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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. 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 $\mu g\ kg^{-1}$, 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.

2. Material and methods

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 in 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 (28th June), flowering (20th August), and senescence (30th 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.

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).

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.

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. (2011). 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.

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 S1. 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^{TM} 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^{TM} 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 S1) 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.

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 S2). 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 S2). 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 S2, 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 S2 and S3).

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 S3 the clones selected for real time qPCR analysis are listed.

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.

2.9 Accession numbers

Sequences obtained in this study have been deposited to Genbank with accession numbers from KX592445 to KX592464 (Table S3).

3. Results

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).

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.

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 S5). 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 S5).

Shewanellaceae were more abundant in rhizosphere soil than in rhizoplane, from flowering to senescence regardless of the water regimes (Fig. 4b and Table S5). According to FISH data, *Shewanellaceae* (probe Shew227) were more active in CF and 2IED rather than in AR (Fig. 3 and Table S4).

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}.

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 S6). 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 S6).

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 S6).

ArsC gene copies ranged from 1×10^5 to 1×10^6 gdw^{-1} (Table S6). 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.

4. Discussion

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).

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).

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 S6). These proportions reflected the values measured in the unplanted soil (Table S6). 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.

4.4 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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Figure captions:

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, $p \leq 0.05$).

Tables

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

10

Figure
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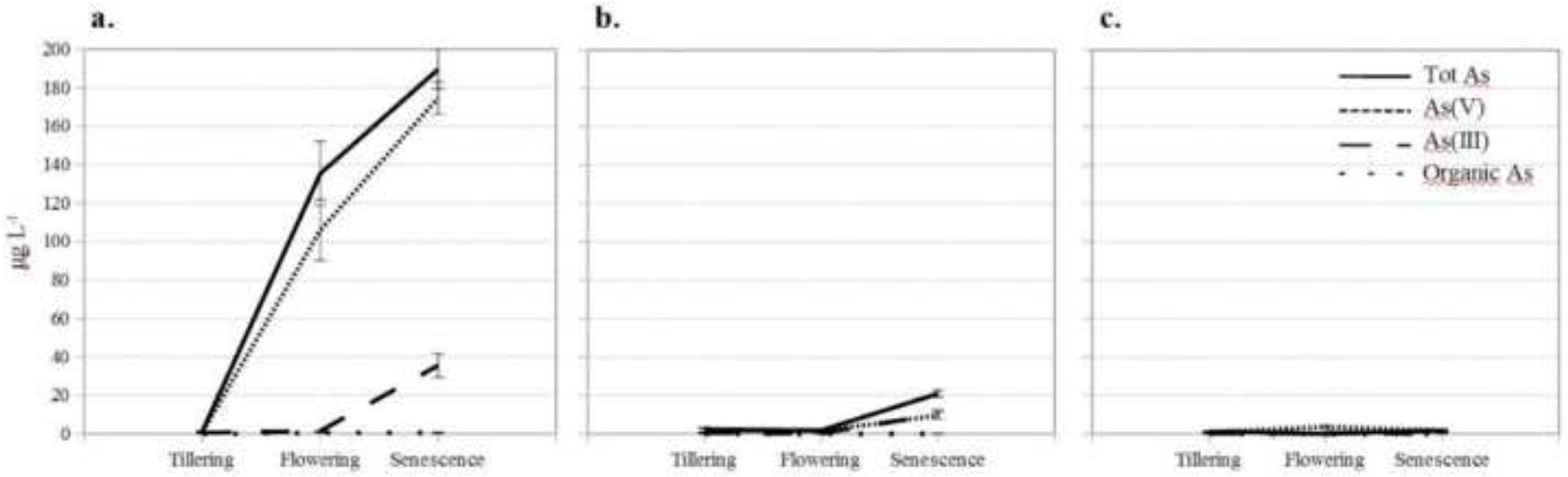

