rhizoplane, whereas DAsRB and DFeRB in the same treatment were closer to the roots (Fig. 4). The observed pattern, with DFeRB more abundant than DAsRB, probably reflects the redox scale predicted by Kocar and Fendorf (2009), who demonstrated that at pH < 6.5 Fe(III)-reduction is favored over dissimilatory As(V)-reduction. Members of the genera *Geobacter* and *Anaeromyxobacter* were confirmed to be common Fe(III)-reducing inhabitants of anoxic paddy fields, promoted by a CF water regime (Hori *et al.*, 2010; Shrestha *et al.*, 2009; Treude *et al.*, 2003).

The best habitat for FeOB was the rhizosphere of D plants. This could be due to a sharp redox interface in CF and CF-D, with only little areas with the optimal concentration of Fe(II) and O_2, and a wider microoxic area in D rhizosphere. Similarly, populations of AsOB were more abundant in D rhizosphere, confirming what observed in previous studies (Das *et al.*, 2016). The presence of both FeOB and AsOB might contribute to the conversion of As(III) to As(V) and its co-precipitaion with Fe oxides. Conversely, in CF DFeRB predominate over FeOB. Therefore, the process of dissolution of Fe oxides and consequent release of As is promoted over its precipitation.

Genera of SOB were more abundant in the rhizoplane of CF plants, in contrast with what observed by Das et al. 2016. The rhizoplane of CF plants could be an optimal habitat for SOB since reduced sulfur compounds are produced by DSRB but, at the same time, little amounts of oxygen needed by these organisms are released by the roots. It has often been reported that SOB are related to ecosystems characterized by sharp opposing gradients of O_2 and reduced sulfur compound (Dahl *et al.*, 2008). Considering that SOB potentially promote sulfide minerals dissolution, their presence in CF rhizosphere might contribute to As release from sulfide minerals. Microbial mineral weathering for nutrient acquisition has already been reported as a cause of As release to soil solution from apatite (Mailloux *et al.*, 2009).

Although in the rhizosphere soil of all the treatments ARB were equally represented, in the rhizoplane these organisms were more abundant in CF plants. This was probably a consequence of the fact that

arsenic was strongly released in that region, where iron oxides and sulfide minerals are present and probably dissolved by DFeRB and SOB. In the rhizoplane, the bacterial diversity was generally lower than in the rhizosphere soil. In the latter, the higher diversity included microorganisms generally present in soil characterized by an average distribution of As resistance. On the other hand, in the rhizoplane the plant selected for microorganisms with plant growth promoting properties. In such communities As resistance would be selected under As pressure, encountered only under CF.
The ability of As methylation was present in rice rhizosphere. Particularly, in CF plants this activity was selected and probably enhanced by the presence of As(III) and organic matter, which are the substrates for this reaction. This is in accordance with recent evidence that under CF more organic As is produced with respect to sprinkler irrigation and aerobic rice (Moreno-Jiménez *et al.*, 2014; Li *et al.*, 2009).

2.5 Conclusions

Together, these outcomes indicate a dramatic effect of water management of rice paddies in shaping the rhizosphere microbiota. Continuous flooding promotes the proliferation of As-releasing bacterial taxa, whereas in aerobic rice microorganisms that reduce the solubility of As in the soil solution are favored. Introducing a 2 weeks-drainage period before the flowering stage within a continuous flooding regime leads to intermediate relative abundances of As-affecting genera.
Reducing the flooding intensity might be helpful for its selection of an As-stabilizing microbial community with the reduction of bioavailable As concentration in the soil solution, thus decreasing As contamination in rice grains.

Acknowledgements

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Table 1 Chemical analyses of the soil solution in the macrocosms managed under continuous flooding (CF), under continuous flooding with 14 days drainage before flowering (CF-D) and under watering every 10 days (D). Data are reported ± standard deviation.

Chemical Parameter	CF	CF-D	D
Tot As (μg L^{-1})	125.3±5.2	1.82±0.41	bdl[1]
Fe(II) (mg L^{-1})	51.1±1.75	0.05±0.01	0.21±0.05
S-SO_4^{2-} (mg L^{-1})	0.06±0.01	4.09±0.27	1.69±0.52
N-NO_3^{2-} (mg L^{-1})	0.04±0.01	0.27±0.05	2.31±0.91
P-PO_4^{2-} (mg L^{-1})	0.33±0.18	0.19±0.001	0.23±0.03
pH	6.2±0.25	5.9±0.25	6±0.34

[1]below detection limit

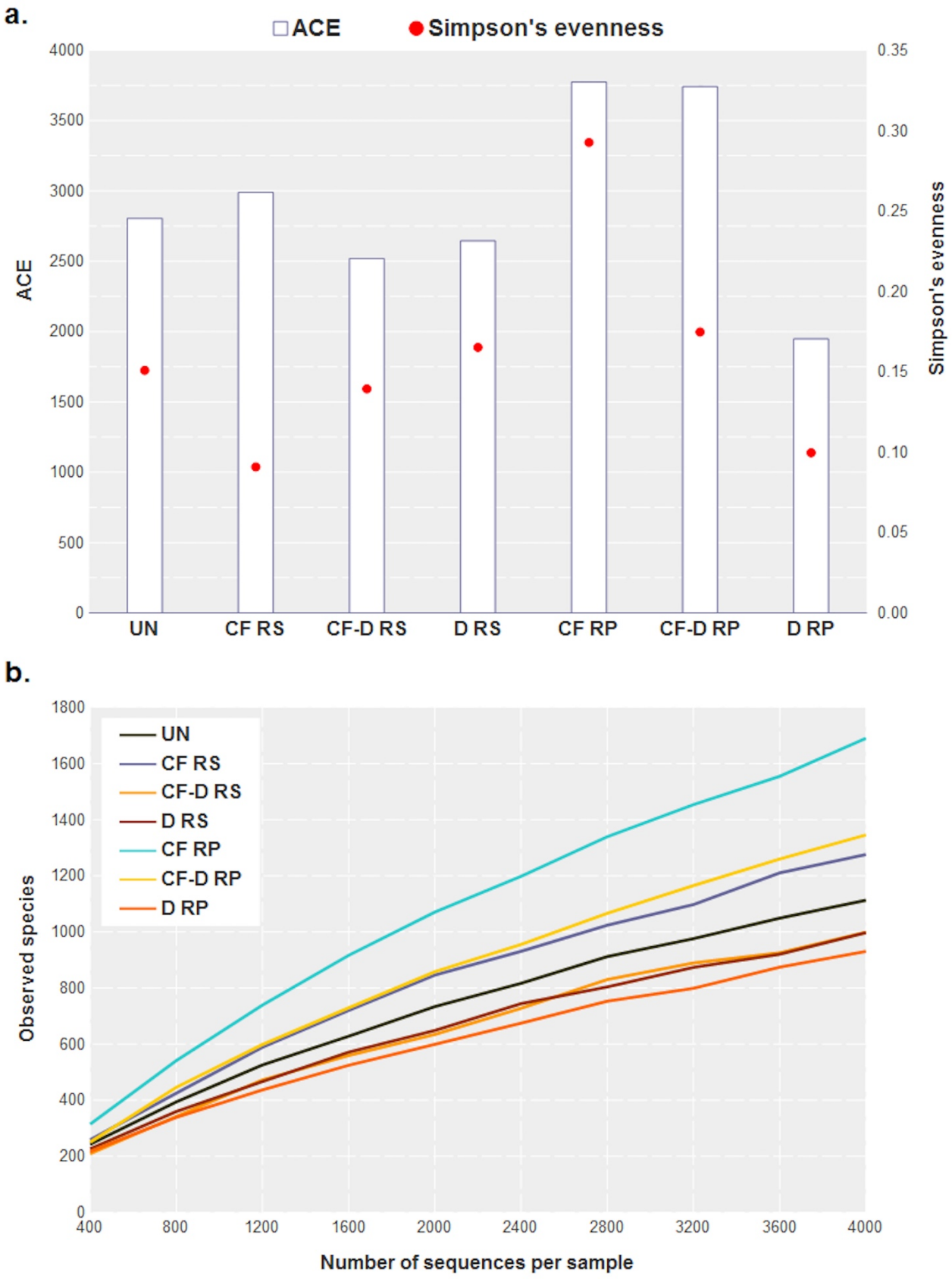

Fig. 1 Alpha diversity evaluated with ACE index and Simpson's evenness measure, assuming a sample size of 2000 reads (a) and by observed species on rarefied samples (b). Values are shown for the unplanted soil (UN), rhizosphere soil and rhizoplane treated under continuous flooding (CF RS and CF RP), under CF with 14 days of drainage before flowering (CF-D RS and CF-D RP) and under watering every 10 days (D RS and D RP).

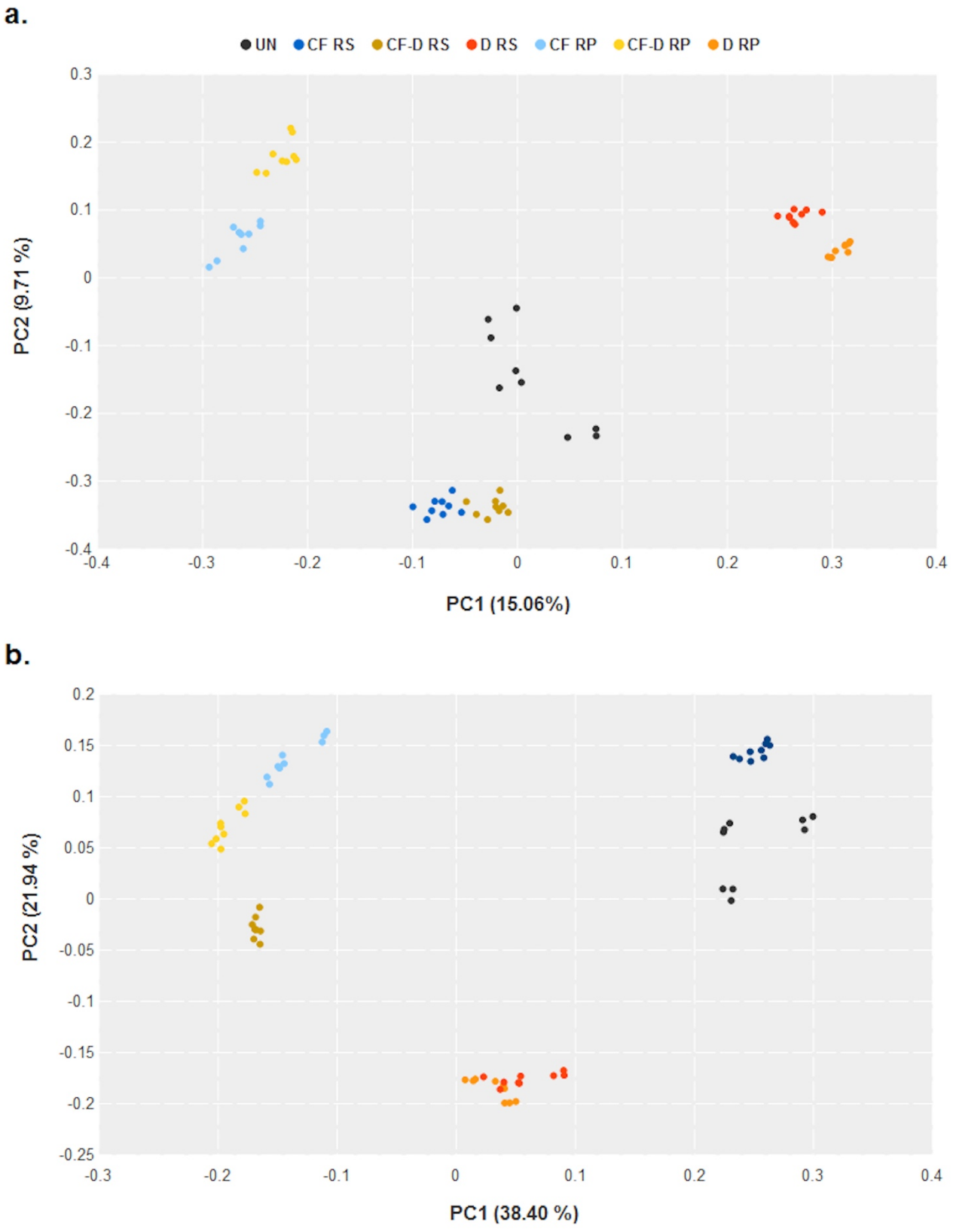

Fig. 2 Beta diversity analysis of all treatments, included unplanted soil (UN), rhisosphere soil and rhizoplane treated with continuous flooding (CF RS and CF RP), with 14 days of drainage before flowering (CF-D RS and CF-D RP) and with watering every 10 days (D RS and D RP). The principal coordinate analysis was performed calculating unweighted (a) and weighted unifrac distances (b) according to Hamady and Knight (2009).

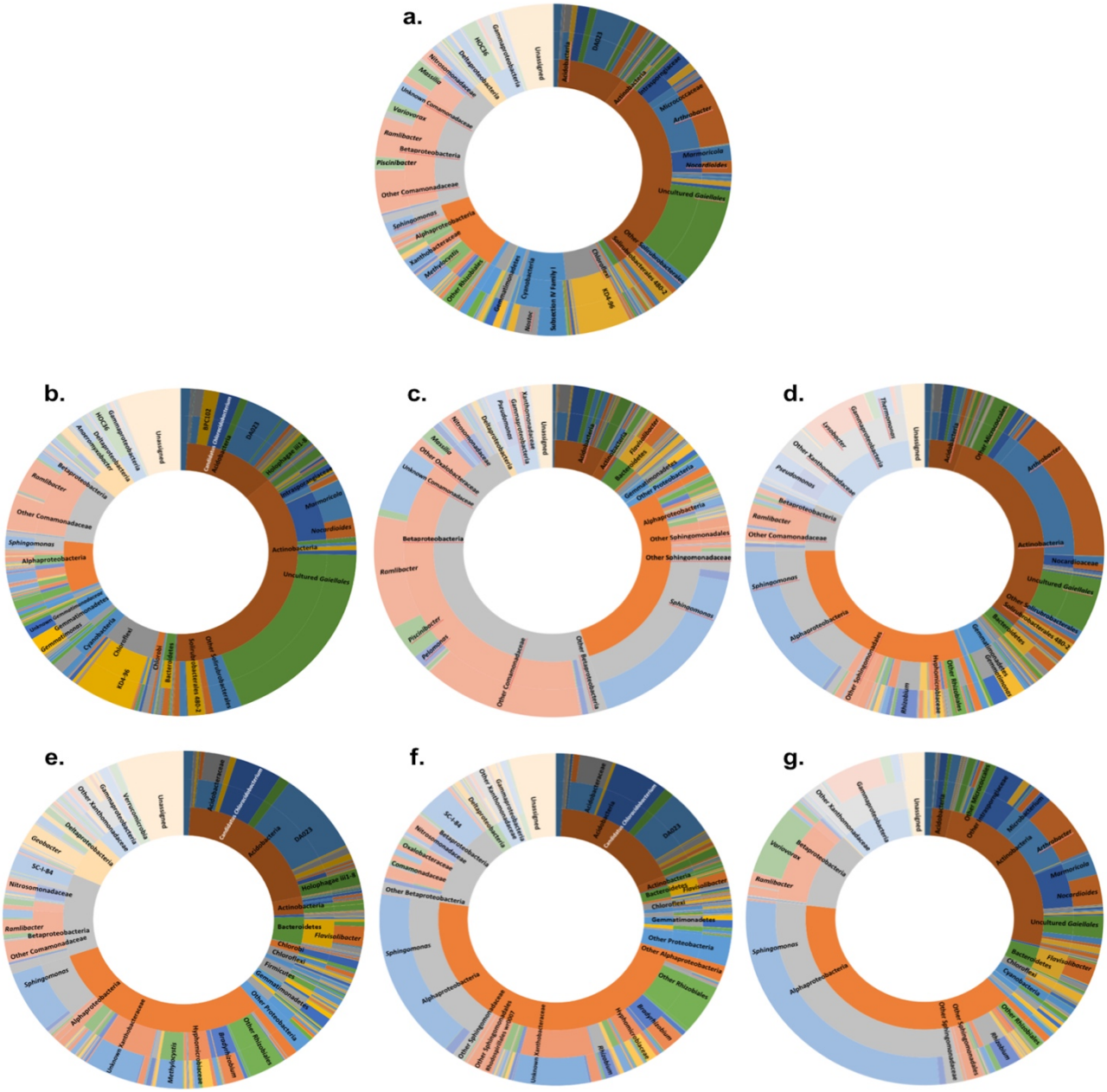

Fig. 3 Relative abundance of phyla and classes (inner ring), families (middle ring) and genera (outer ring) retrieved in the treatments: unplanted soil (a), continuously flooded rhizosphere soil and rhizoplane (b, e), with 14 days of drainage before flowering (c, f) and with watering every 10 days (d, g).

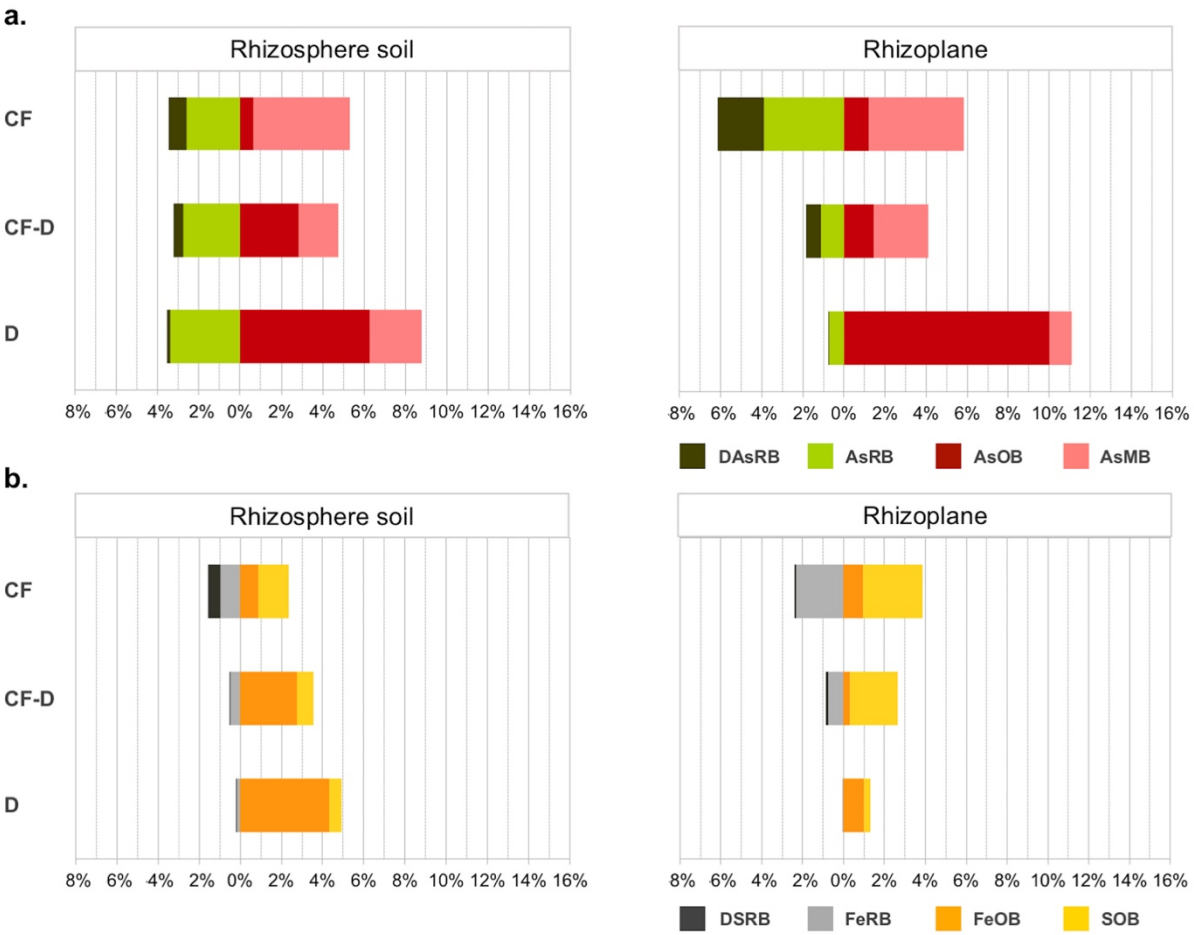

Fig. 4 Relative abundance of species potentially able to process arsenic directly (a) or indirectly as a consequence of their metabolism (b) in rhizosphere soil and rhizoplane of plants cultivated under continuous flooding (CF RS and CF RP), under continuous flooding with 14 days drainage before flowering (CF-D RS and CF-D RP) or with watering every 10 days (D RS and D RP). The metabolic groups considered in this analysis were dissimilatory As(V)-reducing bacteria (DAsRB), As-resistant bacteria (AsRB), As(III)-oxidizing bacteria and As(III)-methylating bacteria (AsMB), dissimilatory Fe(III)-reducing bacteria (DfeRB), Fe(II)-oxidizing bacteria (FeOB), dissimilatory SO_4^{2-}-reducing bacteria (DSRB) and sulfur-oxidizing bacteria (SOB)

3.

Water regime affects iron- and sulfur-oxidizing bacteria living on rice roots

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Abstract

Water management in rice paddies greatly influence arsenic uptake, with continuous submersion responsible for high metalloid concentrations in rice grains. Iron plaques formation on rice roots surface contributes to decrease arsenic uptake and translocation into rice grains. At the same time, reduced sulfur compounds surrounding the roots reduce arsenic mobility and are associated to iron minerals. Research on the effect of different water regimes on iron- and sulfur-oxidizing bacteria associated to iron plaques still needs to be improved.

Rice roots from plants cultivated either under continuous flooding or with watering every 10 days (aerobic rice) were incubated in iron-oxidizing bacteria-specific enrichment cultures, using FeS gradient tubes. In order to test the effect of As exposure on these microorganisms, different enrichment cultures from both water regimes were exposed to either arsenate or arsenite. After 3 transfers, the biomass developed in the visible iron-oxidation region of the gradient tubes was analyzed with Scanning Electron Microscopy (SEM) coupled with Energy-dispersive spectroscopy (EDS). Furthermore, from the same biomass, DNA was isolated and 16S rRNA genes were amplified and cloned. Different *Proteobacteria* communities were selected in roots grown under different water regimes. Under continuous flooding, the presence of arsenate favored the proliferation of versatile iron-oxidizing species affiliated to the genera *Dechloromonas* and *Azospira*. These microorganisms were associated to putative iron (hydr)oxide crystalline structures. Exposure to arsenite decreased drastically the diversity in the enrichment cultures, with the development of the sulfur-oxidizing species of *Thiobacillus thioparus*, likely growing on sulfide released by FeS. In aerobic rice roots, iron-oxidizing microorganisms represented only a minor fraction in the enrichment cultures, and

disappeared completely when exposed to arsenic, indicating a higher susceptibility of these bacteria to arsenite.

These results indicate a strong impact of water regime on iron-oxidizing bacteria associated to the iron plaques. Continuous flooding selects on rice roots surface both iron- and sulfur-oxidizing bacteria resistant to different arsenic species, contrary to what happens in aerobic rice.

3.1 Introduction

Irrigation management of rice paddies is a matter of debate for several reasons. Rice plants usually grow under complete flooding for the whole life cycle. This represent an issue in drought-affected areas (Spanu *et al.,* 2009). Moreover, complete flooding has a strong impact on rice contamination by arsenic (As), with an increase of metalloid accumulation in rice grains with respect to aerobic rice (Das *et al.,* 2016; Ma *et al.,* 2014; Somenahally *et al.,* 2011 a and b; Spanu *et al.*, 2012; Li *et al.,* 2009). Rice plants accumulate arsenic (As) more efficiently with respect to other food crops (Su *et al.,* 2010). Furthermore, continuous flooding leads to redox potential decrease in soil solution, promoting As-releasing processes such as ferric iron [Fe(III)] (hydr)oxides dissolution (Kögel-Knabner, 2010) and arsenate [As(V)] reduction to arsenite [As(III)] (Takahashi *et al.*, 2004; Yamaguchi *et al.*, 2014). Different rice varieties have been selected to resist with lower flooding intensity (Peng *et al.,* 2006; Pinheiro *et al.,* 2006; Kato *et al.,* 2009). However, finding a balance between water supply, rice contamination and yield is still quite challenging.

Under continuous flooding, biogeochemical reactions occurring in rice rhizosphere are sharply influenced by oxygen release from roots aerenchyma (Colmer, 2003). This promotes the oxidation of ferrous iron [Fe(II)], with the development of Fe mineral plaques, that form a compact thin layer enveloping the roots (Zhao *et al.,* 2009). Arsenate has a high affinity for Fe(III) (hydr)oxides (Roberts *et al.,* 2004). In fact, Fe plaques adsorb a significant amount of As(V), preventing its assimilation by the plant (Zhao *et al.,* 2009; Seyfferth *et al.,* 2010).

The role of Fe(II)-oxidizing bacteria (FeOB) in rice rhizosphere Fe cycle is crucial (Kögel-Knabner, 2010; Emerson *et al.,* 2010; Oremland *et al.,* 2005). Under continuous flooding, FeOB contribute to the formation of Fe plaques around the roots (Weiss *et al.,* 2003) using Fe(II) as elector donor in their energy metabolism (Dubinina and Sorokina, 2014). FeOB include a wide range of species in terms of ecological niche: neutrophilic, acidophilic, aerobes and anaerobes (Hedrich *et al.,* 2011). Some FeOB are characterized by extracellular deposition of Fe(III)-oxides either as stalks, like in *Gallionella ferruginea* (Emerson *et al.,* 2010), or as sheaths that cover the entire cells like in *Acidovorax* sp. (Hohmann *et al.,* 2010).

The enrichment cultivation of FeOB based on the "gradient tubes" was firstly described in 1957 by Kucera and Wolfe for the specific isolation of *Gallionella* sp.. This technique was modified to extend the specificity to other betaproteobacterial FeOB as *Sideroxydans* sp. and *Leptothrix* sp. (Emerson and Moyer, 1997; Weiss *et al.,* 2007; Emerson *et al.,* 2010). These organisms are neutrophilic and microaerophilic and grow at the redox interface where O_2 concentration is below 10 μM (Emerson *et al.,* 2010). Despite their recalcitrance in the laboratory, the employment of FeOB for As removal has been taken into account in a variety of applications, such as drinking water biological filtration and groundwater remediation (Pokhrel and Viraraghavan, 2009; Katsoyiannis and Zouboulis, 2006; Mouchet, 1992).A first step towards a better knowledge on their applicability for As remediation purposes in rice paddies was taken by assessing the influence of water regimes on FeOB species living on rice roots.

3.2 Material and methods

3.2.1 Rice roots sampling

The roots used for inoculation were sampled from rice plants (*Oryza sativa* subsp. *japonica*, var. Loto) cultivated in open-air macrocosms managed either with continuous flooding (CF) or with watering every 10 days (aerobic rice, AR). The soil used for the cultivation was an acidic (pH 6) sandy-loam rice field soil from Pavia (Italy), with the total Fe and As concentrations 33.1 ± 1.04 g kg^{-1} and 11.4 ± 0.74 mg kg^{-1} respectively. Rice plants were sampled at flowering stage, which occurred after 100 days from germination. The roots were detached from the epigeal portion, washed with sterile distilled water, ground to 2-3 cm length fragments and used for inoculation.

3.2.2 Iron-oxidizing bacteria enrichment cultures

Iron-oxidizing bacteria enrichment cultures were performed with the gradient tube method according to Emerson and Moyer (1997). Briefly, sterile glass tubes (16 x 120 mm) with screw caps were filled 2/3 (v/v) with a bottom layer containing 0.75 mL of iron sulfide (FeS) and 0.75 mL of Modified Wolfe Mineral Medium (Hanert *et al.*, 1992) added with 1 % agarose, and a top layer with 4.5 mL of the same medium containing 0.15 % of agarose. Before sterilization, 0.5 mM $NaHCO_3$ was added to the top layer. For the bottom layer, FeS was prepared adding 46.2 g of ferrous sulfate ($FeSO_4$) and 39.6 g of sodium sulfide (Na_2S) to 300 mL of distilled water at 50 °C under shaking. After 3 min of continuous stirring, the black FeS sludge was decanted into a narrow-mouthed 500 mL dark glass bottle. The bottle was filled to the top with distilled water to limit oxygen influx and capped. To wash FeS, this was allowed to settle for several hours, replacing the overlaying water at least five times.

After washing, the pH of the FeS solution was 7. After hardening, 1 mL L^{-1} of Wolfe's Vitamin Solution (WVS) (Wolin *et al.*, 1963) was added to the medium. For As amendment, 500 mM stock solutions of $Na_2HAsO_4 \cdot 7H_2O$ and of $NaAsO_2$ (Sigma-Aldrich, St. Louis, MO, USA) were prepared for As(V) and As(III), respectively. The solutions were sterilized with cellulose-acetate filters (Sartorium Stedim Biotech, Germany) and included in the autoclaved top layer immediately before filling the tubes.

In total, 6 enrichment categories were set up, including CF and AR without As amendment, CF and AR amended with 400 μM As(V) (CFV and ARV) and CF and AR amended with 4 μM As(III) (CFIII and ARIII). To compare biological and chemical Fe(II) oxidation, abiotic tubes were prepared for each category.

For each treatment, 10 g of root fragments were inoculated in the top layer. The tubes were incubated for 20 days at room temperature in the dark. Every 20 days, 100 mg of biomass grown within the orange Fe(II) oxidation band was transferred to a fresh tube.

3.2.3 Scanning Electron Microscopy

To analyze microorganisms and Fe (hydr)oxides developed in the gradient tubes, the biomass grown within the orange Fe(II) oxidation band after 3 transfers was sampled and observed with Scanning Electron Microscopy (SEM). From each treatment, 1 g of material was suspended in 5 mL of 2 % glutaraldehyde (dissolved in 1x PBS) for 24 h. The suspensions were centrifuged at 10000 g for 5 min and subsequently washed in phosphate-buffered saline (PBS) solution (0.1 M, pH 7.2) for 8 h, resuspended in 1 % OsO_4 (dissolved in H_2O) for 1 h at room temperature and progressively dehydrated in EtOH from 2 % to 100 %. After final dehydration in $[(CH_3)_3Si]_2NH$ (HMDS), samples were sputter-covered with gold with High Vacuum Coater (Leica Mycrosystems, Wetzlar, Germany). The observations were performed with a microscope Leo 1430 (Zeiss) equipped with energy-dispersive spectroscopy (EDS) with INCA probe and analyzed at the IBis platform at the Centro Interdipartimentale di Microscopia Avanzata (CIMA) of the University of Milano.

3.2.4 16S rRNA gene clone library preparation

From 0.25 g of biomass collected within the orange Fe(II) oxidation band after 3 transfers, DNA was isolated using the UltraClean® Microbial DNA Isolation kit (MO BIO), according to manufacturer's instructions.

To prepare 16S rRNA gene clone libraries, this target was amplified mixing 0.3 μM of primers GM-3F (5'-AGAGTTTGATCMTGGC-3') and GM-4R (5'-TACCTTGTTACGACTT-3') (Muyzer *et al.,* 1995) with 1x Taq PCR Master Mix (QIAGEN), 20 ng of template DNA and PCR-grade water

(Sigma-Aldrich) to a final volume of 25 µL. The thermal protocol was carried out on T-Gradient Biometra thermocycler and included 5 min of denaturation at 95°C, 35 cycles of denaturation for 1 min at 95°C, 40 sec of annealing at 55°C and 1 min and 40 sec of elongation at 72°C, and a final elongation for 10 min at 72°C. The PCR products were cloned on TOP10 chemically competent *E. coli* cells using TOPO® TA Cloning® Kit (Invitrogen) and pCR™2.1-TOPO® vector following manufacturer's instructions. From the positive clones, the plasmid was extracted with UltraClean™ 6 minutes Mini Plasmid Prep Kit (MO BIO).

3.2.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

To cluster the different clones in Operational Taxonomic Units based on their insert, 200 µg of the extracted plasmids were digested over night at 37 °C with 0.5 U of HaeIII restriction enzyme and 1X REact®2 buffer (Invitrogen) in a total volume of 10 µL. Digestion products were loaded on a 3% agarose gel prepared with Tris-acetate-EDTA (TAE) 1X buffer and separated applying 50 mV for 3 h. Inserts showing the same ARDRA profile were clustered in unique OTUs and one representative for each OTU was sequenced.

3.2.6 Sequence analysis

Sequences were edited and aligned using MEGA software version 6 (Tamura *et al.*, 2013) and compared to the GenBank database with BLASTn. Clone and reference sequences where aligned with MUSCLE (Edgar, 2004) and trees were built using the maximum likelihood method based on the Tamura-Nei model (Tamura *et al.*, 1993).

3.3 Results and Discussion

3.3.1 Fe(II)-oxidizing bacteria enrichment cultures

Within few days after gradient tubes preparation and inoculation, an orange band was formed in the top layer (Fig. 1). In the abiotic tubes, this band was more diffuse and at 1 cm from the black FeS bottom layer. In the inoculated tubes, the orange band was sharper, more intense and at 1 cm from the surface. In some inoculated tubes, a second band was present at 1 cm from the bottom layer, similarly to the abiotic samples, probably indicating anaerobic oxidation of Fe(II).

3.3.2 Scanning Electron Microscopy

To characterize microorganisms and Fe (hydr)oxides developed in the gradient tubes, SEM-EDS analysis was carried out in selected samples from the third transfer of AR, CFV and CFIII, and from the abiotic control.

In the abiotic samples, amorphous structures were visualized (Fig. 2a), in which Fe was present at low concentrations (Fig. 2b). In CFV enrichments, irregular crystalline structures enveloping putative round-shaped cells with diameter below 1 μm and nano-filaments were detected (Fig. 3a and 3b). The EDS analysis revealed the presence in the irregular crystals of Fe, C and O_2 (Fig. 3c and 3d, Table 1), leading to the hypothesis that these structures were made of Fe-oxides minerals. In concomitance with these structures, a little amount of As was also detected. In CFIII enrichments, similar crystalline structures were retrieved (data not shown). In AR enrichment cultures, cells with 2 μm diameter with nano-globules on the surface were present (Fig. 4a and 4b). The presence of Fe on these structures was confirmed by the EDS analysis. In previous studies, the presence of Fe encrustations on microbial cell surface has been reported (Hohmann *et al.,* 2010). However, the structures observed in this study were different with respect to the above-mentioned encrustations.

3.3.3 Identity of enriched Fe(II)-oxidizing bacteria

From ARV, ARIII and CF enrichment cultures after 3 transfers no DNA was obtained. These results were reproducible, since several attempts were performed.

From all enrichments, only 16S rRNA genes of microorganisms belonging to the class *Proteobacteria* were retrieved (Fig. 5). Enrichments CFV and CFIII displayed a lower diversity were with respect to AR (Fig. 6). In fact, while in AR enrichments bacteria affiliated to *Alpha-*, *Beta-* and *Gammaproteobacteria* were retrieved, in CF most of the clones were classified within the *Betaproteobacteria*, with only one representative within the *Gammaproteobacteria*. However, AR enrichments from which microbial DNA was retrieved were not exposed to As pressure. This might explain the higher diversity observed in this enrichment line.

CFV was dominated by few species, 73% of which belonging to the genera *Azospira, Dechlorosoma* and *Pseudomonas*, known to be able to oxidize Fe(II) for metabolic purposes (Fig. 6, see Table 2 for references). The remaining community was represented by *Thiobacillus thioparus* and *Massilia timonae*. In CFIII enrichments, only *T. thioparus* and *M. timonae* were retrieved. None of these species are known to oxidize Fe(II) for metabolic purposes. However, *T. thioparus* is an obligate chemolithoautotroph, typical of Italian rice fields (Stubner *et al.*, 1998), which uses sulfide (S^{2-}) as electron acceptor. These microorganisms might have survived by the oxidation of S^{2-} present in the FeS bottom layer. Its presence might have supported the growth of *M. timonae*, which is an aerobic

heterotroph (Table 2). The ability of *M. timonae* to resist to As(V) and to As(III) is suggested by the presence of genes homologous with As(V) reductase and with *arsB*, deposited in GenBank with accession numbers EKU80358.1 and EKU80575.1 respectively by Earl and colleagues (unpublished work). This could explain the survival of these microorganisms in the enrichments added with As.

Members of the genera *Azospira* and *Pseudomonas* are known to be able to oxidize As(III) (Cavalca *et al.,* 2013). However, these species did not survive in CFIII. In this case, the concentration of AsIII might have been too high for these microorganisms in these conditions.

A weird thing to explain is the absence of biomass in the CF enrichment line. The presence of As in CFV and CFIII enrichments likely selected different consortia with respect to CF during the first transfers. This community might have been based on the chemolithoautotrophic growth FeOB and/or sulfide-oxidizing microorganisms difficult to maintain in laboratory cultures. This is quite a common issue in the history of FeOB isolation (Emerson and Moyer, 1997). Another hypothesis is that the absence of As pressure allowed the proliferation of heterotrophic bacteria supported by organic matter derived from the inoculum, leading to the loss of FeOB at the beginning of the incubation. As a consequence, organic matter decreased over the transfers, leading to the decline of the whole community.

In AR enrichment cultures, several typically rhizospheric species were enriched, but only *Pseudomonas* sp. and *Lysobacter* sp. were related to species known to oxidize Fe(II) (Table 2). Together, these two species represented 15% of the total community. Among the genera retrieved in AR, *Caulobacter* and/or *Asticcacaulis* might resemble the microorganisms observed in Fig. 3 (see references in Table 2). Curiously, although As-resistant species were present in AR, none of these survived in the As-added enrichments ARV and ARIII. For example, *P. putida* has been reported to resist to As(V) by reducing it to As(III), which is then extruded outside the cell using the As(III)-efflux pump encoded by the *arsB* gene (Achour *et al.,* 2007). In the present study, the community could have been supported by few autotrophic organisms such as *Sulfuricella denitrificans*, which, however, did not find their optimal substrates (Table 2). With the selective pressure of As, the general diversity might have decreased too much to allow nutrient recycling.

Sulfur-oxidizing microorganisms, typical of ecosystems with opposing gradients of S^{2-} and oxygen, play an important role in rice rhizosphere sulfur-cycling (Stubner *et al.*, 1998; Dahl *et al.,* 2008). With their activity, these organisms promote As release from S^{2-} minerals, probably having an important role in As mobilization in CF plants.

3.4 Conclusion

These outcomes revealed a huge diversity in the root communities of rice plants selected by different water regimes. Continuous flooding promoted the presence of versatile FeOB belonging to *Azospira* and *Dechlorosoma* genera as well as the sulfide-oxidizing species *Thiobacillus thioparus*. In the gradient tubes, sulfide-oxidizing microorganisms as well as FeOB might sustain long-lasting communities resistant to As.

Table 1 Content of C, O, Fe and As in the EDS spectra shown in Fig. 2, 3 and 4.

Element	*		1		2		3		4	
	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %
C	63.07	70.81	28.9	43.00	35.17	48.10	64.27	72.64	63.02	71.20
O	31.13	27.08	42.34	47.15	44.20	45.37	28.57	24.24	31.39	26.62
Fe	1.15	0.28	25.33	8.08	18.17	5.34	1.89	0.46	2.68	0.65
As	-	-	1.01	0.24	0.86	0.19	-	-	-	-

Table 2 Main phenotypic and physiologic features of the taxa found in the enrichment cultures.

Species	Shape	Size	E^- acceptor	E^- donor	Reference
Asticcacaulis taihuensis	Rod	0.5-0.7 x 1.4-2.0 µm	Oxygen, nitrate	Sugars, starch	Vasilyeva *et al.,* 2006 Liu *et al.,* 2005
Caulobacter sp.	Vibrioid, fusiform, rod	0.4-0.5 x 1-2 µm	Oxygen, nitrate	Sugars, aminoacids	Henrici and Johnson, 1935 Poindexter, 1964 Abraham *et al.,* 1999
Azospirillum sp.	Spiral, vibrioid	1.0 x 1.5-5 µm	Oxygen, nitrate	Sugars	Xie and Yokota, 2005
Kaistia sp.	Rod, coccoid	0.6-1 µm	Oxygen, nitrate	Sugars, aminoacids, acetate, small organic acids	Jin *et al.,* 2011
Pseudolabrys sp.	Rod	-	Oxygen, nitrate	Acetate, aconitate, small organic acids	Kämpfer *et al.,* 2006
Rhizobium sp.	Rod	1 µm	Oxygen	Small sugars and organic acids, acetate	van Berkum *et al.,* 1998
Achromobacter sp.	Rod, coccoid	1-5 µm	Oxygen, nitrate	Sugars, small organic acids	Coenye *et al.,* 2003 Gray *et al.,* 2010
Massilia timonae	Straight rod	1-3 µm	Oxygen	Sugars, aminoacids, small organic acids	La Scola *et al.,* 1998
Sulfuricella denitrificans	Rod	0.8-2.0 x 0.4-0.6 µm	Oxygen, nitrate, nitrite	Thiosulfate, elemental sulfur	Kojima and Fukui, 2010
Thiobacillus thioparus	Rod	1.0-2.0 µm	Oxygen	Sulfide, thiocyanate	Kelly and Wood, 2000
Azospira sp.	Curved rod	0.4-0.6 µm	Oxygen, nitrate, perchlorate	Small organic acids, ferrous iron	Reinhold-Hurek and Hurek, 2000 Dubinina and Sorokina, 2014
Dechlorosoma sp.	Rod	1.0 x 0.3 µm	Oxygen, nitrate, perchlorate	Short organic fatty acids, ferrous iron	Achenbach *et al.,* 2001 Dubinina and Sorokina, 2014
Pseudomonas sp.	Rod	0.9-1.5 µm	Oxygen, nitrate	Sugars, organic acids, aminocids, aromatic compounds, ferrous iron	Stanier *et al.,* 1966, Neumann *et al.,* 2005, Dubinina and Sorokina, 2014

Lysobacter sp.	Rod	0.3-0.4 x 2.5-5 µm	Oxygen	Sugars, organic acids, aminocids, ferrous iron	Emerson and Moyer, 1997 Sullivan *et al.,* 2003, Siddiqi and Im, 2016

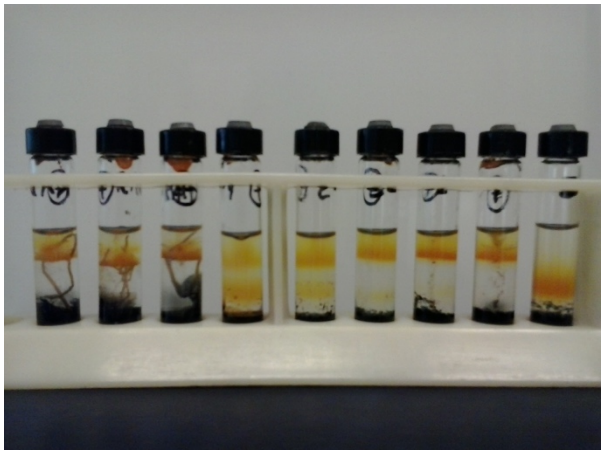


Figure 1 Iron oxidizing bacteria enrichment cultures. From the left, first inoculation of CFV, CFIII and AR, second transfer of CF, ARV, AR, CFV, CFIII and abiotic control.