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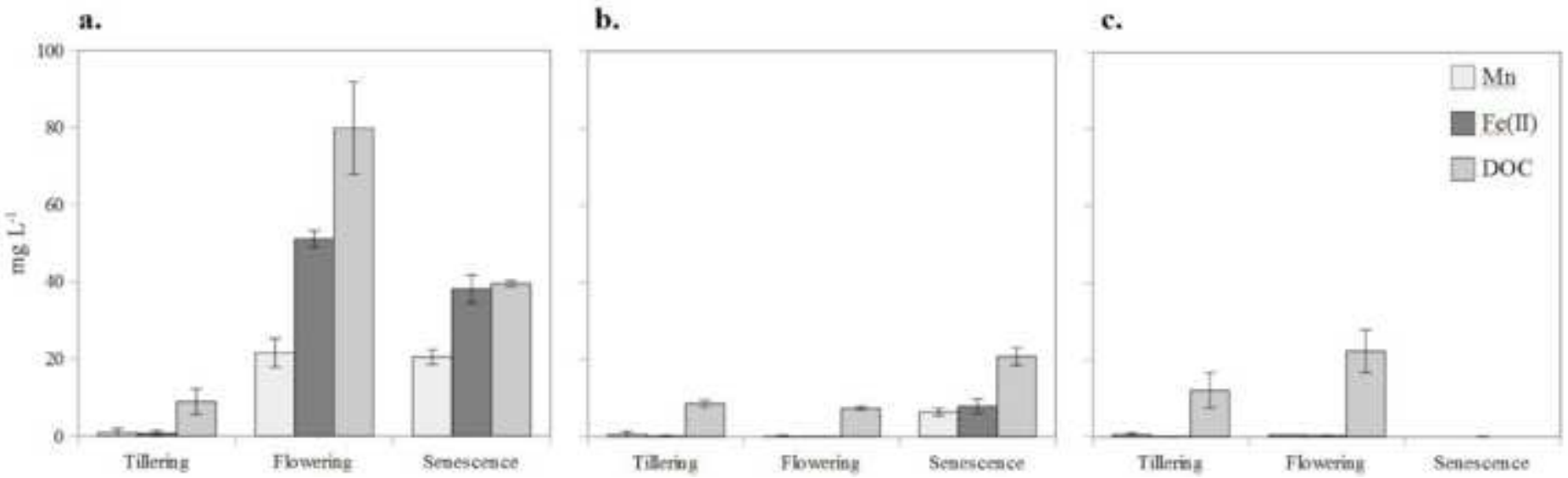