a. **b.**

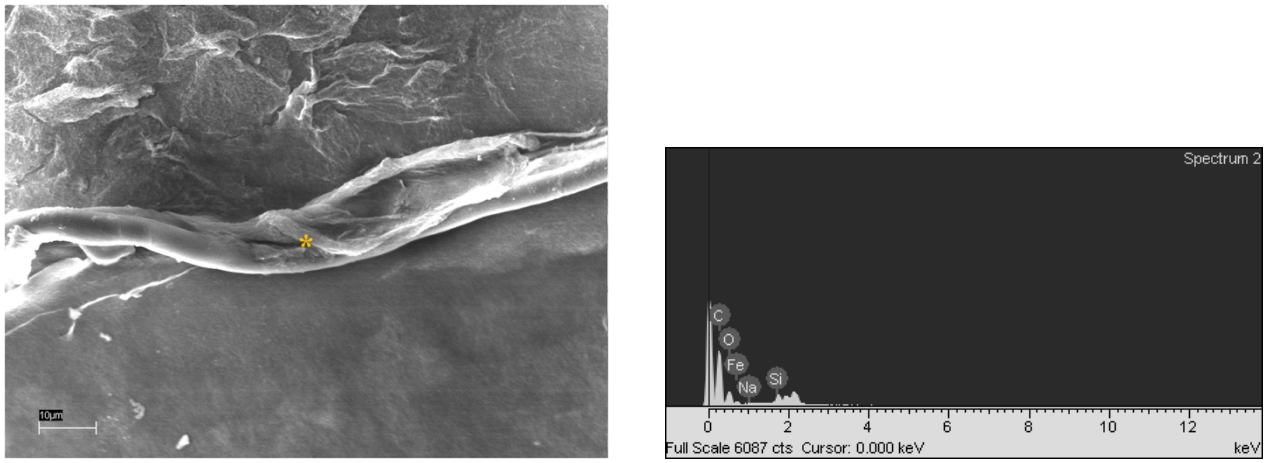
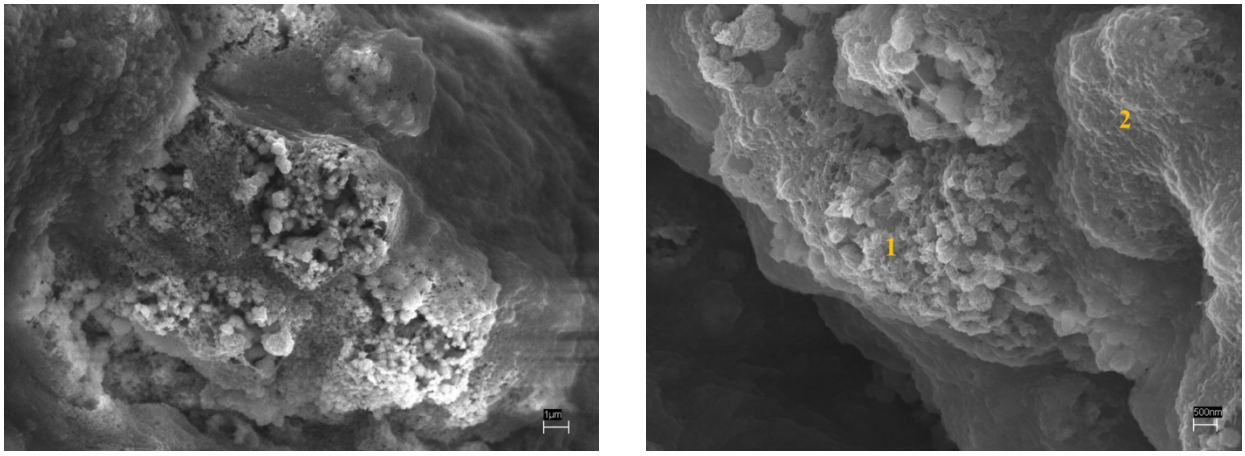


Figure 2 Scanning Electron Microscopy photograph at 1200 magnifications on a portion of the orange Fe-oxidation band sampled from an abiotic gradient tube (a) and EDS spectrum (b) performed in the point indicated by the star.

a. **b.**



c. **d.**

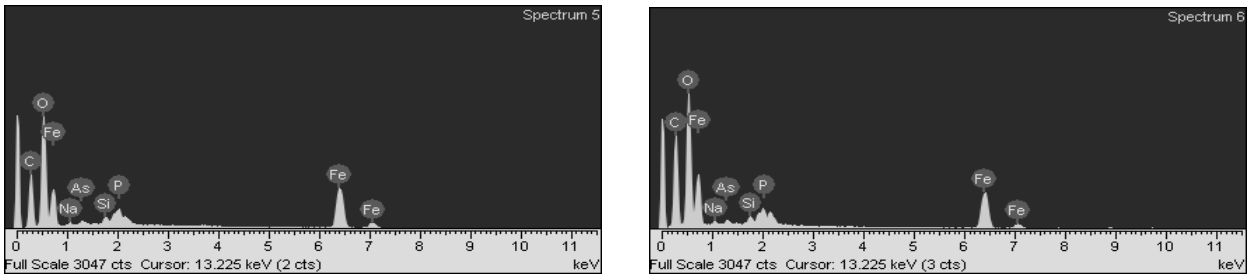
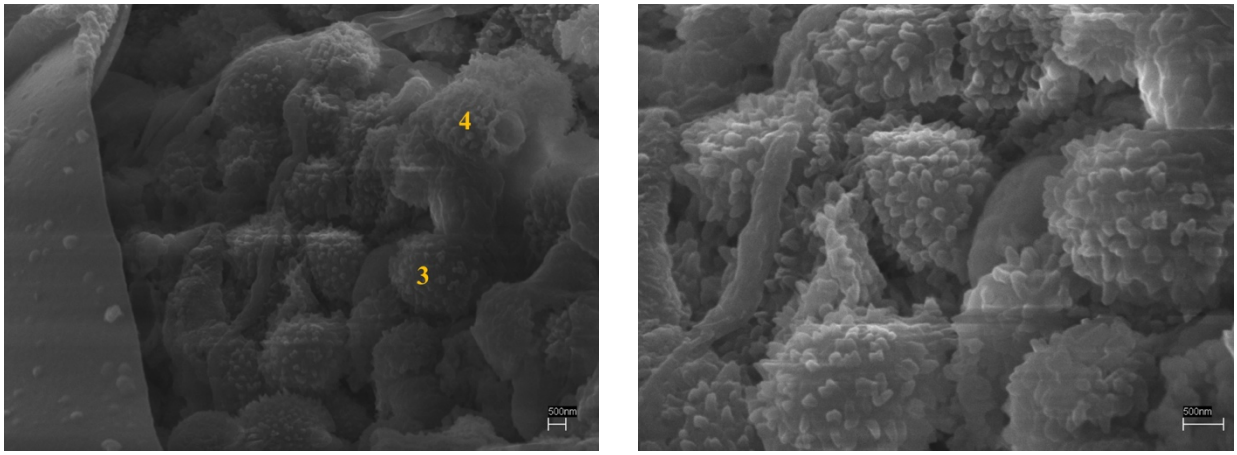


Figure 3 Images obtained with SEM performed on a portion of the orange Fe-oxidation band sampled from CFV enrichment cultures at 5300 (a) and 10000 (b) magnifications, and related EDS spectra (c, d).

a. **b.**



c. **d.**

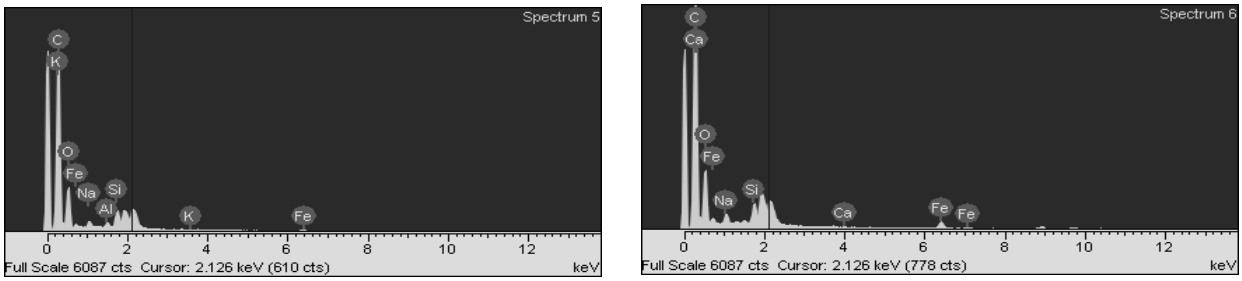


Figure 4 Images obtained with SEM performed on a portion of the orange Fe-oxidation band sampled from AR enrichment cultures at 7500 (a) and 17000 (b) magnifications, and related EDS spectra (c, d).

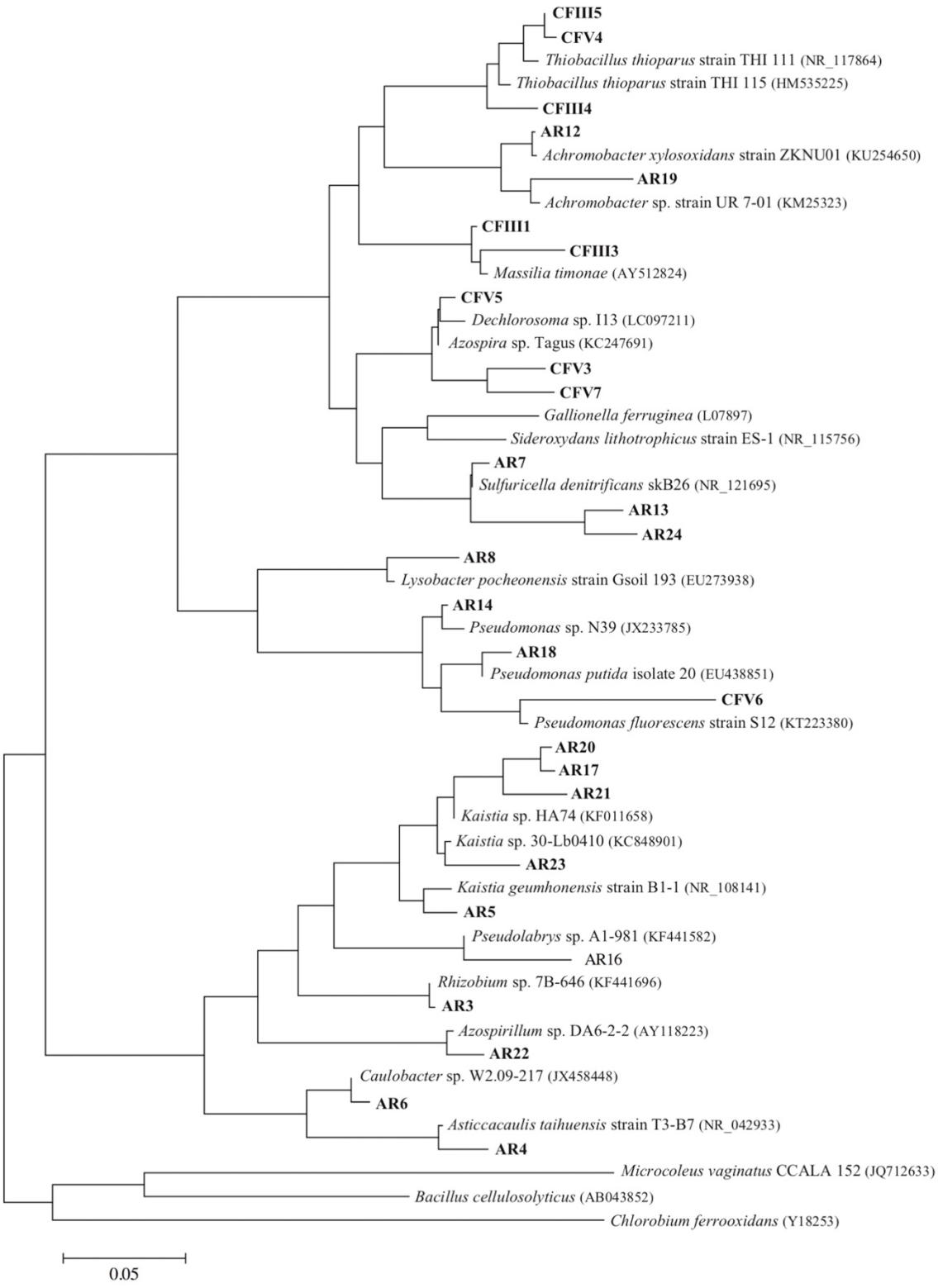


Figure 5 Phylogenetic affiliation of the 16S rRNA gene clones obtained from AR, CFV and CFIII enrichment cultures. The evolutionary history was inferred with the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al.*, 1993). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

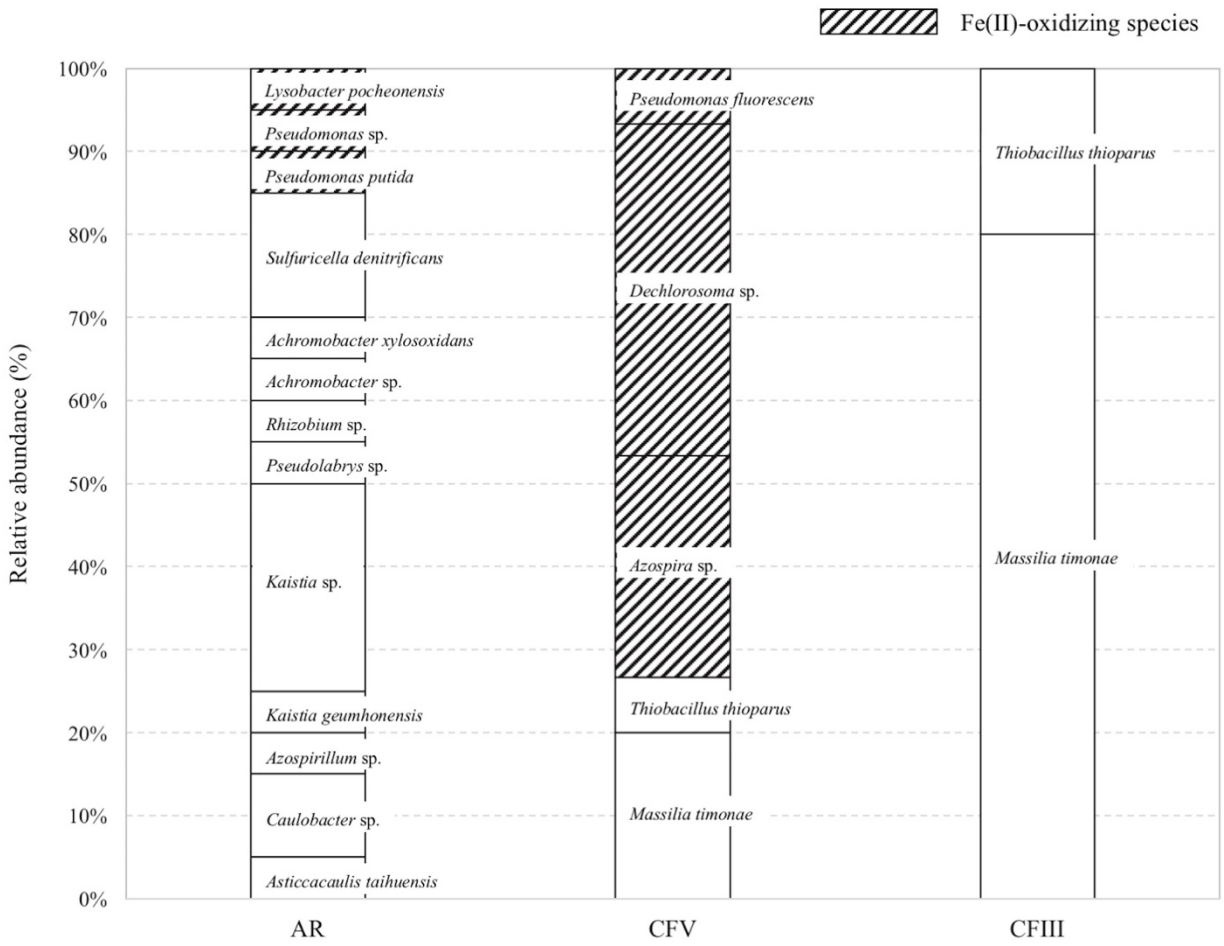


Figure 6 Relative abundance of the *Proteobacteria* species retrieved in the 16S rRNA gene clone libraries obtained from AR, CFV and CFIII enrichment cultures.

4.

Sulfate amendment in rice field soil affects arsenic translocation in rice

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Abstract

Arsenic translocation from soil to rice grains is a serious issue, since anoxic conditions established in rice paddies favor metalloid mobilization. Previous work demonstrated that the use of sulfate might decrease significantly arsenic concentration in rice grains. The aim of this study was to evaluate the effect of calcium sulfate ($CaSO_4$) addition on arsenic bioavailability in rice rhizosphere and on rhizosphere microbiota involved in arsenic cycle. Unplanted and planted mesocosms with and without sulfate addition were set up, where rice plants were cultivated in contaminated rice field soil (arsenic concentration 29.41 mg kg^{-1}). Different physic-chemical parameters were evaluated in the soil solution and in roots and leaves of the plants. Furthermore, microbial genes involved in arsenite oxidation (*aioA*) and in arsenite methylation (*arsM*) were quantified with real time qPCR.

In the unplanted mesocosms arsenic concentration in soil solution increased over time in concomitance with the decrease of the redox potential, whereas in the planted treatments metalloid dissolution was significantly lower. Sulfate addition significantly reduced arsenic mobilization in the planted mesocosms. Although arsenic concentration in roots did not vary with sulfate amendment, treated plants contained significantly lower amounts of metalloid and iron with respect to the control. Microbial genes for arsenite oxidation were highly abundant in all treatments, with a significant decrease in planted mesocosms with respect to the original soil. A similar trend was observed for genes for arsenite methylation, although these genes represented a minor fraction of the total microbial community.

In addition to an effective decrease in arsenic dissolution from soil mineral, this study demonstrated that sulfate amendment decreases arsenic translocation from roots to leaves at early growth stage of

the plants. This phenomenon was apparently not related to microbial arsenite oxidation potential observed in rice rhizosphere.

4.1 Introduction

Rice cropping is highly affected by arsenic (As) contamination all over the world (Meharg *et al.*, 2009). Arsenic is naturally present in all soils, but a combination of physic-chemical factors and the efficiency of rice plants in As assimilation lead to the accumulation of this metalloid in grains in both contaminated and non-contaminated sites (Abedin *et al.*, 2002; Xie and Huang, 1998; Chapter 1). In fact, prolonged flooding in rice paddies leads to As release from iron (Fe) minerals to the pore water, as well as to the reduction of arsenate [As(V)] to arsenite [As(III)], which is more efficiently taken up by the plants.

In the environment, As chemistry is strictly related to that of Fe and sulfur (S) (Bowell *et al.*, 2014). In fact, sulfide (S^-) produced by dissimilatory sulfate-reducing bacteria co-precipitate with As(III), decreasing its bioavailability in the environment (Upadhyaya *et al.*, 2010, 2012). Sulfide also promotes As(III) oxidation in sulfide-oxidizing microorganisms in alkaline lake waters and in laboratory enrichment cultures (Fisher *et al.*, 2008).

Sulfur is an essential nutrient for plants, and different forms of sulfur fertilization have been demonstrated to increase rice yield (Yasmin *et al.*, 2007). Sulfate fertilization is commonly used in sodic and alkaline rice field soils (Bhumbla and Abrol, 1978). This practice has been suggested as an option to decrease the huge methane (CH_4) amounts released annually into the atmosphere by rice paddies (van der Gon, 2001; Linquist *et al.*, 2012). Furthermore, Hu and colleagues demonstrated that sulfate (SO_4^{2-}) amendment in rice field soil decreased As accumulation in rice by enhancing Fe plaque formation (Hu *et al.*, 2007). Sulfate fertilization has an impact on dissimilatory sulfate-reducing bacteria (Wörner *et al.*, 2016), however, information on their actual role on the decrease of As contamination induced by SO_4^{2-} amendment is still poorly understood (Jia *et al.*, 2015). In general, studies on the biogeochemical mechanisms behind the beneficial effect of SO_4^{2-} fertilization against As contamination are currently lacking.

The aim of this study was to evaluate the effect of SO_4^{2-} addition on As bioavailability and uptake in rice plants grown on a contaminated rice field soil. Rhizospheric microbial populations involved in As biogeochemistry where also investigated, in order to ascertain their potential role in this processes.

4.2 Material and methods

4.2.1 Soil characterization

For this experiment, a sandy-loam soil with pH 7.2 was sampled in a rice field in Carpiano (MI) in May 2016. For the physic-chemical characterization, 3 replicates of 5 g of soil were mineralized with Anton Paar MULTIWAVE-ECO mineralizer in teflon tubes with 10 mL of 65% HNO_3. Total As and ion content was measured with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent). Multistandard solution containing Scandium (^{45}Sc), Yttrium (^{89}Y) and Terbium (^{159}Tb)] was included, with a final concentration of 20 μg L^{-1}.

4.2.2 Experimental setup

The soil was sieved at 2 mm and 8 kg were distributed in plastic pots (40 x 50 cm) with no drainage holes in the bottom, in order to maintain anoxic conditions in the bulk soil. Three replicates of four mesocosms were set up as follows: unplanted with and without SO_4^{2-}, planted with and without SO_4^{2-}. The source of SO_4^{2-} was anhydrite ($CaSO_4$), which was ground to powder and uniformly distributed in the soil to a final concentration of 0.13% (w/w). Rice seeds (*Oryza sativa* type japonica var. Koral, provided by Ente Nazionale Risi) were soaked in distilled water on paper filter in Petri dishes and incubated at room temperature in the dark for 14 days. The germinated seedlings were transferred in the pots at 10 cm distance. In each planted mesocosm, 3 plants were present. After 7 days the plants were flooded to 3 cm water level and all pots received N:P:K (4:2:1, w/w) fertilization. After 10 days of plant acclimatization, the water level was increased to 10 cm, and the day after was set as time point 0 for the chemical analyses of the porewater. After 28 days from time 0, the plants were sampled and the pots were left flooded for additional 28 days. In total, complete flooding lasted 56 days.

4.2.3 Chemical analysis of soil solution

Starting from time point 0, the soil solution was sampled every 14 days with rhizon soil moisture samplers ("Rhizon, SMS 5 cm, Rhizosphere"). The solution was filtered with 0.45 μm pores cellulose acetate filters (Ministart® NML Syringe Filter, Sartorius). Immediately after sampling and filtering, pH and redox potential were measured (PHS-25CW pH/mV meter, Bante instruments). Arsenic species were separated with WATER Sep-Pak_Plus Acell Plus QMA cartridges (Waters Corporation) according to Corsini *et al.* (2010). For As and Fe detection, 5 mL of filtered soil solution was acidified to pH 2 HNO_3 and As and Fe standards were added. Total arsenic and ions content was measured ICP-MS. Sulfate was measured with Nanocolor® sulfate 200 and Nanocolor®

sulfate 1000 kits (Macherey-Nagel) according to manufacturer's instructions. A 1 g L^{-1} $NaSO_4$ solution was used for standards preparation.

4.2.4 Plant sampling, fraction separation and chemical analysis

At 28 days from complete flooding, most of the plants showed stress symptoms, such as slow growth and brown spots on the leaves. Therefore, at that time point all plants were sampled. From each planted mesocosms replicate, the bulk soil was sampled at 5-10 cm from the roots. Epigeal and hypogeal fractions of the plants were separated and portions of thoroughly washed roots and leaves were dried at 50°C for one week, ground and mineralized as described above for total As and Fe quantification with ICP-MS. Translocation factors were calculated as shoot to root concentration ratio. Rhizosphere soil was obtained as described by Cavalca *et al.* (2010).

4.2.5 Quantification of arsenic genes

Microbial DNA was isolated from the original soil used for the experiment (T0), from soil sampled from unplanted mesocosms without and with sulfate amendment at 28 days from complete flooding (UC and US) and from bulk and rhizosphere soil of plants without and with sulfate addition (PC-BS, PC-RS, PS-BS and PS-RS). For DNA isolation, PowerSoil®DNA Isolation kit (MOBIO) was used according to manufacturer instructions.

Total 16S rRNA genes and genes encoding the arsenite oxidase (*aioA*) and the arsenite methyltransferase (*arsM*) were quantified by real time quantitative PCR (RT-qPCR). Respectively, Eub338F/Eub518R (Fierer *et al.*, 2005), aoxBM1-2F/aoxBM2-1R (Quemeneur *et al.*, 2008, 2010) and arsMF1/arsMR2 (Jia *et al.*, 2013; Chapter 1) primer couples were used to amplify the targets. For eubacterial 16S rRNA and *aioA* genes, DNA from *Achromobacter* sp. strain 1L was used for standard curve preparation, whereas ArsM6 clone (Chapter 1) was used for the same purpose for *arsM* gene.

4.2.6 Statystical analysis

Statistical analysis of the results was performed with SPSS software version 21. One-way analysis of variance (ANOVA), Tukey's b, Duncan and t test at $p < 0.05$ and $p < 0.01$ were used for comparisons.

4.3 Results and Discussion

4.3.1 Soil characterization and porewater chemical analysis

Ion characterization of the soil used for the experiment is shown in Table 1. Concentrations of As and Zn were 29.41 and 420.98 mg (kg dw)$^{-1}$ respectively, both exceeding the law limits (20 and 150 mg (kg dw)$^{-1}$ respectively, D. Lgs 152/2006).

As typically observed in flooded mineral soils, pH in soil solution significantly increased over time, with a concomitant decrease of the redox potential (Tables 2a and 2b; Kögel-Knabner, 2010). Total As in soil solution increased over time in all mesocosms (Figure 1), according to the establishment of anoxic conditions. After 56 days from complete flooding, in the unplanted pots the concentrations of As were 58.25 µg L^{-1} without sulfate and 46.17 µg L^{-1} with sulfate, significantly higher with respect to the planted pots ($p < 0.005$). Measured values in planted pots where 19.66 µg L^{-1} and 6.82 µg L^{-1} without and with sulfate respectively (Table 3). In all treatments, most of total As was in the form of As(III) (Fig. 1 and Table 3).

In unplanted mesocosms, Fe concentrations in soil solution increased from 2 mg L^{-1}, immediately after complete flooding, to 7 mg L^{-1} and to 5 mg L^{-1} without and with sulfate respectively. Likely, anoxic conditions led to the reduction of Fe(III) to Fe(II), more soluble in the soil solution. In planted mesocosms without and with sulfate amendment, Fe concentrations were 1.2 mg L^{-1} and 0.9 mg L^{-1} at day 56, respectively. In these treatments, oxygen leakage from the roots might have inhibited Fe(III) reduction, decreasing Fe(II) release from soil minerals. Furthermore, part of the soluble Fe might have been taken up by the plants.

Immediately after complete flooding (day 0), SO_4^{2-} concentrations in pore waters ranged from 650 to 750 mg L^{-1} in mesocosms with SO_4^{2-} amendment, whereas it was 250 mg L^{-1} in non-treated controls (Fig. 2). In all mesocosms SO_4^{2-} significantly decreased over time ($p < 0.05$ and $p < 0.01$, Fig. 2). In the unplanted mesocosms, SO_4^{2-} decrease was more pronounced, probably because the lower redox potential favored SRB activity with respect to the planted mesocosms. Furthermore, oxygen leakage from rice roots might have allowed the recycling of SO_4^{2-} in the rhizosphere in planted mesocosms.

4.3.2 Plant growth and chemical characterization

In mesocosms amended with SO_4^{2-}, 6 out of 18 plants persisted until sampling, whereas, without SO_4^{2-}, only 3 out of 18 plants survived. The total plant dry biomass sampled at 28 days was 4.3 g and 96.8 g in the mesocosms without and with SO_4^{2-}, respectively. Although the biomass weight varied, As and Fe concentrations in roots did not vary significantly with SO_4^{2-} amendment ($p < 0.005$, Fig. 3). However, the same elements in the leaves significantly decreased from 9 to below 1 mg/g (As/Fe)

kgdw^{-1} when SO_4^{2-} was applied ($p < 0.01$). Arsenic translocation factors were 0.65 ± 0.03 and 0.03 ± 0.01 without and with SO_4^{2-}, respectively. Iron translocation factors were 0.64 ± 0.02 and 0.03 ± 0.00 without and with SO_4^{2-}, respectively.

Previous studies demonstrated that rice plant exposed to high As concentrations (12 – 149 mg kg^{-1}) accumulate 200 to 1300 mg kg^{-1} of Fe in the leaves, resulting in plants death (Tsutsumi, 1980). Therefore, the stress that occurred in control plants was likely due to As and/or Fe toxicity. A decrease in As translocation with SO_4^{2-} amendment was also observed by Zhang and colleagues (Zhang *et al.*, 2011). However, non-protein thiols, phytochelatins as well as glutathione concentrations did not change concomitantly. Therefore, the mechanisms driving As and Fe translocation reduction with SO_4^{2-} fertilization are still unclear.

4.3.3 Quantification of microbial arsenic processing potential

The whole bacterial community was represented by 16S rRNA gene copy numbers in the order of 10^8-10^9 copies per gdw^{-1} in all treatments (Fig. 4). Total bacteria were significantly less abundant in planted bulk and rhizosphere soil with respect to the original non-treated soil (T0), except for the rhizosphere soil of plants amended with sulfate (PC-RS, $p < 0.05$). A similar trend was observed for *aioA* gene copies, that were in the order of 10^8 copies gdw^{-1} in all treatments. Gene copy numbers of *arsM* ranged within the order of 10^3-10^4 gdw^{-1} and were significantly lower with respect to the original soil in the bulk soil of planted mesocosms amended with sulfate (PS-BS, $p < 0.05$).

Arsenic genes likely varied in relation to total community variations, therefore not influenced by SO_4^{2-} amendment. Notably, *aioA* genes were highly abundant in this soil, potentially representing a key metabolism in this ecosystem. Similar observations resulted in previous work described in Chapter 1. This is in accordance with the evidence that As(III) is the predominant species in the pore water, which may be, therefore, an important substrate for As-oxidizing microorganisms in these mesocosms.

4.4 Conclusion

With this study, a short-term effect on As and Fe translocation in rice plants due to SO_4^{2-} fertilization was evidenced. Concomitantly, As dissolution from soil mineral decreased in accordance to previous studies. These variations were apparently not related to changes in the microbial As(III) oxidation and methylation potential observed in rice rhizosphere. Further investigation should be addressed to highlight a possible role of Fe- and S-cycling microorganisms in rhizosphere soil as well as of bacterial populations inhabiting rice endosphere.

Table 1 Ion composition in the soil used for the experiment.

Element	Concentration	Measure unit	Law limits (D. Lgs 152/2006)	Measure unit
As	29.41 ± 0.82	mg (kg dw)$^{-1}$	20	mg (kg dw)$^{-1}$
Ca	5.52 ± 0.26	g (kg dw)$^{-1}$	-	-
Cd	1.18 ± 0.03	mg (kg dw)$^{-1}$	2	mg (kg dw)$^{-1}$
Co	11.37 ± 0.76	mg (kg dw)$^{-1}$	20	mg (kg dw)$^{-1}$
Cr	101.99 ± 5.47	mg (kg dw)$^{-1}$	150	mg (kg dw)$^{-1}$
Cu	72.34 ± 3.49	mg (kg dw)$^{-1}$	120	mg (kg dw)$^{-1}$
Fe	24.91 ± 1.59	g (kg dw)$^{-1}$	-	-
K	2.88 ± 0.18	g (kg dw)$^{-1}$	-	-
Mg	6.12 ± 0.47	g (kg dw)$^{-1}$	-	-
Mn	1.48 ± 0.15	g (kg dw)$^{-1}$	-	-
Na	230.85 ± 14.52	mg (kg dw)$^{-1}$	-	-
Ni	53.8 ± 5.24	mg (kg dw)$^{-1}$	120	mg (kg dw)$^{-1}$
Pb	77.73 ± 5.46	mg (kg dw)$^{-1}$	100	mg (kg dw)$^{-1}$
Se	0.52 ± 0.08	mg (kg dw)$^{-1}$	3	mg (kg dw)$^{-1}$
Zn	420.98 ± 30.65	mg (kg dw)$^{-1}$	150	mg (kg dw)$^{-1}$

Table 2 Values of pH (a) and redox potential (mV, b) measured in soil solution over time. Letters indicate statistically significant groups according to one-way ANOVA Tukey b ($p < 0.05$) over time.

a.

Treatment / Time (days)	UC	US	PC	PS
0	6.5 a	6.5 a	6.8 a	6.7 ab
14	6.8 b	6.6 ab	6.8 a	6.6 a
28	7.1 bc	6.8 abc	7.0 ab	6.8 b
42	6.9 bc	6.9 bc	6.9 a	6.9 c
56	7.2 c	7.0 c	7.2 b	7.0 c

b.

Treatment / Time (days)	UC	US	PC	PS
0	316.67 b	320.00 d	284.00 b	323.33 b
14	503.00 c	402.33 c	451.67 c	440.00 c
28	108.00 b	89.33 b	87.67 a	100.67 a
42	43.67 a	61.33 ab	67.00 a	80.33 a
56	23.33 a	14.67 a	17.67 a	71.67 a

Table 3 Arsenic concentration (µg L^{-1}) in porewater 56 days after complete flooding in the unplanted treatments without and with sulfate (UC and US) and in the planted treatments without and with sulfate (PC and PS). Letters indicate statistically significant groups within the same arsenic species according to ANOVA based on Tukey's b test ($P > 0.05$); stars indicate statistically significant difference due to the presence of the plant according to t test (UC vs PC and US vs PS, $p < 0.05$); crosses indicate statistically significant difference due to sulfate addition according to t test (UC vs US and PC vs PS, $p < 0.05$).

Arsenic species / **Treatment**	**Tot As**	**As(III)**	**As(V)**
UC	58.25 b	36.94 b	21.31 b
US	46.17 b	39.11 b	7.07 a
PC	19.66 a	18.34 a	1.48 a
PS	6.82 a*†	8.14 a*†	0.32 a*

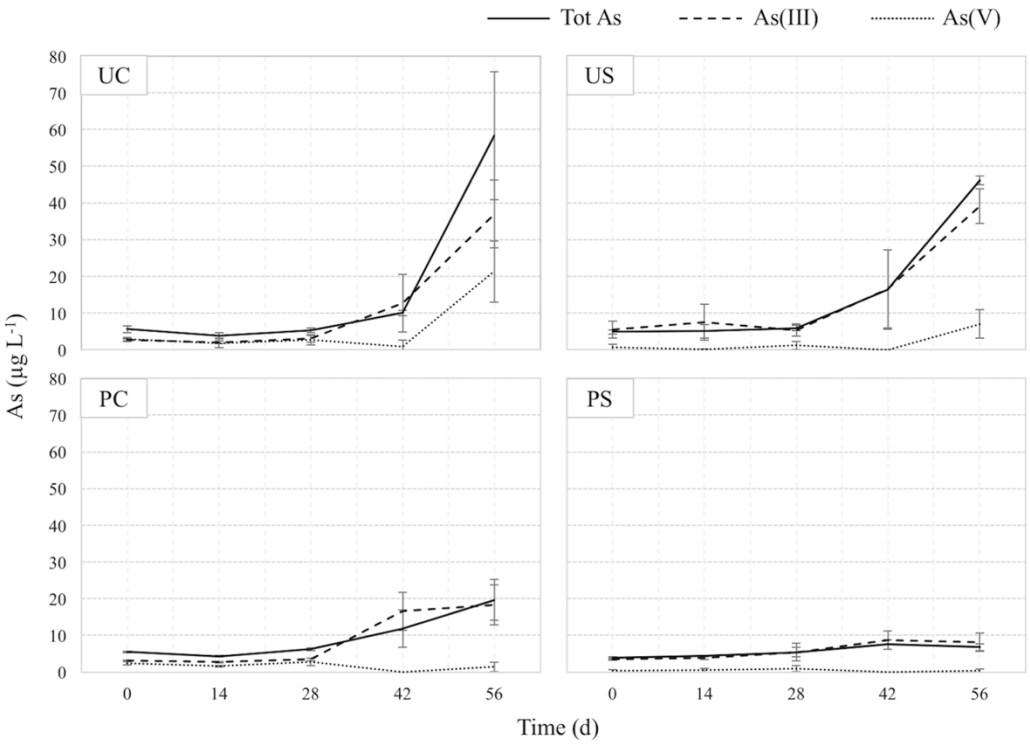

Figure 1 Tot As, As(III) and As(V) in the porewater over time in unplanted without sulfate (UC), unplanted with sulfate (US), planted without sulfate (PC) and planted with sulfate (PS).

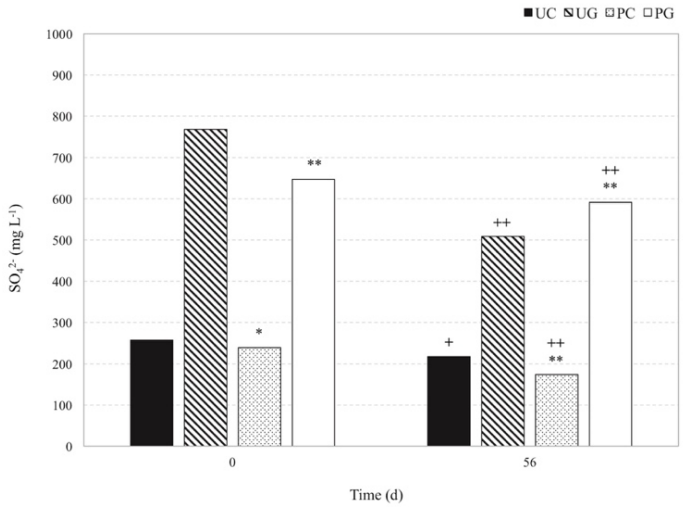

Figure 2 Sulfate measured in the porewater at time 0 and at the end of the incubation (after 56 days from complete flooding) in the unplanted mesocosms without and with sulfate addition (UC and US) and in the planted mesocosms without and with sulfate addition (PC and PS). Stars indicate statistically significant difference in the mesocosms due to the presence of the plant (UC vs PC and US vs PS) (t test, one star $p < 0.05$, two stars $p < 0.01$); crosses indicate statistically significant difference within the same mesocosm at the end of incubation (t test, one cross $p < 0.05$, two crosses $p < 0.01$).

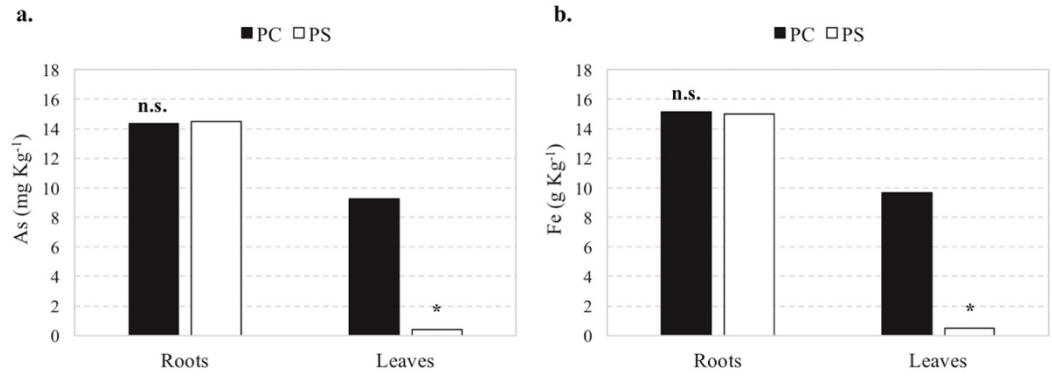

Figure 3 Total arsenic and iron in roots and leaves of plants without and with sulfate addition (PC and PS). Stars indicate statistically significant difference due to sulfate addition according to t test ($p < 0.01$).

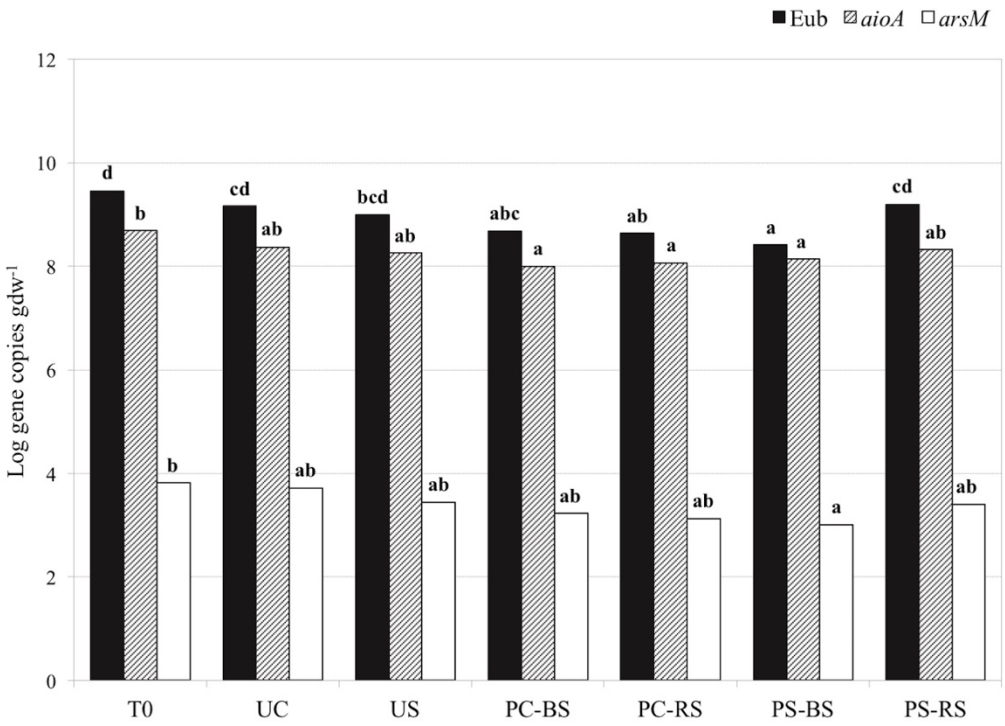

Figure 4 Real time qPCR of eubacterial 16S rRNA genes (Eub), *aioA* and *arsM* genes in the soil used for the experiment (T0), in the unplanted mesocosms without and with sulfate addition (UC and US) and plants bulk and rhizosphere soil without and with sulfate addition (PC-BS, PC-RS, PS-BS, PS-RS). Letters indicate statistically significant groups according to one-way ANOVA Tukey b ($p < 0.05$) within the same target gene.

5.