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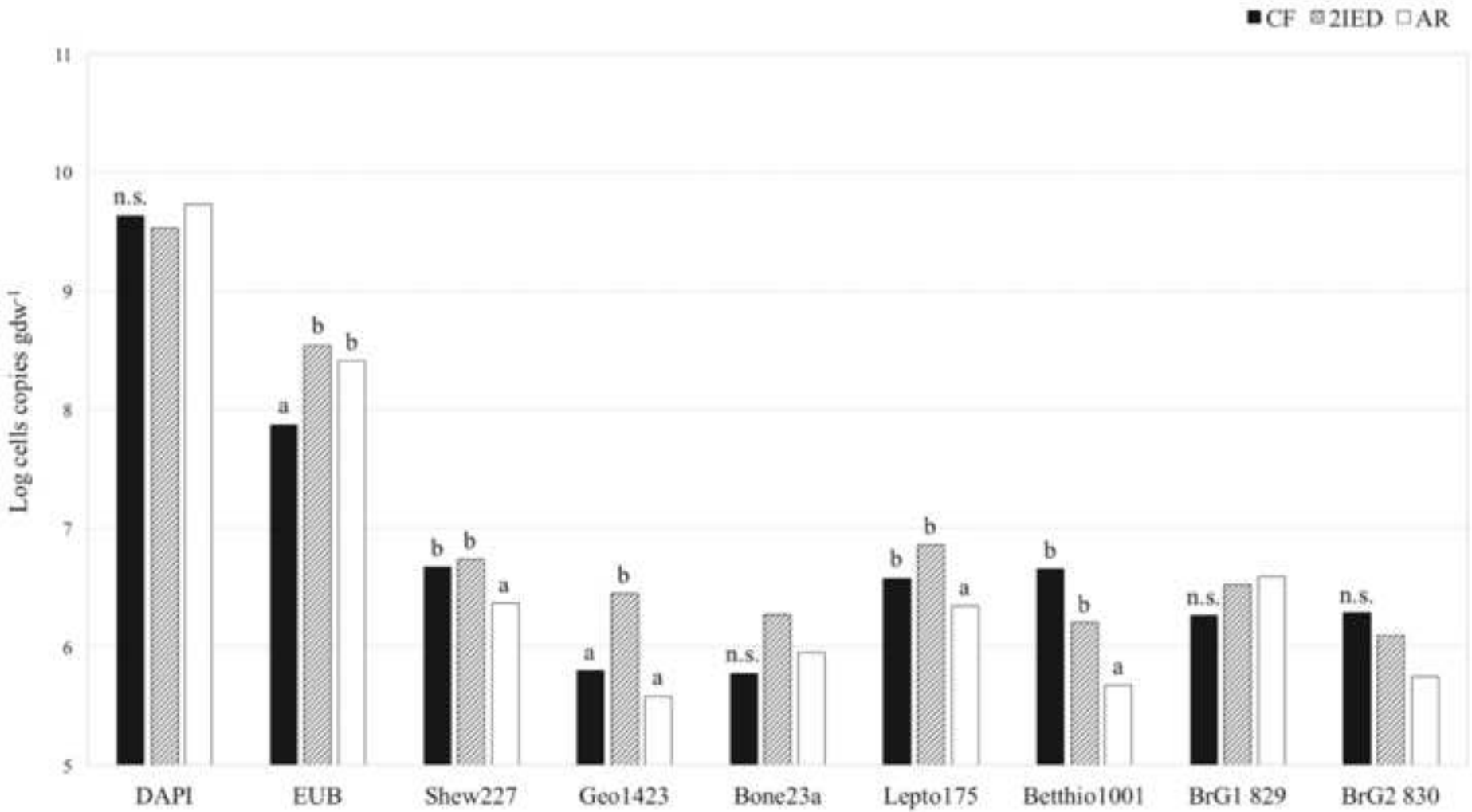

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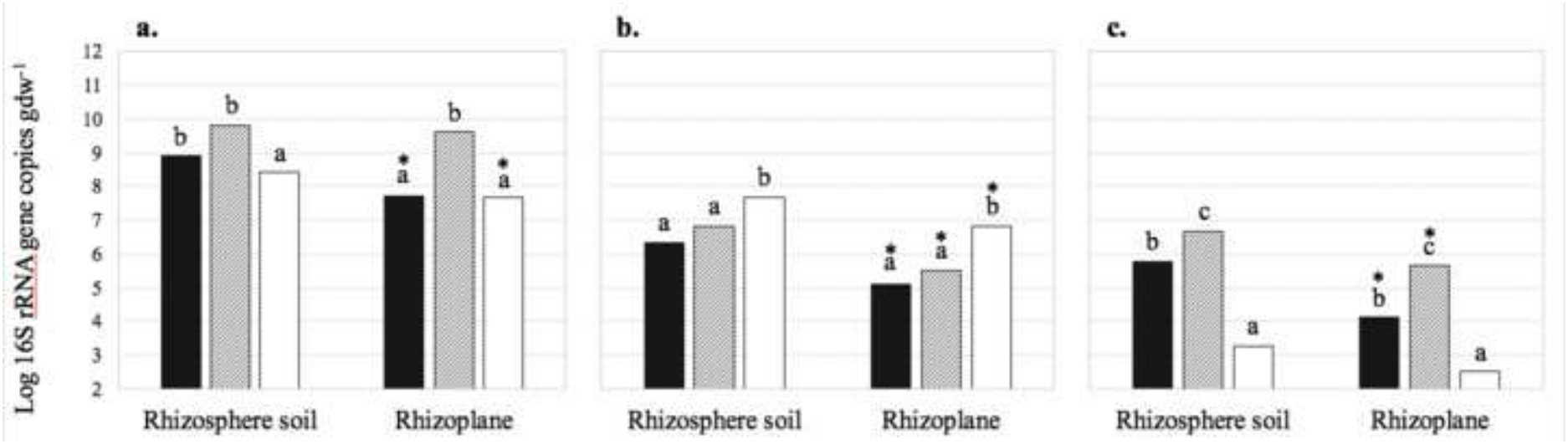