Putative novel sulfate-reducing members of the phylum *Nitrospirae* living in rice paddy soil, enhanced by gypsum addition

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Abstract

The relevance of microbial dissimilatory sulfate reduction in rice paddies has emerged over the last decades, with the detection of relatively high sulfate reduction rates considering the low sulfate concentrations available. In this study, the presence of putative sulfate-reducing bacteria affiliated to a new deep branching genus/species of the *Nitrospirae* phylum living in the bulk soil of rice paddies is reported. This evidence derived from the isolation of a metagenomic bin obtained from bulk soil samples of rice plants treated with gypsum addition, containing the *dsrAB* genes. A 2770094 bp genome with < 3% contamination was assembled and resulted to be closely related to the magnetotactic candidate genera *Candidatus* Magnetobacterium and *Candidatus* Magnetoovum. These organisms appeared to be more abundant and active in the bulk soil than in the rhizosphere. The metabolic reconstruction and the analysis of *in situ* protein expression revealed a versatile metabolism based on sulfate respiration, promoted by gypsum addition, and nitrate respiration, performed in the control treatments without gypsum. Acetate is most likely used as substrate via the oxidative Wood-Ljungdahl pathway and evidence for butyrate degradation was observed as well. Strikingly, complex carbohydrates degradation was expressed in concomitance with the expression of sulfate reduction. These outcomes provide new insights on the hidden sulfur cycle in rice paddies,

driven by still uncharacterized microbial species. The unusual correlation between dissimilatory sulfate-reduction and carbohydrate degradation might revolutionize our knowledge on the role of microbial sulfate respiration in rice rhizosphere.

5.1 Introduction

Sulfate reducing microorganisms (SRM) in rice paddies have been paid considerable attention in the last years (Wind *et al.*, 1999; Scheid and Stubner, 2001; Stubner, 2004; Lin *et al.*, 2010; He *et al.*, 2010; Liu *et al.*, 2014; Wörner *et al.*, 2016), due to their role in fueling an active sulfur cycling in these ecosystems. In fact, despite the average low concentrations of sulfate (4-150 μM, Wind and Conrad, 1997), high sulfate reduction rates have been recorded within rice rhizosphere (12 – 500 nmol cm^{-3} day^{-1}, Wind and Conrad, 1997). The importance of microbial sulfate reduction in rice paddies relies in its potential in decreasing methane emissions (Wörner *et al.*, 2016) as well as in contrasting heavy metals uptake (Lin *et al.*, 2010; Somenahally *et al.*, 2011a) by rice plants. These potentials are nearly becoming certainties, since several authors reported the suppression of methane emissions by adding a source of sulfate to rice field soil and related this phenomenon to the promotion of SRM (Wind *et al.*, 1999; Scheid and Stubner, 2001; Wörner *et al.*, 2016).

Dissimilatory sulfate reduction characterizes a wide range of phylogenetic taxa, and it is a common trait in some *Deltaproteobacteria* and *Firmicutes* families (topic comprehensively reviewed by Rabus *et al.*, 2006 and 2015). The only known SRM in the *Nitrospirae* phylum are bacteria belonging to the genus *Thermodesulfovibrio* (Henry *et al.*, 1994; Sonne-Hansen and Ahring, 1999; Haouari *et al.*, 2008; Sekiguchi *et al.*, 2008). These microorganisms are typically thermophilic anaerobes living in natural hotsprings or waste and wastewater treatment plants, and couple sulfate reduction to the oxidation of small organic compounds or H_2. The phylum *Nitrospirae* is highly variable in terms of metabolic pathways, including chemolithoautotrophic aerobic nitrite-oxidizing species of the genus *Nitrospira* sp. (Watson *et al.*, 1986; Ehrich *et al.*, 1995; Spieck *et al.*, 2006; Lebedeva *et al.*, 2011; Ushiki *et al.*, 2013) and acidophilic aerobic iron oxidizing bacteria of the genus *Leptospirillum* sp. (Golovacheva *et al.*, 1992; Hippe, 2000; Coram and Rawlings, 2002; Tyson *et al.*, 2005). A group of still uncultured *Nitrospirae* is represented by magnetotactic bacteria belonging to the putative genera *Candidatus* Magnetobacterium, *Candidatus* Thermomagnetovibrio and *Candidatus* Magnetoovum (Lefèvre *et al.*, 2010; Lin *et al.*, 2011; Lefèvre *et al.*, 2011). These microorganisms typically live at the oxic-anoxic interface in sediments and aquatic environments and their magnetosomes are made of magnetite (Fe_3O_4) (Lefèvre and Bazylinski, 2013). Although very little is known about the metabolism of these microorganisms, the presence of sulfur-rich inclusions in the cells of *Ca.*

Magnetobacterium bavaricum and of *Ca.* Magnetoovum mohavensis indicates a probable metabolism based on sulfur oxidation (Lefèvre and Bazylinski, 2013).

With this work the metabolism of a new putative sulfate reducing genus/species belonging to the phylum *Nitrospirae* living in rice rhizosphere has been reconstructed from its genome and the protein expression pattern was analyzed, in order to elucidate its lifestyle and role in the rice paddies sulfur cycle.

5.2 Materials and methods

5.2.1 Greenhouse experiment

The greenhouse experiment was carried out at the Max Planck Institute for Terrestrial Microbiology of Marburg (Germany). Briefly, four sets of pots, each in quadruplicate, were filled with 2 kg of dry sieved rice field soil provided by the Italian Rice Research Institute of Vercelli (Italy). In two of the four sets the soil was mixed with 0.15% (w/w) gypsum ($CaSO_4 \cdot 2H_2O$). One rice seed (*Oryza sativa* var. Koral type japonica) for each pot was placed into 80 g of soil surrounded by a 24 μm mesh bag, to separate rhizosphere soil from bulk soil. After one week from seeding, the pots were flooded and incubated for 58 to 59 days under a natural day/night cycle. Rhizosphere and bulk soil, respectively inside and outside the mesh bags, were collected in 15 mL tubes, shock frozen in liquid N_2 and stored at -80°C.

5.2.2 DNA isolation and metagenome sequencing

After grinding of soil samples in liquid N_2, DNA was isolated using the RNA Powersoil® DNA Elution Accessory Kit (MO BIO). Qualitative and quantitative check of DNA was performed with agarose gel electophoresis and NanoDrop spectrophotometer respectively. For each treatment, 2 μg of DNA were used for metagenomic library preparation. Paired-end sequencing (read length 2 x 100 bp) was carried out on an Illumina HiSeq 2000 platform at the King Abdullah University of Science and Technology (KAUST) in Thuwal (Saudi Arabia).

5.2.3 Sequence processing and de novo assembly

Raw reads were quality screened and trimmed in the CLC Genomic Workbench 5.5.1 (CLC bio, Aarhus, Denmark) using only paired-end reads longer than 50 bp with no more than 1 ambiguity and a quality score equal or above 0.03 (99% accuracy). *De novo* assembly was performed with CLC using a word size of 41 and a minimum contig length of 2000 bp. Gene prediction was performed using Prodigal (Hyatt *et al.*, 2012). Essential single-copy genes (ESS) were identified with Hmmsearch of the HMMer package (Eddy, 1998) and the predicted genes were taxonomically

classified with MEGAN (Hudson *et al.*, 2007). Scaffolds containing genes encoding the dissimilatory bi-sulfite reductase subunits A and B (DsrAB) were identified by a blastp search (Altschul *et al.*, 1990) against a DsrAB reference database (Müller *et al.*, 2015).

5.2.4 Nitrospirae bin extraction

Assembled scaffolds including their read coverage and taxonomic classification were used for binning using a combination of the R package mmgenome (Albertsen *et al.*, 2013) and SPAdes (Bankevich *et al.*, 2012). The scaffolds were plotted according to their differential coverage data and scaffolds clustering to putative population genomes were binned out. GC-content and k-mer usage were used to further refine the bins. The quality check of the bins was performed considering the total and average length of the scaffolds, the total number of ESS and the ratio (total ESS)/(unique ESS) (measure of contamination). Thereafter, completeness, contamination and strain heterogeneity were further evaluated using CheckM (Parks *et al.*, 2015).

5.2.5 Relative abundance of the bin in the treatments

Read coverage of individual scaffolds of the final *Nitospirae* bin were imported into R and processed with the package phyloseq. The largest scaffold of the bin was investigated for abundance differences between soil regions and treatments with a two-way analysis of variance (ANOVA).

5.2.6 Phylogenetic analysis

Since sequences related to 16S rRNA gene could not be retrieved in the *Nitrospirae* bin, the 23S rRNA gene was used as phylogenetic marker. The 23S rRNA gene were aligned and a maximum likelihood tree was inferred with GTRGAMMA model in RaxML v8.2.8 (Stamatakis, 2006) as implemented on the CIPRES webserver (Miller *et al.*, 2010). For the *dsrAB* genes, a maximum likelihood tree was inferred from 411 distinct alignment patterns using RaxML v8.2.8 (Stamatakis, 2006) as implemented on the CIPRES webserver (Miller *et al.*, 2010). For the phylogenomics, the alignment was based on a set of 43 phylogenetically informative marker genes (ribosomal proteins S12/S23; S7p/S5e; L2, RNA binding domain; S3, C-terminal domain; S19; L22p/L17e; L14p/L23e; L16p/L10e; L23; L5; L3; L11, RNA binding domain; S15; S2; S5, N-terminal domain; S17; S9/S16; S8; S11; L10; L13; L4/L1; L5P, C-terminus; L1p/L10e; L29; L18p/L5e; S5, C-terminal domain; L11, N-terminal domain; L2, C-terminal domain; RNA polymerases Rpb2, domain 6; Rpb1, domain 2; Rpb6; Rpb2, domain 7; Rpb2, domain 2; beta subunit; Rpb2, domain 3; Rpb1, domain 1; Rpb1, domain 4; TruB family pseudouridalyate synthase; signal peptide binding domain; translation-initiation factor 2; alanine-tRNA ligases; valine-tRNA ligase) (Parks *et al.*, 2015). A maximum likelihood tree was inferred using RaxML v8.2.8 on the CIPRES webserver.

5.2.7 Annotation and manual curation

A preliminary automatic annotation of the *Nitrospirae* bin was obtained using the on-line annotation service RAST (Rapid Annotation using Subsystem Technology, http://rast.nmpdr.org; Aziz *et al.*, 2008; Overbeek *et al.*, 2014; Brettin *et al.*, 2015). The manual curation of the annotation was performed after uploading the genome on the annotation platform Microscope provided by MaGe (Magnifying Genomes, https://www.genoscope.cns.fr/agc/microscope/home/; Vallenet *et al.*, 2013; Vallenet *et al.*, 2009; Vallenet *et al.*, 2006).

5.2.8 Proteomic analysis

Protein extraction in-gel tryptic digestion and LC-MS/MS measurements were performed as described in Starke *et al.*, 2016. Briefly, 2 g of soil were used for phenol extraction procedure with a subsequent ammonium acetate precipitation. Tryptic peptides were analysed using a UPLC-LTQ Orbitrap Velos MS/MS (Herbst *et al.*, 2013). Obtained raw data were processed using Proteome Discoverer v1.4 (Thermo Fisher Scientific, Waltham, MA, USA). Searches were performed using MASCOT algorithm with the following parameters: tryptic cleavage with maximal two missed cleavages, a peptide tolerance threshold of ±10 ppm and an MS/MS tolerance threshold of ± 0.5 Da, and carbamidomethylation at cysteines as static and oxidation of methionines as variable modifications. The metagenome of rice paddy bulk soil and the *Nitrospirae* bin was used as sample-specific database. Protein identification was established for sequences with at least one unique high confidence similar peptide (false discovery rate, FDR<0.01).

5.3 Results

5.3.1 Relative abundance and genome features of the Nitrospirae bin

Of the 159 population genome bins found in the metagenome, one bin closely related to the phylum *Nitrospirae* contained the *dsrAB* genes. The largest scaffold of the *Nitrospirae* bin was significantly more abundant in the bulk soil and in the initial soil with respect to rhizosphere soil (Fig. 1). Gypsum amendment did not lead to significant variations. The bin contained 151 scaffolds, with a total length of 2770094 bp and a contamination level below 3%. The genomic features of the bin are listed in Table 1.

5.3.2 Taxonomic affiliation

According to the phylogenetic analysis performed on the 23S rRNA gene, the *dsrAB* genes and to the phylogenomic analysis, the *Nitrospirae* bin was closely related with members of the uncultured

species *Candidatus* Magnetobacterium bavaricum, *Candidatus* Magnetobacterium casensis and *Candidatus* Magnetoovum chiemensis (phylum *Nitrospirae*, Fig. 2). The *Nitrospirae* bin likely represents a novel deep branching species/genus.

5.3.3 Sulfate and nitrate respiration

The complete array of enzymes involved in dissimilatory sulfate reduction were identified (Fig. 3, Table C1). These included dissimilatory (bi)sulfite reductase A and B (*dsrAB*, KEGG 1.8.99.3), adenylylsulfate reductase B (*aprB*, KEGG 1.8.99.2) and sulfate adenylyltransferase (*sat*, KEGG 2.7.7.4). Genes encoding the DsrC protein and part of the membrane complex DsrMKJOP were located downstream the *dsrAB*. Genes crucial for sulfate reduction as *dsrA* and *sat* were expressed at high rate in bulk soil with gypsum amendment (Fig. 5). The protein DsrA was also retrieved in rhizosphere soil with gypsum addition. Genes encoding for enzymes involved in sulfur oxidation typically present in sulfur-oxidizing organisms *(sox* genes) were not retrieved.

Two inner membrane complexes and one periplasmic complex involved in three steps of the denitrification process were present (Fig. 3). These included the periplasmic nitrate reductase (NapAGHBD, KEGG 1.7.99.4), which catalyze the reduction of NO_3^- to NO_2^-, and the two cytochromes NrfH and NrfA (KEGG 1.7.2.2, 1.9.6.1), which form the nitrite reductase complex, catalyzing the reduction of NO_2^- to NH_3. Two copies for each gene encoding the nitric oxide reductase (NorBC, KEGG 1.7.2.5), involved with the conversion of NO to N_2O, were also retrieved. However, the *nir* and *nos* operons, which connect the first two reactions and lead to the final production of N_2 were not present in the genome. The NapA and NorC proteins were expressed in the bulk soil without gypsum addition, whereas NapG was expressed in rhizophere soil without gypsum (Fig 5).

5.3.4 Acetate degradation

Genes involved in the Wood-Ljungdhal pathway were retrieved (Fig. 4, Table C1). Within the carbonyl branch, these included AMP and ADP-forming acetyl-CoA synthetase (KEGG 6.2.1.1, 6.2.1.13), acetyl-CoA hydrolase (KEGG 3.1.2.1) and CO dehydrogenase (KEGG 2.3.1.169, 1.2.7.4). These genes were located within the same operon. For the methyl branch, mehtyl-THF:CoFeSP CO-methyltransferase (KEGG 2.1.1.258), methylene-THF reductase (KEGG 1.5.7.1), bi-functional methylene-THF dehydrogenase/methenyl-THF cyclohydrolase (KEGG 3.5.4.9), formyl-THF cycloligase (KEGG 6.3.3.2), formate-THF ligase (KEGG 6.3.4.3) and formate dehydrogenase (KEGG 1.2.1.2) were retrieved and were dispersed throughout the genome. Some of these genes were expressed mostly in the bulk soil, with and without gypsum fertilization.

5.3.5 Other pathways

Part of the genes required for butyrate degradation were present and expressed in rhizosphere soil (Table C1), including butyryl-CoA dehydrogenase (KEGG 1.3.8.1), 3-hydroxybutyryl-CoA dehydratase (KEGG 4.2.1.17), 3-hydroxybutyryl-CoA dehydrogenase (KEGG 1.1.1.35), acetyl-CoA acetyltransferase (KEGG 2.3.1.16) and acyl-CoA dehydrogenase (KEGG 1.3.8.7).

The capacity of degrading complex carbohydrate molecules was indicated by the expression of a glycosyl hydrolase (glycosidases class, KEGG number 3.2.1.3) in the bulk soil when gypsum was amended.

The presence and expression of key genes of the pentose phosphate pathway (ribose-5-P isomerase (KEGG 5.3.1.6), ribulose-5-P epimerase (KEGG 5.1.3.1), transketolase (KEGG 2.2.1.1), transaldolase (KEGG 2.2.1.2)) likely allowed the formation of precursors for biosynthesis of nucleic acids, LPS and aminoacids.

Although genes encoding for proteins indirectly related to magnetotactic properties like Nap (nitrite reductase), NorBC (nitric oxide reductase), Fur (Fe-dependent transcriptional regulator), Fnr (O_2-dependent transcriptional regulator), FeoB (Fe^{2+} iron membrane transporters), FtsZ (C-terminally truncated tubulin-like protein) (Uebe and Schüler, 2016) were retrieved, genes specifically involved in magnetosome formation and in magnetotactic properties (*mam*, *mms*, *mtx*, *lim*, *amb*) were not present in the genome.

5.4 Discussion

Although sulfate and nitrate reduction in rice paddies have been widely studied both with culture-dependent and independent methods (Ishii *et al.*, 2011; Pester *et al.*, 2012), members of the phylum *Nitrospirae* were never directly associated to these processes in these ecosystems. Only in the case of *Nitrospira* sp., often found in rice field soil (Matsuyama *et al.*, 2007; Cahyani *et al.*, 2008; Ishii *et al.*, 2009; Wu *et al.*, 2011; Ke *et al.*, 2013; Chen *et al.*, 2015), a potential role on nitrification has been proposed (Ishii *et al.*, 2011).

The genome isolated in this study represents an evolutionary connection between the sulfate-reducing genus *Thermodesulfovibrio* sp. and the still uncultured magnetotactic *Nitrospirae*. Intriguingly, the position of this *Nitrospirae* bin in the different phylogenies (Fig. 2) is similar to that of *Candidatus* Thermomagnetovibrio paiutensis (Lefèvre *et al.*, 2010). However, for the latter putative species, only 16S rRNA gene sequence is available, therefore a comparison with the *Nitrospirae* bin is at the moment not applicable.

Although this bin is closely related to the candidate genera Magnetobacterium and Magnetoovum, no genes specifically related to magnetosome formation were retrieved in the genome. On the other hand, *drs* genes highly similar to those found in *Thermodesulfovibrio yellowstonii* were present. These genes were also found in both magnetotactic genera (Table C2), however for these microorganisms the hypothesis of a metabolism based on sulfur oxidation rather than sulfate reduction is favored among the authors (Lin *et al.*, 2014).

Microorganisms represented by the *Nitrospirae* bin appeared to live preferentially in the bulk soil (Fig. 1), supporting the hypothesis of an anaerobic metabolism. Gypsum addition did not affect the relative abundance, neither in bulk nor in rhizosphere soil. This, together with the retrieval and expression of genes involved in both sulfate and nitrate respiration, indicates the versatility of the respiratory processes. This feature is quite common in other SRM (Rabus *et al.*, 2015). The expression of sulfate reduction occurred when gypsum was added, whereas nitrate was likely reduced only in the control treatments (Fig. 5). These observations were in line with the fact that the production of the periplasmic nitrate reductase (Nap) is usually repressed by the presence of sulfate (Rabus *et al.*, 2015). The promotion of sulfate respiration as a consequence of gypsum addition highlights the role of these microorganisms in the competition with acetate-degrading methanogenic Archaea, and therefore in the reduction of methane emissions from rice paddies when a source of sulfate is amended.

The metabolic capacities identified in the *Nitrospirae* bin are comparable to the ones found in the genome of *Ca.* Magnetobacterium spp. (Lin *et al.*, 2014). Here, similar genes involved in sulfur, nitrogen and carbon metabolisms were found, however, as previously mentioned, different interpretations were provided. Lin and coworkers stated that *Ca.* Magnetobacterium casensis could perform both sulfur oxidation and sulfate reduction (Lin *et al.*, 2014). Similarly, to the *Nitrospirae* bin analyzed in the present study, in the genome of *Ca.* M. casensis *sox* genes, typically used for sulfur oxidation, were not identified. In the same study, the same genes involved in the Wood-Ljungdhal pathway were retrieved and the authors, although tending for the hypothesis of CO_2 fixation, did not exclude the possibility of using this pathway for acetate oxidation. According to Ikeda-Ohtsubo *et al.*, (2016), acetyl-CoA synthetase (KEGG 6.2.1.), present in both genomes, is a typical feature of acetate-oxidizing SRM. On the contrary, phosphotransacetylase (KEGG 2.3.1.8) and acetate kinase (KEGG 2.7.2.1), absent both in the *Nitrospirae* bin and in *Ca*. Magnetobacterium casensis, characterize acetate-producing SRM. These findings likely indicate that this pathway may run towards the oxidation of acetate to CO_2. The oxidation of acetate trough the Wood-Ljungdahl pathway is rather common in various SRM (reviewed by Rabus *et al.*, 2015), although in some species this pathway is reversible according to the environmental conditions (Amann *et al.*, 2010).

Acetate degradation was catalyzed independently of gypsum addition (Fig. 5). In fact, both sulfate and nitrate respiration can be coupled to the oxidation of this molecule (Paul *et al.*, 1989; Ishii *et al.*, 2011).

Surprisingly, microorganisms affiliated to the *Nitrospirae* bin appeared to be able to degrade complex carbohydrates. This metabolic feature has never been observed in SRM, given the low energy provided by sulfate respiration (Rabus *et al.*, 2015). Although observations made in this study may result from *Nitrospirae* bin contamination, complex carbohydrate degradation cannot be excluded *a priori*, since no pure cultures are available to study the physiology of these microorganisms.

5.5 Conclusion

The presence of a new putatively sulfate-reducing *Nitrospirae* genus/species in rice fields was revealed by a metagenomic approach. These microorganisms could play an important role in sulfate and nitrate reduction as well as in organic matter degradation in rice paddies, adapting their metabolism to the highly variable environmental conditions. Furthermore, evidence for complex carbohydrates degradation, enhanced by sulfate fertilization, suggest a wider ecological relevance of SRM. Eventually, their role in suppressing methane emission from rice paddies is strongly suggested by the promotion of sulfate respiration when a sulfate source like gypsum is provided.

Acknowledgements

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Table 1 *Nitrospirae* bin genome features.

Feature	Value
Chromosome size (bp)	2770094
GC content (%)	48.78
Number of scaffolds	151
Number of CDS	2855
Average CDS length (bp)	854.72
Protein coding density (%)	87.12
Number of rRNA genes	1
Number of tRNA genes	21

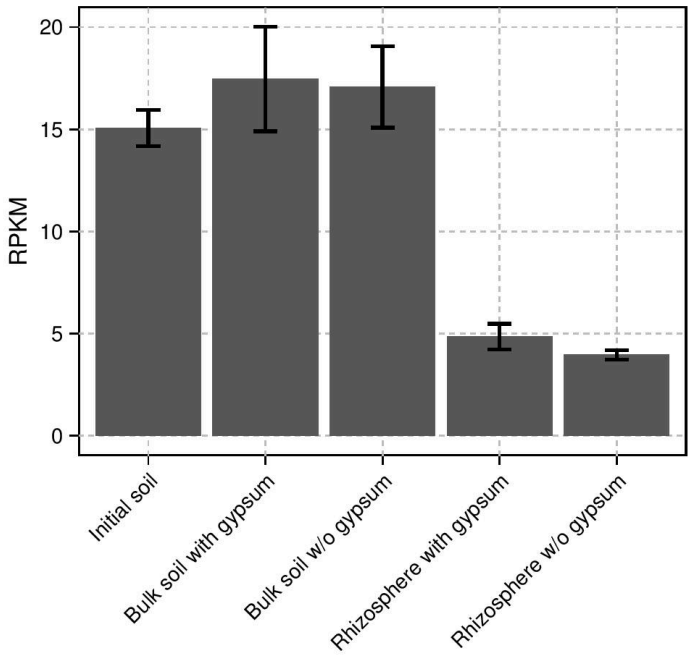
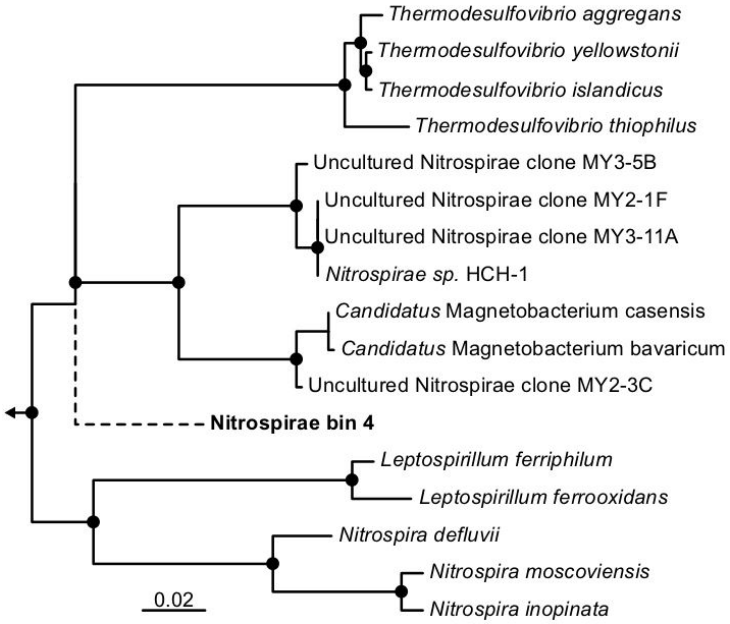
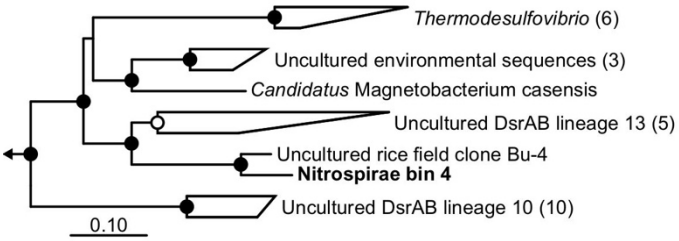


Figure 1 Relative abundance of the *Nitrospirae* bin in the treatments.

a.



b.



c.

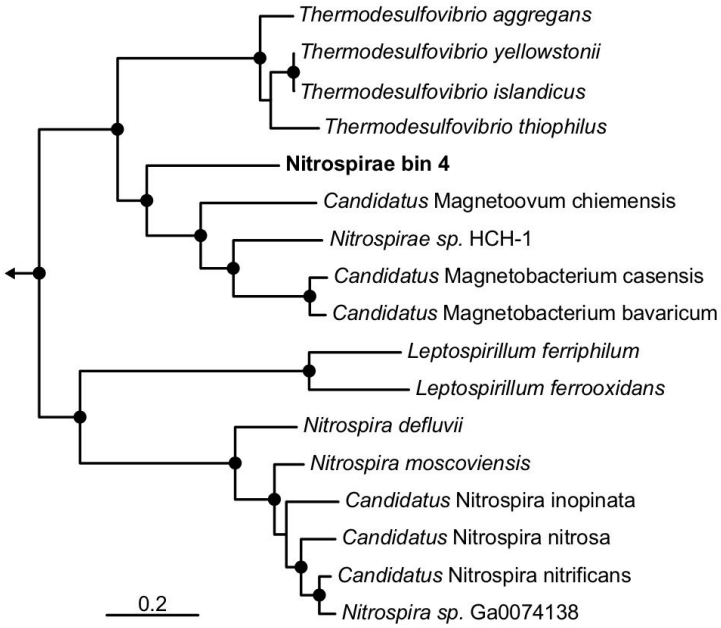


Figure 3 Phylogeny of the *Nitrospirae* bin based on the 23S rRNA gene (a), dsrAB genes (b) and phylogenomics (c).

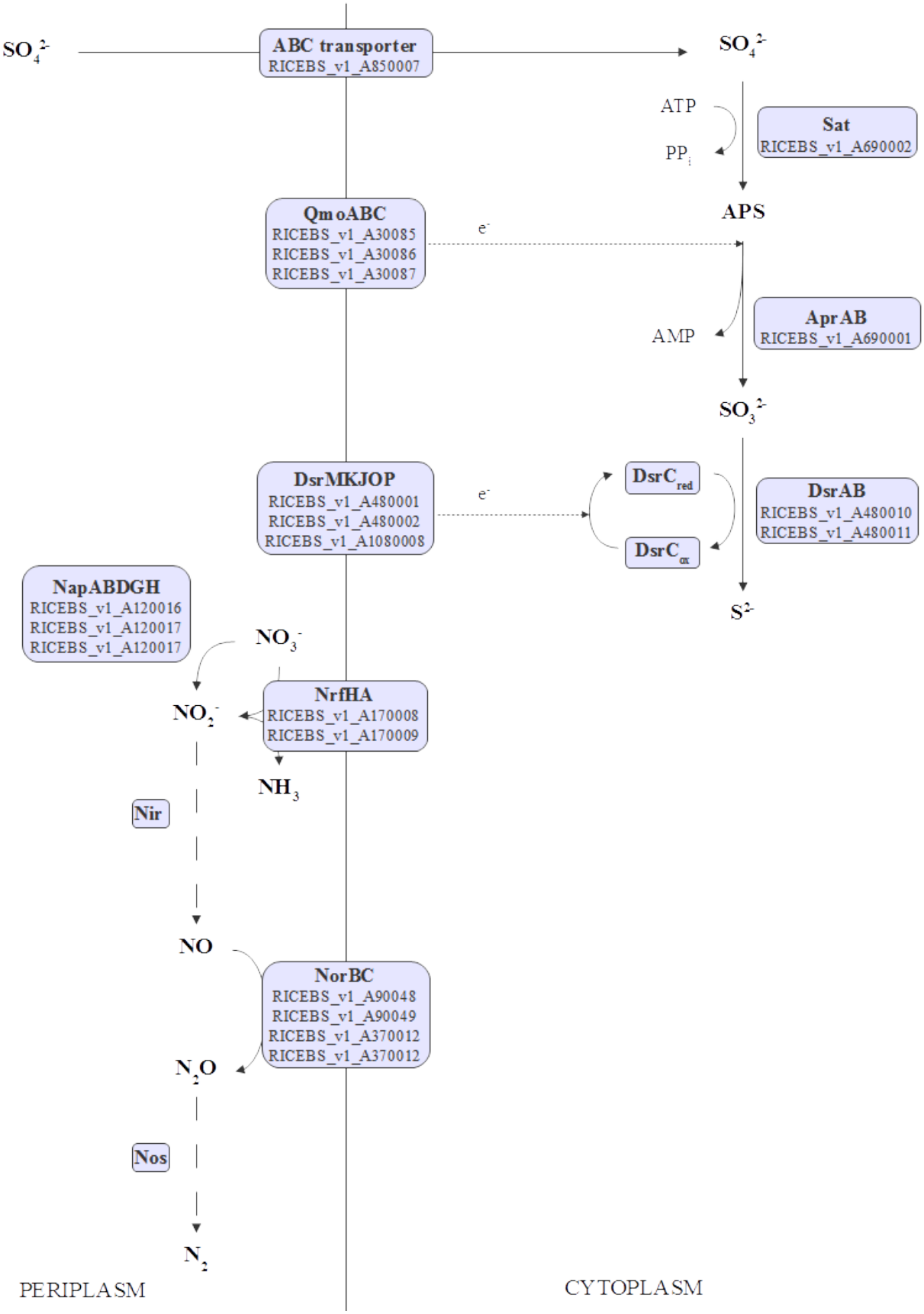


Figure 3 Sulfate and nitrate reduction pathways retrieved in the *Nitrospirae* bin. Dashed arrows: genes not retrieved in the genome; dotted arrows: hypothetical.

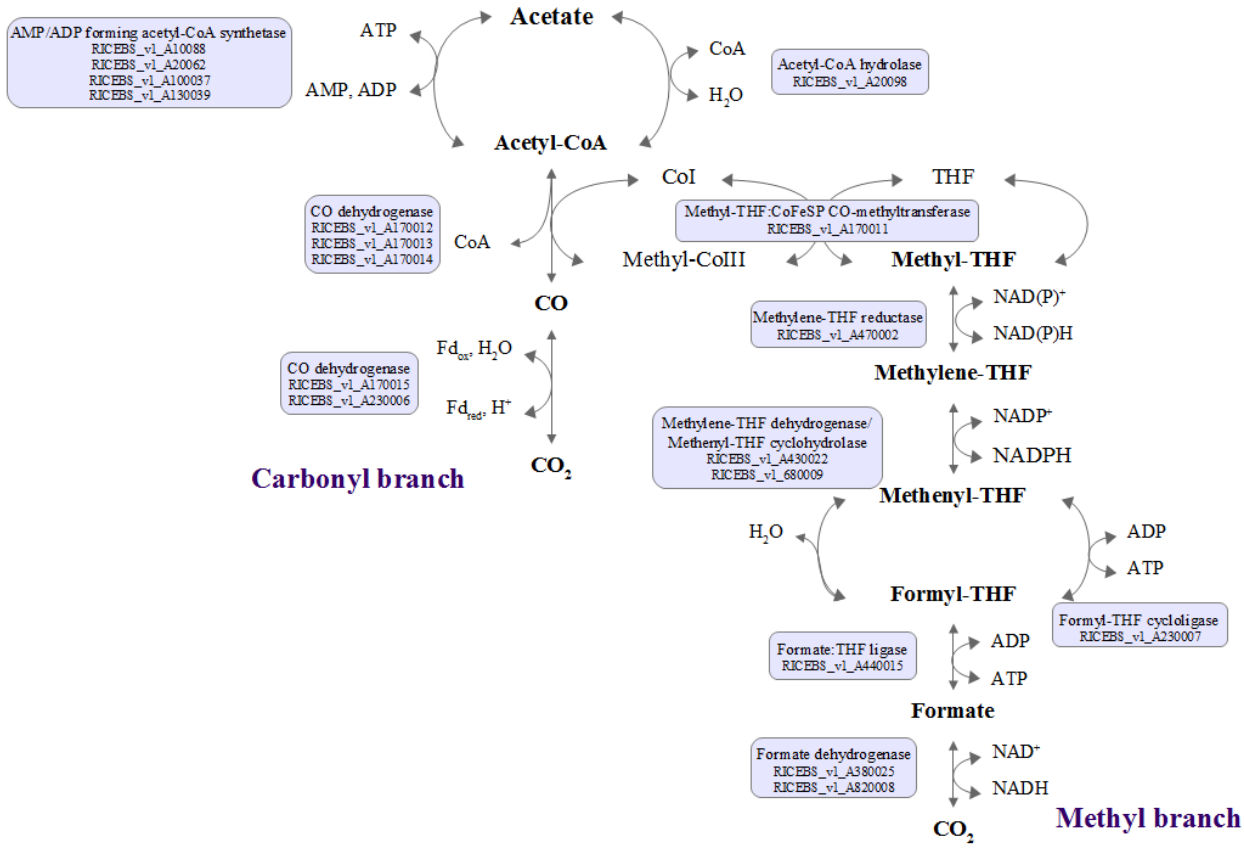


Figure 4 Genes retrieved in the *Nitrospirae* bin encoding for the Wood-Ljungdahl pathway.

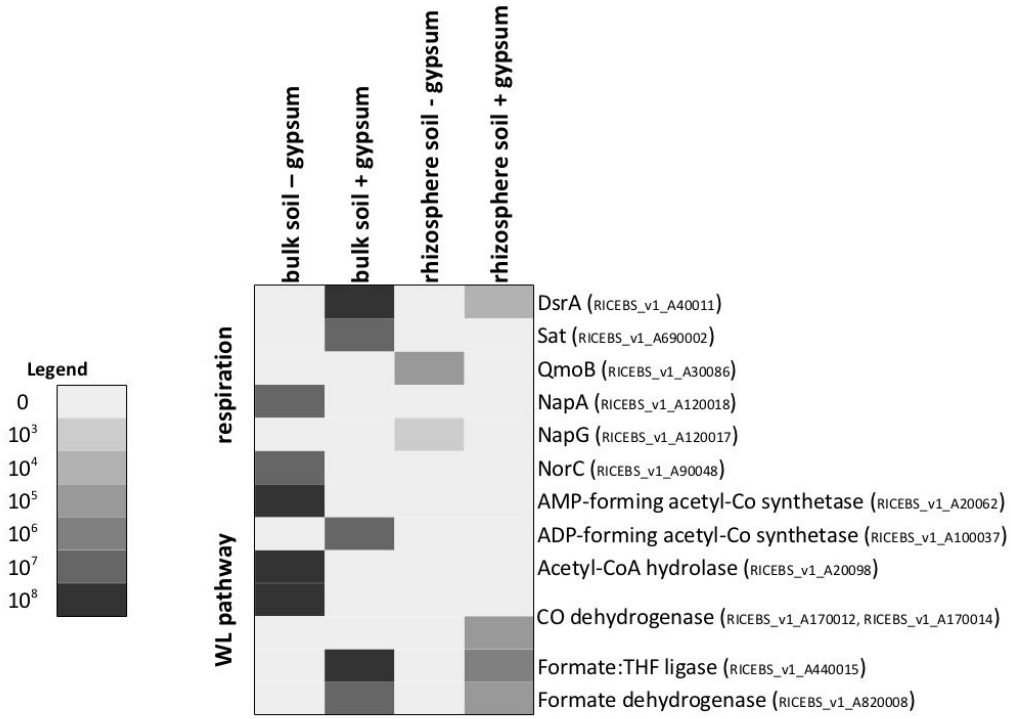


Figure 5 Expression of enzymes involved in sulfate reduction, nitrate reduction, nitric oxide reduction and in the Wood-Ljungdahl pathway in the different treatments. Measure unit: LFQ intensity.

Conclusions

Overall, the use of microbial ecology in the study of food systems could give an important contribute in deciphering the mechanisms on the bases of several food-environment issues. Rice rhizosphere is an extremely complex environment, where physic-chemical as well as biological parameters greatly vary within few millimeters of distance. Although data interpretation in microbial ecology is still quite challenging, these studies contributed to unravel different aspects of As cycle, revealing the identity of bacterial species putatively involved in As contamination of rice grains.

Among the first outcomes, it was demonstrated that continuous flooding promotes the proliferation of bacterial species putatively associated to As-releasing processes, such as dissimilatory Fe(III)-reducing bacteria, sulfur-oxidizing bacteria, dissimilatory As(V)-reducing bacteria and As(III)-methylating bacteria. Within these, members of the *Geobacteraceae* family appeared to be strongly influenced by water regime in the rhizosphere soil. However, continuous flooding was also related to a higher diversity in the active bacterial community (Chapters 2, 3), which might be indicative that these conditions are more favorable for rice plant growth, as already suggested by previous data on rice yield using different water regimes (Pinheiro *et al.*, 2006; Kato *et al.*, 2009). Therefore, an intermediate option such as a 14-days period drainage before flowering (Chapters 1, 2) should be recommendable in order to preserve plant health and diversity as well as low As uptake.

Another important outcome was that sulfate addition promotes As decrease in rice plants in an early life stage, which was not correlated with microbial potential for As(III) oxidation and/or methylation (Chapter 4). Further studies are needed in order to highlight possible mechanisms occurring in the endosphere involving the activities of endophytic plant symbionts.

Finally, we found indications for the presence of a new putatively sulfate-reducing *Nitrospirae* species inhabiting Italian rice paddies (Chapter 5). These microorganisms were enhanced by sulfate addition, possibly contributing to fuel sulfate reduction in rice rhizosphere. Indirectly, these microorganisms potentially contribute to As(III) co-precipitation with sulfide minerals. Further studies are needed in order to succeed in the isolation of these microorganisms, on the basis of their metabolic reconstruction, and confirm the existence and relevance of this putative new species/genus.

With the progress in the Next Generation Sequencing (NGS) techniques, these outcomes put the basis for the development of further investigations aimed to improve our knowledge on the metabolisms of these microorganisms in rice paddies and prove their actual metabolic rates *in situ*. In the future, refining our knowledge on their metabolic properties, we cannot exclude that even hardly lab-cultivable microorganisms could be employed for bioaugmentation purposes in rice paddies.

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Appendix

A. Supplementary information Chapter 1

Table A1 Probes used for fluorescence in situ hybridization (FISH).

Probe	Sequence (5'-3')	Target organism	Function	FA(%)	NaCl (mM)	Reference
EUB338 I	GCT GCC TCC CGT AGG AGT	Bacteria	-	35	70	Amann et al. 1990
EUB338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales and some OP11	-			Daims et al. 1999
EUB338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobia	-			
nonEUB	ACT CCT ACG GGA GGC AGC	Negative control	-			Wallner et al. 1993
Bone23a	GAA TCC CAT CCC CCT CT	Beta1 group of *Betaproteobacteria* (*Gallionella* sp., *Leptothrix* sp.)	Microaerobic Fe(II)-oxidation	35	70	Amann et al. 1996
cBone23a	GAA TTC CAC CCC CCT CT	Competitor of Bone23a				
Lepto175	ATC CAC AGA TCA CAT GCG	*Leptothrix ochracea*	Microaerobic Fe(II)-oxidation	15	318	Fleming 2011
BrG1-829	AAA GTG AAT TCC CAA CAA C	Iron-oxidizing denitrifiers *Betaproteobacteria*	Anaerobic Fe(II)-oxidation	0	900	Straub and Buchholz-Cleven 1998
BrG2-830	TTG CCA GTA TCC AGT GCC A			0	900	
Betthio1001	CTT AGC ACG TCA TTT GGG ACC	Betaproteobacterial Thiobacilli	Anaerobic Fe(II)-oxidation	25	149	Haaijer et al. 2006
Geo1423	TCA CGC ACT TCG TCG GGA CCA	*Geobacter* spp.	Fe(III)-reduction	25	149	Haaijer et al. 2008
SHEW227	AGC TAA TCC CAC CTA GGT WCA TC	*Shewanella* sp.	Fe(III)-reduction	40	46	Hugget et al. 2006
cSHEW227	AGC TAA TCC CAC CTA GGC WTA TC	Competitor of SHEW227	-			

Table A2 Primers used for quantitative real time PCR.

Target	Primer	Sequence	Concentration (ng μL^{-1})	Annealing T (°C)	Amplicon (bp)	Standard DNA	Reference
Eubacteria (total Bacteria)	Eub338F	ACT CCT ACG GGA GGC AGC AG	0.3	53	200	Strain 7 AsIII Res (*Ochrobactrum tritici*)	Fierer et al. 2005
	Eub518R	ATT ACC GCG GCT GCT GG					
Geobacteraceae (Fe^{3+}-reduction)	Geo564F	AAG CGT TGT TCG GAW TTA T	0.3	60	276	Clone GEO6	Cummings et al. 2003
	Geo840R	GGC ACT GCA GGG TCA ATA					
Shewanellaceae (Fe^{3+}-reduction)	She120F	GCC TAG GGA TCT GCC CAG TCG	0.3	60	100	Clone SHEW1	Himmelheber 2009

	She220R	CTA GGT TCA TCC AAT CGC G					
Gallionella-like (Fe^{2+}-oxidation)	628F	GBM AGG CTA GAG TGT AGC	0.3	56	370	Clone GAL1	Wang et al. 2011
	998R	CTC TGG AAA CTT CCT GAC					Wang et al. 2009 a
arsC Bacillus sp. (Arsenate reductase)	ArsC52F	AGC CAA ATG GCA GAA GC	0.4	55	275	Clone ArsC14	Bachate et al. 2009
	ArsC323R	GCT GGR TCR TCA AAT CCC CA					
aioA (Arsenite oxidase)	aoxBM1-2F	CCA CTT CTG CAT CGT GGG NTG YGG NTA	0.3	59	550	Strain 1L (*Achromobacter* sp.)	Quemeneur et al. 2008
	aoxBM2-1R	GGA GTT GTA GGC GGG CCK RTT RTG DAT					Quemeneur et al. 2010
arsM (Arsenite S-methyltransferase)	arsMF1	TCY CTC GGC TGC GGC AAY CCV AC	0.3	74	346	Clone ArsM6	Jia et al. 2013
	arsMR2	CGW CCG CCW GGC TTW AGY ACC CG					
arrA (Dissimilatory arsenate reductase)	ArrAF	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYT T	0.2 - 0.5	48 - 68	160 - 200	-	Malasarn et al. 2004
	ArrAR	CCT GTG ATT TCA GGT GCC CAY TYV GGN GT					
	ArrAF1	CCC GCT ATC ATC CAA TCG	0.2 - 0.5	48 - 68	187	-	Upadhyaya et al. 2012
	ArrAR1	GGT CAG GAG CAC ATG AG					
	ArrAF2	CAT CGC TTC TCG CTG TG	0.2 - 0.5	48 - 68	201	-	
	ArrAR2	GAG GTA GTT GCA GTT TCG					
	HAArrA-D1F	CCG CTA CTA CAC CGA GGG CWW YTG GGR NTA	0.2 - 0.5	48 - 68	500	-	Kulp et al. 2006
	HAArrA-G2R	CGT GCG GTC CTT GAG CTC NWD RTT CCA CC					

Table A3 Clones obtained for the construction of qPCR standard curve. Identities of ArsC and ArsM translated proteins were evaluated for *arsC* and *arsM* genes respectively.