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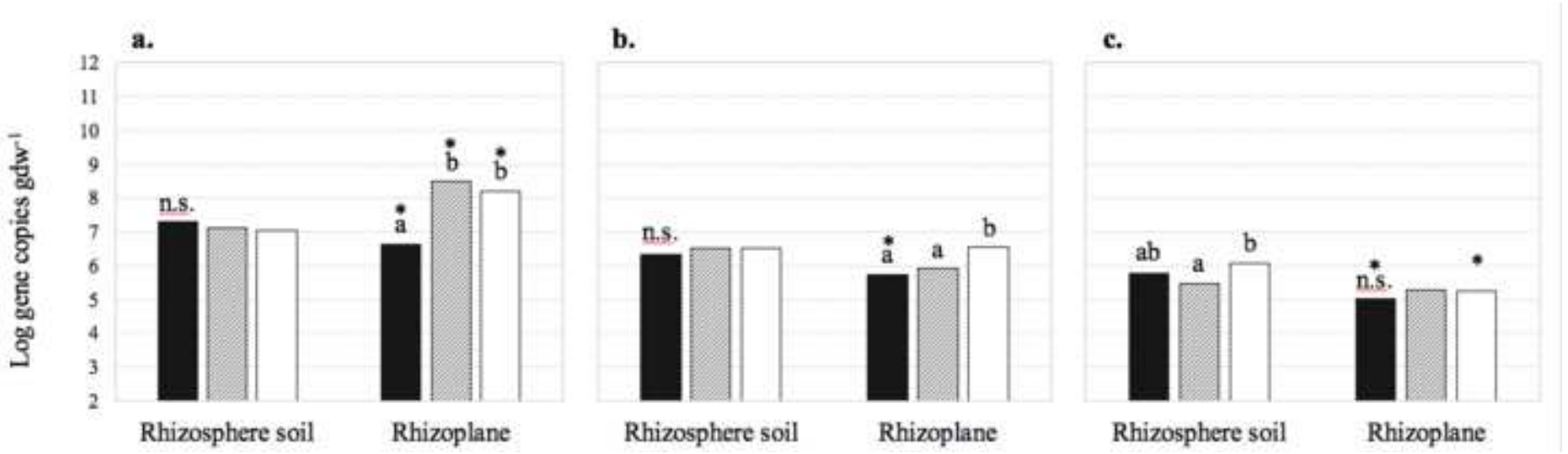

Rhizospheric iron and arsenic bacteria affected by water regime: implications for metalloid uptake by rice

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Soil Biology and Biochemistry

Supplementary Material

Table S1 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 S2 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.	ArsC52F	AGC CAA ATG GCA GAA GC	0.4	55	275	Clone ArsC14	Bachate et al.

(Arsenate reductase)	ArsC323R	GCT GGR TCR TCA AAT CCC CA					2009
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 S3 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 S4 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 S5 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 †
Shew	-	3.26E+05	-	-	-	-	-	-
	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**
Gall	-	3.37E+05	-	-	-	-	-	-
	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 S6 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
aioA	-	2.14E+07	-	-	-	-	-	-
	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, *, †**
arsC	-	4.76E+05	-	-	-	-	-	-
	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, *, †**

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