Primers	Source	Clone	Length (bp)	Match	Identity	Accession number
Geo564F Geo840R	Rice rhizosphere reproductive phase continuous flooding	GEO6	208	*Geobacter bremensis*	99%	KX592448
		GEO11	217	*Geobacter* sp.	99%	KX592449
Shew120F Shew220R	Iron bacteria enrichment culture from rice roots	SHEW1	116	*Shewanella* sp. S8	100%	KX592450
		SHEW2	121	*Shewanella baltica* OS223	95%	KX592451
		SHEW3	121	*Shewanella baltica* OS106	94%	KX592452
		SHEW4	121	*Shewanella* sp. S8	100%	KX592453
		SHEW6	121	*Shewanella* sp. S8	100%	KX592454
		SHEW7	121	*Shewanella* sp. S8	100%	KX592455

		SHEW8	115	*Shewanella* sp. S8	99%	KX592456
		SHEW9	121	*Shewanella* sp. S8	100%	KX592457
Gal628F Gal998R	Rice rhizosphere ripening continuous flooding	GAL1	372	*Gallionella* sp. enrichment culture clone MWE_C19	99%	KX592445
		GAL2				KX592446
		GAL3				KX592447
ArsC52F ArsC323R	Rio Rosso salix rhizosphere soil	ArsC14	204	ArsC of *Arthrobacter* sp. CH72	92%	KX592458
ArsMF1 ArsMR2	Unplanted rice field soil (used for continuous flooding)	ArsM6	278	ArsM of *Cohnella laeviribosi*	79%	KX592459
		ArsM7	275	ArsM of *Dehalogenimonas* sp. WBC-2	69%	KX592460
		ArsM10	268	ArsM of *Terriglobus roseus*	79%	KX592461
		ArsM15	277	ArsM of *Rhodopseudomonas palustris*	89%	KX592462
		ArsM16	277	ArsM of *Rhodopseudomonas palustris*	86%	KX592463
		ArsM20	288	ArsM of *Rhodopseudomonas palustris*	85%	KX592464

Table A4 Cell numbers gdw^{-1} obtained with DAPI and FISH at flowering. Lowercase letters indicate significantly different treatments (Tukey's test, $p \leq 0.05$), whereas non-significant differences were denoted with n.s..

Probe	CF	2IED	AR
DAPI	4.55E+09 **n.s.**	3.41E+09	8.13E+09
EUB	8.47E+07 **a**	3.48E+08 **b**	2.66E+08 **b**
Bone23a	7.59E+05 **n.s.**	2.05E+06	4.42E+05
Shew227	4.74E+06 **b**	5.59E+06 **b**	2.40E+06 **a**
Geo1423	6.96E+05 **a**	2.87E+06 **b**	4.42E+05 **a**
Lepto175	3.99E+06 **a**	7.21E+06 **b**	2.19E+06 **a**
Betthio1001	4.87E+06 **b**	1.61E+06 **b**	5.68E+05 **a**
BrG1 829	2.15E+06 **n.s.**	3.30E+06	3.99E+06
BrG2 830	1.93E+06 **n.s.**	1.25E+06	7.22E+05

Table A5 Quantitative real time PCR of the 16S rRNA genes of total and Fe-cycling Bacteria. Values are expressed as gene copy number gdw^{-1}. Lowercase letters and n.s.: respectively, significantly different treatments in one timepoint (Tukey's test, $p \leq 0.05$) and not significant; star: significant difference of senescence with respect to flowering (t test, $p \leq 0.05$); cross: significant difference in the rhizoplane with respect to rhizosphere soil (t test, $p \leq 0.05$).

Target	Compartment	Unplanted soil	Flowering			Senescence		
			CF	2IED	AR	CF	2IED	AR
Eub	-	9.60E+10	-	-	-	-	-	-
	RS	-	1.54E+11 **a**	3.47E+11 **ab**	5.30E+11 **b**	4.13E+11 **b, ***	4.09E+11 **b, ***	2.12E+11 **a**
	RP	-	2.41E+08 **a, †**	4.75E+10 **c, †**	1.12E+10 **b, †**	2.62E+10 **a, *, †**	1.12E+11 **b, *, †**	6.24E+11 **c, *, †**
Geo	-	4.90E+08	-	-	-	-	-	-

	RS	-	1.17E+09 **b**	6.37E+09 **b**	2.95E+08 **a**	1.10E+09 **b**	6.23E+09 **b**	5.31E+07 **a**
	RP	-	6.60E+07 **a,** †	4.32E+09 **b**	5.38E+07 **a,** †	3.59E+08 †	8.73E+07 ***,** †	1.98E+08 †
	-	3.26E+05	-	-	-	-	-	-
Shew	RS	-	2.64E+06 **a**	6.72E+06 **a**	5.06E+07 **b, ***	1.01E+06 **a**	7.78E+05 **a, ***	7.65E+06 **b**
	RP	-	1.35E+05 **a,** †	3.22E+05 **a,** †	6.94E+06 **b,** †	6.74E+05 **a,** †	1.70E+05 **a, *,** †	5.12E+06 **b**
	-	3.37E+05	-	-	-	-	-	-
Gall	RS	-	6.84E+05 **b**	5.02E+06 **c**	2.34E+03 **a**	3.17E+06 **c, ***	1.01E+06 **b, ***	1.86E+03 **a**
	RP	-	1.36E+04 **b,** †	4.76E+05 **c,** †	1.22E+03 **a**	1.24E+03 ***,** †	1.96E+03 ***,** †	4.97E+03 *

Table A6 Quantitative real time PCR of arsenic genes. Values are expressed as gene copy number gdw^{-1}. Lowercase letters and n.s.: respectively, significantly different treatments in one timepoint (Tukey's test, $p \leq 0.05$) and not significant; star: significant difference of senescence with respect to flowering (t test, $p \leq 0.05$); cross: significant difference in the rhizoplane with respect to rhizosphere soil (t test, $p \leq 0.05$).

Target	**Compartment**	**Unplanted soil**	**Flowering**			**Senescence**		
			CF	**2IED**	**AR**	**CF**	**2IED**	**AR**
	-	2.14E+07	-	-	-	-	-	-
aioA	RS	-	2.12E+07	1.42E+07	1.15E+07	2.47E+07 **b**	2.41E+07 **b**	4.07E+06 **a, ***
	RP	-	4.31E+06 **a,** †	3.18E+08 **b,** †	1.57E+08 **b,** †	9.87E+07 **b, *,** †	1.01E+07 **a, ***	2.96E+09 **c, *,** †
	-	4.76E+05	-	-	-	-	-	-
arsC	RS	-	6.50E+05 **ab**	3.15E+05 **a**	1.22E+06 **b,**	1.03E+06	1.10E+06	6.37E+05
	RP	-	1.04E+05**,** †	2.20E+05	2.08E+05 †	9.76E+04 **a,** †	3.17E+05 **a,** †	3.53E+06 **b, *,** †
	-	1.25E+06	-	-	-	-	-	-
arsM	RS	-	2.35E+06	3.52E+06	3.97E+06	1.47E+06	8.56E+05 *	3.86E+05 *
	RP	-	5.28E+05 **a**, †	8.71E+05 **a**	3.50E+06 **b**	5.34E+05 **b**, †	2.95E+04 **a, ***, †	6.08E+06 **c, *,** †

B. Supplementary information Chapter 2

Table B1 Bacterial taxa retrieved in this study for which arsenic-processing and/or iron- and sulfur-processing have been experimentally demonstrated. The related references are reported below the table.

Taxon	Fe(III) reduction	Fe(II) oxidation	Sulfur oxidation	Diss. sulfate reduction	Diss. As(V) reduction	As resistance (*ars* or *ACR*)	As(III) oxidation	As(III) methylation
Candidatus Solibacter	-	-	-	-	-	-	-	20
Geothrix	26, 30, 32	-	-	-	-	-	-	-
Mycobacterium	-	-	-	-	-	1	-	-
Amycolatopsis	-	-	-	-	-	-	-	20
Streptomyces	-	-	-	-	-	1	-	-
Conexibacter	-	-	-	-	-	-	-	20
Aurantimonas	-	-	33	-	-	-	-	-
Bradyrhizobium	-	-	33	-	-	-	-	-
Rhodopseudomonas	-	8, 10, 16, 32	33	-	-	12	-	7, 20, 47
Rhodomicrobium	-	8, 16	-	-	-	-	-	20
Methylobacterium	-	-	11	-	-	-	-	-
Mesorhyzobium	-	8	-	-	-	1	14	-
Rhizobium	-	-	-	-	-	-	14	-
Magnetospirillum	-	-	-	-	-	12	-	-
Rhodobacter	-	8, 10, 16, 32	33	-	-	-	-	-
Rhodovulum	-	-	8, 16	-	-	-	-	-
Sphingopyxis	-	-	-	-	-	1	-	-
Achromobacter	-	-	-	-	-	12	4, 14	7
Burkholderia	-	-	-	-	-	12	4	7
Limnobacter	-	-	-	-	-	-	4	-
Polynucleobacter	-	-	33	-	-	-	-	-
Ralstonia	-	-	33	-	-	-	4	-
Acidovorax	-	8, 10, 16, 32	-	-	-	-	4	-
Aquabacterium	-	8, 16	-	-	-	-	-	-
Comamonas	-	-	33	-	-	-	-	-
Leptothrix	-	10, 16, 32	-	-	-	-	-	-
Polaromonas	-	-	33	-	-	12	4	-
Variovorax	-	-	-	-	-	-	14	-
Herminiimonas	-	-	33	-	-	-	4, 14	-
Thiobacillus	30	8, 10, 16, 32	33	-	-	-	-	20
Sideroxydans	-	8, 10, 16, 32	-	-	-	12	-	-
Dechloromonas	-	8	33	-	-	12	4	-
Acidiferrobacter	-	16	-	-	-	-	-	-
Acinetobacter	-	-	-	-	-	12	4	-
Pseudomonas	-	8	-	-	-	1	4	-
Stenotrophomonas	-	-	-	-	-	-	4	-

Thermomonas	-	8, 16	-	-	-	-	-	-
Desulfobacteraceae	-	-	-	34	-	-	-	-
Pelobacter	30	-	-	-	-	12	-	-
Geobacter	26, 30, 32	8	-	-	4, 12	12	-	20
Geothermobacter	21	-	-	-	-	-	-	-
Anaeromyxobacter	19	-	33	-	-	12	-	-
Desulfomonile	-	-	-	34	-	-	-	-
Syntrophus	-	-	-	-	-	12	-	-
Desulfovirga	-	-	-	34	-	-	-	-
Syntrophobacter	-	-	-	34	-	-	-	-
Cytophaga	-	-	-	-	-	-	-	20
Flavobacterium	-	-	-	-	-	-	-	7
Chitinophaga	-	-	-	-	-	-	-	20
Niastella	-	-	-	-	-	-	-	20
Nostoc	-	-	-	-	-	-	-	46
Bacillus	30	-	-	-	4, 12	1	-	-
Clostridium	-	-	-	-	-	-	-	3
Desulfitobacterium	-	-	-	-	4, 12	-	-	20
Desulfosporosinus	-	-	-	34	4, 12	-	-	-
Pelotomaculum	-	-	-	-	-	-	-	20
Gemmatimonas	-	-	-	-	-	-	-	20
Nitrospira	-	-	-	-	-	-	-	20
Spirochaeta	-	-	33	-	-	-	-	-
Opitutus	-	-	-	-	-	12	-	20

Table B2 Number of reads produced with 16S rRNA amplicon pyrosequencing that passed the quality check, with related diversity information. Values are shown for macrocosms that underwent continuous flooding (CF), continuous flooding with 2 weeks of drainage before flowering (CF-D) and watering every 10 days (D). The operational taxonomic units (OTUs) were defined at 97% sequence identity. To compare the diversity in the different treatments, values for a standardized sampling effort of 2000 reads are provided.

Compartment	Sample ID	N. of reads	Seq. length (bp)	N. of OTUs	N. of OTUs in 2000 reads	N. of phyla
Soil	UN	18428	364 ± 26	5598	898 ± 55	32
Rhizosphere soil	CF	16497	364 ± 26	5934	984 ± 28	28
	CF-D	28005	363 ± 28	7108	777 ± 31	24
	D	26309	364 ± 25	7097	836 ± 33	18
Rhizoplane	CF	20370	363 ± 27	8088	1158 ± 51	25
	CF-D	19346	363 ± 26	7579	1062 ± 67	19
	D	21424	363 ± 27	5122	699 ± 61	15

Table B3 Relative abundance of taxa involved in either direct or indirect arsenic processing (see Table S1). Values are shown for the different compartments, managed either with continuous flooding (CF), with continuous flooding with 2 weeks of drainage before flowering (CF-D) or with watering every 10 days (D).

Taxon		Rhizosphere soil			Rhizoplane		
	UN	CF	CF-D	D	CF	CF-D	D
Candidatus *Solibacter*	0.67±0.25	0.71±0.3	0.56±0.17	0.34±0.06	0.97±0.21	0.92±0.21	0.35±0.17
Geothrix	0	0.01±0.02	0.02±0.02	0	0.02±0.02	0	0
Mycobacterium	0.01±0.01	0.01±0.02	0.01±0.01	0.01±0.01	0	0	0.01±0.02

Amycolatopsis	0.01±0.01	0	0	0	0	0	0
Streptomyces	0.14±0.19	0.03±0.02	0.01±0.02	0.12±0.01	0.01±0.02	0	0.21±0.1
Conexibacter	0.09±0.09	0.1±0.06	0	0	0.02±0.04	0	0
Aurantimonas	0	0	0	0.31±0.11	0	0	0.12±0.08
Bradyrhizobium	0.34±0.27	0.01±0.02	0.11±0.1	0.09±0.04	1.76±0.6	2.11±0.05	0.03±0.03
Rhodopseudomonas	0	0	0.01±0.01	0.01±0.01	0.09±0.06	0	0
Rhodomicrobium	0.05±0.04	0.05±0.06	0	0	0.02±0.02	0	0.01±0.02
Methylobacterium	0.02±0.03	0.01±0.02	0.01±0.01	0.01±0.03	0.03±0.03	0	0.14±0.02
Mesorhizobium	0.01±0.01	0	0	0.04±0.001	0.02±0.02	0.02±0.02	0.01±0.01
Rhizobium	0.27±0.35	0.04±0.001	0.07±0.04	2.06±0.63	0.44±0.16	1.21±0.25	1.90±0.47
Magnetospirillum	0.01±0.02	0.1±0.06	0.19±0.07	0	0.19±0.12	0.15±0.12	0
Rhodobacter	0	0.2±0.21	0.13±0.04	0.06±0.02	0.1±0.03	0.01±0.02	0
Rhodovulum	0	0.13±0.05	0.05±0.03	0	0.05±0.06	0	0
Sphingopyxis	0	0	0	0.05±0.03	0	0.06±0.02	0.16±0.09
Achromobacter	0.01±0.01	0.01±0.02	0.01±0.01	0.04±0.03	0.01±0.02	0.02±0.02	0.04±0.06
Burkholderia	0.03±0.02	0.05±0.02	0.01±0.01	0.15±0.03	0	0	0.04±0.03
Limnobacter	0	0	0.03±0.02	0	0	0	0
Polynucleobacter	0	0	0	0	0.01±0.02	0.01±0.02	0
Ralstonia	0	0	0.01±0.01	0.07±0.03	0.01±0.02	0	0.05±0.05
Acidovorax	0.02±0.03	0.03±0.02	0.07±0.07	0.06±0.02	0.14±0.16	0.08±0.1	0.01±0.01
Aquabacterium	0	0.07±0.05	0.05±0.01	0.01±0.01	0.03±0.03	0	0
Comamonas	0.01±0.02	0.01±0.02	0.34±0.09	0.04±0.01	0.01±0.02	0.04±0.02	0
Leptothrix	0	0.1±0.05	0.07±0.02	0	0.02±0.02	0.01±0.02	0
Polaromonas	0.02±0.02	0	0	0	0	0	0.01±0.01
Variovorax	1.1±0.51	0.23±0.06	0.69±0.04	0.22±0.06	0.16±0.11	0.08±0.02	6.76±1.44
Herminiimonas	0.01±0.02	0	0.01±0.01	0	0	0	0
Thiobacillus	0±0.01	0	0	0	0.05±0.04	0.03±0.03	0
Sideroxydans	0.02±0.03	0	0.04±0.02	0	0.03±0.03	0.02±0.02	0
Dechloromonas	0	0	0.03±0.03	0	0.06±0.03	0.02±0.02	0
Acidiferrobacter	0	0.01±0.02	0	0	0	0.02±0.04	0
Acinetobacter	0	0	0	0.12±0.05	0.02±0.04	0	0.01±0.01
Pseudomonas	0.04±0.03	0.28±0.01	1.82±0.35	2.69±0.59	0.34±0.19	0.01±0.02	0.18±0.07
Stenotrophomonas	0.01±0.01	0	0.07±0.08	0.83±0.22	0.01±0.02	0	0.98±0.38
Thermomonas	0.12±0.12	0.05±0.06	0.26±0.16	1.48±0.53	0.10±0.07	0.09±0.04	0.77±0.1
Desulfobacteraceae	0.03±0.03	0.59±0.3	0.01±0.01	0	0.05±0.04	0.07±0.07	0
Pelobacter	0	0	0.01±0.01	0	0.01±0.02	0	0
Geobacter	0.03±0.02	0.65±0.13	0.45±0.16	0.02±0.04	2.04±0.54	0.55±0.25	0
Geothermobacter	0	0.05±0.05	0	0	0	0	0
Anaeromyxobacter	0.13±0.11	1.11±0.35	0.13±0.02	0	0.64±0.32	0.12±0.09	0
Desulfomonile	0	0	0	0	0.02±0.02	0.06±0.04	0
Syntrophus	0.01±0.01	0.05±0.06	0	0	0.11±0.04	0	0
Desulfovirga	0	0	0	0.01±0.01	0	0	0
Syntrophobacter	0.02±0.03	0.03±0.02	0	0	0.03±0.03	0	0
Cytophaga	0	0	0	0	0	0.01±0.02	0
Flavobacterium	0.02±0.01	0	0	0	0	0	0
Chitinophaga	0	0	0	0	0	0.01±0.02	0.03±0.02
Niastella	0.01±0.01	0	0.03±0.01	0	0.05±0.05	0	0.04±0.02
Nostoc	2.11±2.01	0.98±0.44	0.01±0.01	0.04±0.02	0.15±0.04	0.18±0.04	0.28±0.03
Bacillus	0.28±0.23	0.23±0.05	0.02±0.04	0.14±0.08	0.18±0.07	0.15±0.1	0.04±0.02
Clostridium	0.04±0.04	0.18±0.02	0.07±0.01	0.15±0.02	0.55±0.13	0.23±0.01	0
Desulfitobacterium	0	0	0	0	0	0.01±0.02	0

Desulfosporosinus	0	0	0.01±0.01	0	0	0	0
Pelotomaculum	0	0.03±0.02	0	0	0.01±0.02	0	0
Gemmatimonas	0.64±0.37	1.89±0.59	0.74±0.11	1.71±0.45	0.43±0.14	0.48±0.24	0.32±0.07
Nitrospira	0.01±0.01	0	0	0.03±0.01	0.08±0.07	0.2±0.15	0.01±0.01
Spirochaeta	0.01±0.02	0	0	0	0.05±0.05	0.01±0.02	0
Opitutus	0.05±0.02	0.01±0.02	0.04±0.04	0.01±0.01	0.15±0.13	0.01±0.02	0

Table B4 Mean counts calculated for the OTUs that vary significantly (according to ANOVA, $p < 0.05$, after Bonferroni's correction) in the macrocosms treated either with continuous flooding (CF), with continuous flooding with 2 weeks of drainage before flowering (CF-D) or with watering every 10 days (D).

Compartment	CF	CF-D	D	Taxon	OTU
Rhizosphere soil	0	0	83.7	*Micrococcales*	denovo23217
	1.7	0.7	39.7	*Arthrobacter*	AB637277
	0	0.3	22.3	*Arthrobacter*	EU221355
	0	0	37.7	*Arthrobacter*	FJ382040
	0.3	0.3	8.7	*Agromyces*	denovo36205
	5.7	0	0	Uncultured *Gaiellales*	denovo9942
	0	22	0.7	*Flavisolibacter*	denovo35275
	0.3	9.3	0.3	*Sphingomonas*	denovo27415
	2	59.3	9.3	*Pelomonas*	FJ269077
	22.7	222.7	12	*Comamonadaceae*	JN869130
	1	18.3	0	*Comamonadaceae*	EF018534
	2.3	29	1	*Comamonadaceae*	FQ658719
	0	10.7	1	*Comamonadaceae*	EU133771
	0	5.3	0	*Comamonadaceae*	JF267702
	0	5.3	0	*Comamonadaceae*	AY491563
	4	110	21	*Ramlibacter*	HQ640565
	0.3	21.3	0.7	*Pseudomonas*	JN038312
	0	0	13.3	*Xanthomonadaceae*	HQ341391
	0	1.3	123	*Lysobacter*	FR682714
	7.3	0.7	0	*Dictyoglomus*	denovo11774
Rhizoplane	0	0	25.3	Bacteria	denovo26504
	4	0.7	48.3	*Arthrobacter*	FQ659744
	0	1	12	*Nocardioides*	denovo13694
	1.3	0	20	Uncultured *Gaiellales*	EU132848
	0	0	13	*Flavisolibacter*	JN409004

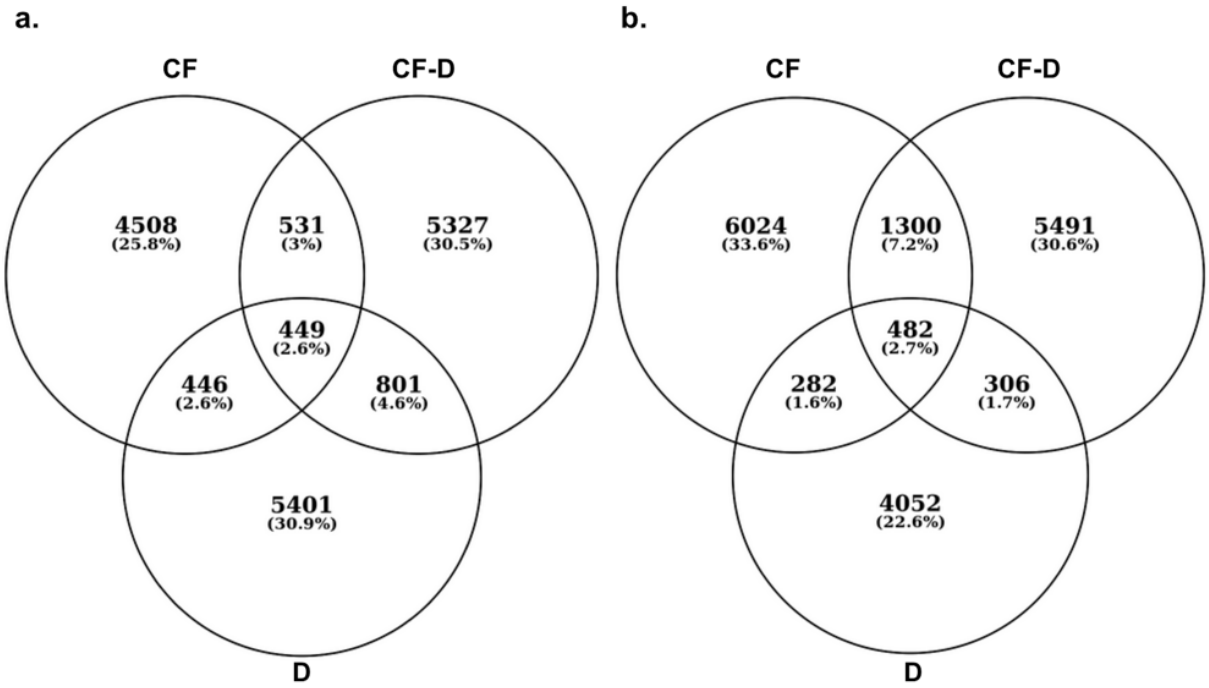

Fig. B1 Shared and exclusive OTUs at 97% similarity retrieved under continuous flooding (CF), under continuous flooding with drainage before flowering (CF-D) and with watering (D) in rhizosphere soil (a) and in the rhizoplane (b)

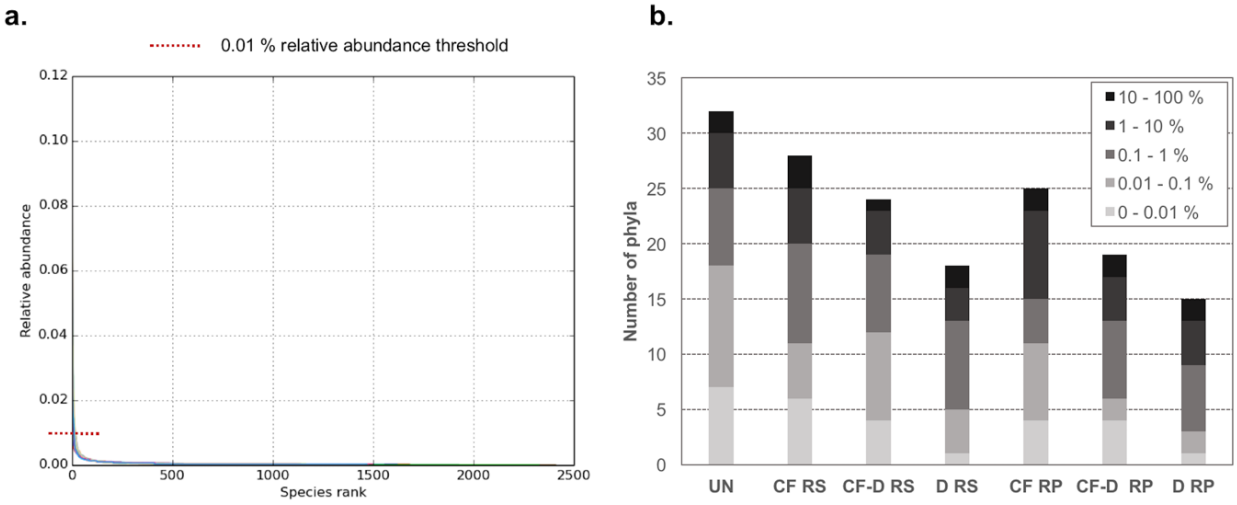

Fig. B2 Rank abundance (a) and number of phyla categorized according to their relative abundance (b). Values are shown for unplanted soil, rhizosphere soil and rhizoplane (UN, RS and RP) managed either with continuous flooding (CF), with continuous flooding with 14 days drainage before flowering (CF-D) or with watering every 10 days (D)

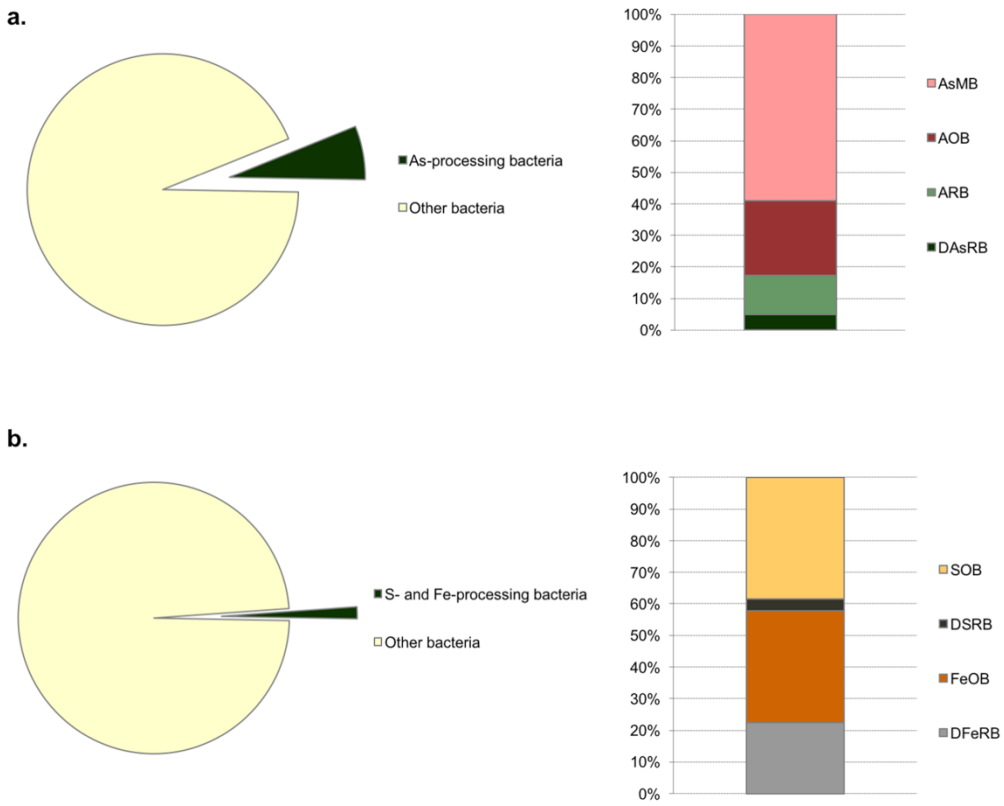

Fig. B3 Relative abundance of species potentially able to process arsenic directly (a) or indirectly as a consequence of their metabolism (b) in the unplanted soil. The metabolic groups considered in this analysis were dissimilatory As(V)-reducing bacteria (DAsRB), As-resistant bacteria (AsRB), As(III)-oxidizing bacteria and As(III)-methylating bacteria (AsMB), dissimilatory Fe(III)-reducing bacteria (DFeRB), Fe(II)-oxidizing bacteria (FeOB), dissimilatory SO_4^{2-}-reducing bacteria (DSRB) and sulfur-oxidizing bacteria (SOB)

C. Supplementary information Chapter 5

Table C1 Genes found in the *Nitrospirae* bin encoding for sulfate and nitrate respiration, for acetate, butyrate and carbohydrate degradation and for the pentose phosphate pathway, with related protein expression scores in the bulk soil without and with gypsum (CBS and SBS) and in rhizosphere soil with and without gypsum (CRS and SRS).

SULFATE RESPIRATION							
Enzyme	**Gene**	**Begin**	**End**	**CBS**	**SBS**	**CRS**	**SRS**
Dissimilatory bisulfite reductase A	*dsrA*	1852030	1853256	0	119640000	0	60535
Dissimilatory bisulfite reductase B	*dsrB*	1850934	1852004	0	0	0	0
Dissimilatory bisulfite reductase C	*dsrC*	1847391	1847726	0	0	0	0
Dissimilatory bisulfite reductase M	*dsrM*	1845603	1846595	0	0	0	0
Dissimilatory bisulfite reductase K	*dsrK*	1844008	1845600	0	0	0	0
Dissimilatory bisulfite reductase P	*dsrP*	2627797	2628821	0	0	0	0
Adenylyl sulfate reductase B	*aprB*	2221252	2221698	0	0	0	0
Sulfate adenylyltransferase	*sat*	2221859	2223073	0	31680000	0	0
Quinone-interacting membrane-bound oxidoreductase complex, subunit A	*qmoA*	300919	302181	0	0	0	0
Quinone-interacting membrane-bound oxidoreductase complex, subunit B	*qmoB*	298553	300913	0	0	116770	0
Quinone-interacting membrane-bound oxidoreductase complex, subunit C	*qmoC*	297373	298542	0	0	0	0
ABC transporter	-	2421476	2422180	0	0	0	0
NITRATE RESPIRATION							
Enzyme	**Gene**	**Begin**	**End**	**CBS**	**SBS**	**CRS**	**SRS**
Nitrate reductase A	*napA*	746977	749748	13501000	0	0	0
Nitrate reductase B	*napB*	744742	745299	0	0	0	0
Nitrate reductase D	*napD*	744434	744745	0	0	0	0
Nitrate reductase G	*napG*	746148	746933	0	0	4868.04	0
Nitrate reductase H	*napH*	745319	746146	0	0	0	0
Cytochrome c nitrate reductase	*nrfH*	962539	964026	0	0	0	0
Ammonia-forming nitrate reductase	*nrfA*	962057	962521	0	0	0	0
Nitric oxide reductase B	*norB*	625030	626421	0	0	0	0
Nitric oxide reductase B	*norB*	1600843	1602165	0	0	0	0
Nitric oxide reductase C	*norC*	624346	625017	21285000	0	0	0
Nitric oxide reductase C	*norC*	1600156	1600830	0	0	0	0
ACETATE DEGRADATION							
Enzyme	**Gene**	**Begin**	**End**	**CBS**	**SBS**	**CRS**	**SRS**
AMP-forming acetyl-CoA synthetase	*acsA*	167202	169172	498390000	0	0	0
ADP-forming acetyl-CoA synthetase	*acdA/B*	817782	820460	0	0	0	0
ADP-forming acetyl-CoA synthetase	*acdA*	667743	669113	0	10613000	0	0
ADP-forming acetyl-CoA synthetase	*acdB*	99766	100470	0	0	0	0
Acetyl-CoA hydrolase	-	209097	210977	323640000	0	0	0
CO dehydrogenase/Acetyl-CoA syntase B	*acsB*	968000	970198	355480000	0	0	0
CO dehydrogenase/Acetyl-CoA syntase D	*acsD*	965564	966505	0	0	0	0
CO dehydrogenase/Acetyl-CoA syntase C	*acsC*	966549	967889	0	0	0	375680
CO dehydrogenase	*cooS*	970218	972203	0	0	0	0

CO dehydrogenase	*cooC*	1181417	1182247	0	0	0	0
Methyl-THF:CoFeSP CO-methyltransferase	*acsE*	964640	965497	0	0	0	0
Methylene-THF reductase	*met*	1822829	1823809	0	0	21126	0
Methylene-THF dehydrogenase/Methenyl-THF cyclohydrolase	*folD*	1752610	1753518	0	412110000	0	0
Methylene-THF dehydrogenase/Methenyl-THF cyclohydrolase	*folD*	2211239	2212183	0	0	0	0
Formyl-THF cycloligase	-	1182297	1182878	0	0	0	0
Formate-THF ligase	*fhs*	1768119	1769882	0	519840000	0	1694700
Formate dehydrogenase	*fdh*	1635004	1636644	0	0	0	0
Formate dehydrogenase	*fdh*	2389318	2391813	0	10852000	0	184350

BUTYRATE DEGRADATION

Enzyme	Gene	Begin	End	CBS	SBS	CRS	SRS
Butyryl-CoA dehydrogenase	-	1075795	1076964	0	0	0	41798
3-hydroxybutyryl-CoA dehydratase	-	1074521	1074946	0	0	0	59470
3-hydroxybutyryl-CoA dehydrogenase	-	1074918	1075766	0	0	2664500	0
Acetyl-CoA acetyltransferase	-	1076966	1078153	0	0	0	0
Acyl-CoA dehydrogenase	-	1078317	1079516	0	0	0	0

CARBOHYDRATE DEGRADATION

Enzyme	Gene	Begin	End	CBS	SBS	CRS	SRS
Glycosyl hydrolase	-	2138322	2140334	0	5480200	0	0

PENTOSE PHOSPHATE PATHWAY

Enzyme	Gene	Begin	End	CBS	SBS	CRS	SRS
6-phosphogluconolactonase	*pgl*	1162526	1163284	0	0	0	0
Transketolase	*tkt*	2776604	2778439	0	0	68624	0
Transketolase	*tkt*	1159837	1161669	0	0	0	0
Transketolase	*tkt*	1423835	1425841	231015000	0	0	0
Ribose-5-P isomerase A	*rpiA*	2330966	2331664	0	0	0	0
Ribose-5-P isomerase B	*rpiB*	872056	872577	0	0	0	0
Ribulose-5-P epimerase	*rpe*	760456	761112	24094000	0	0	0
Transaldolase	*tal*	2557126	2557785	0	0	0	0
Transaldolase	*tal*	1161666	1162529	0	0	0	0

Table C2 Main physiologic features of different species affiliated to the phylum *Nitrospirae*.

Species	e⁻ acceptors	e⁻ donors	Fermentation	T (°C)	pH	Morphology	G+C	*dsr*	*nap*	*nrf*	*nor*	Reference
Thermodesulfovibrio aggregans	SO_4^{2-}, thiosulfate, Fe(III)	Lactate, acetate, formate, , H_2, pyruvate	Pyruvate	45-70	6.0-8.5	Curved rod, vibrio	35.2	y	n	y	n	38
T. hydrogeniphilus	SO_4^{2-}, SO_3^{2-}, thiosulfate, As(V)	Formate, acetate, pyruvate	Pyruvate	50-70	7.1	Curved, vibrio	36.1	n	n	n	n	15
T. islandicus	SO_4^{2-}, thiosulfate, NO_3^-	H_2, frmate, lactate, pyruvate	Pyruvate	45-70	7.0	Vibrio shaped rod	38.0	y	n	y	n	39
T. thiophilus	SO_4^{2-}, SO_3^{2-},thiosulfate, Fe(III)	Lactate, acetate, formate, pyruvate	Pyruvate	45-60	6.0-8.5	Curved rod, vibrio	34.0	y	n	y	n	38
T. yellowstonii	SO_4^{2-}, SO_3^{2-},thiosulfate, S0, fumarate, NO_3^-	H_2, acetate, formate, lactate, pyruvate, propionate, butyrate, ethanol, malate	Pyruvate, lactate	65	6.8-7.0	Vibrio	29.5	y	n	y	n	2, 17
Leptospirillum ferriphilum	O_2	Fe(II), pyrite	-	30-45	1.4-1.8	Rod, spirillum	56.5	n	n	n	n	5
L. ferrodiazotrophum	O_2	Fe(II)	-	37	1.2	Spirillum	-	n	n	n	n	42
L. ferroxidans	O_2	Fe(II)	-	20-45	1.5-4.0	Rod, vibrio	51.7	n	n	n	n	18, 35, 37
Nitrospira calida	O_2	NO_2^-	-	37-58	7.0-8.8	Spirillum, rod	-	n	n	n	n	25
N. japonica	O_2	NO_2^-, formate	-	10-46	7.5-7.8	Rod	-	n	n	n	n	43
N. moscoviensis	O_2, NO_3^-	NO_2^-, H_2	-	33-40	7.6-8.0	Rod	56.9	n	y	n	n	9
C. Magnetobacterium casensis	NO_3^-, NO_2^-, NO, N_2O	S^-, S_0, SO_3^{2-}	-	-	-	Giant rod	48.9	y	y	n	n	29
C. M. bavaricum	NO_3^-, NO_2^-	S^-, S_0	-	-	-	Giant rod	47.4	y	y	n	n	23, 41, 44
C. Magnetoovum chiemensis	NO_3^-, NO_2^-	S^-, SO_3^{2-}	-	20-32	-	Ovoid	40.4	y	n	n	n	23, 28
C. Nitrospira bockiana	O_2	NO_2^-	-	28-48	7.16	Curved, vibrio	-	n	n	n	n	24
C. N. defluvii	O_2	NO_2^-	-	28-32	-	Rod	59.0	n	n	n	y	31, 40
C. N. inopinata	O_2	NH_4, NO_2^-	-	46-56	7.5-7.8	Spirillum	-	n	y	y	y	6
C. N. nitrificans	O_2	NH_4, NO_2^-	-	23	7.0	-	56.6	n	n	n	n	45
C. N. nitrosa	O_2	NH_4, NO_2^-	-	23	7.0	-	54.8	n	n	y	n	45
C. Thermomagnetovibrio paiutensis	-	-	-	25-63	-	Vibrioid	-	n	n	n	n	27

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Products

Publications

Sarah Zecchin, Anna Corsini, Maria Martin, Marco Romani, Gian Maria Beone, Raffaella Zanchi, Elena Zanzo, Daniele Tenni, Maria Chiara Fontanella, Lucia Cavalca (2017). Rhizospheric iron and arsenic bacteria affected by water regime: Implications for metalloid uptake by rice. *Soil Biology and Biochemistry* **106**: 129-137. DOI: 10.1016/j.soilbio.2016.12.021.

Susanne Wörner, Sarah Zecchin, Jianguo Dan, Nadezhda Hristova Todorova, Alexander Loy, Ralph Conrad, Michael Pester (2016). "Gypsum amendment to rice paddy soil stimulated bacteria involved in sulfur cycling but largely preserved the phylogenetic composition of the total bacterial community". *Environmental Microbiology Reports* **8**(3). DOI: 10.1111/1758-2229.12413

Oral presentations

"Assessing the contribution of the rhizosohere microbiome in arsenic biogeochemical cycle in rice fields". 21th Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology, Portici (Italy), 14-16th September 2016.

"Nella rizosfera del riso la sommersione continua seleziona popolazioni batteriche coinvolte nel ciclo dell'arsenico". Arsenico nelle catene alimentari, Roma (Italy), 4-5th June 2015.

"Microbial arsenic cycle in rice rhizosphere in relation to water management". Cortona Procarioti, Cortona (Italy), 15-17th May 2014.

Poster presentations

"Rhizospheric iron and arsenic bacteria influence metalloid uptake by plant in rice paddies". 3rd Thünen Symposium on Soil Metagenomics, Braunschweig (Germany), 14-16th December 2016.

"Bacterial resources for assisted phytostabilization of acid mine drainage- affected mountain stream bank", Rhizosphere 4, Maastricht (The Netherlands), 21-25th June 2015.

"Iron-reducing bacteria in rice rhizosphere contribute to arsenic mobilization under flooded conditions". Rhizosphere 4, Maastricht (The Netherlands), 21-25th June 2015.

"Continuous flooding selects for bacterial populations involved in arsenic cycle in rice rhizosphere". Bacterial Genetics and Ecology (BAGECO) 13, Milano (Italy), 14-18th June 2015.