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Corresponding Author: Dr. Simona Rossetti,

Corresponding Author's Institution: Water Research Institute IRSA-CNR

First Author: Simona Crognale

Order of Authors: Simona Crognale; Barbara Casentini; Stefano Amalfitano; Stefano Fazi; Maurizio Petruccioli; Simona Rossetti

Abstract: Arsenic (As) contamination in drinking water represents a worldwide threat to human health. During last decades, the exploitation of microbial As-transformations has been proposed for bioremediation applications. Among biological methods for As-contaminated water treatment, microbial As(III)-oxidation is one of the most promising approaches since it can be coupled to commonly used adsorption removal technologies, without requiring the addition of chemicals and producing toxic by-products. Despite the As(III) oxidation capability has been described in several bacterial pure or enrichment cultures, very little is known about the real potentialities of this process when mixed microbial communities, naturally occurring in As contaminated waters, are used. This study highlighted the contribution of native groundwater bacteria to As(III)-oxidation in biofilters, under conditions suitable for a household-scale treatment system. This work elucidated the influence of a variety of experimental conditions (i.e., various filling materials, flow rates, As(III) inflow concentration, As(III):As(V) ratio, filter volumes) on the microbially-mediated As(III)-oxidation process in terms of oxidation efficiency and rate. The highest oxidation efficiencies (up to 90% in three hours) were found on coarse sand biofilters treating total initial As concentration of 100 ug L-1. The detailed microbial characterization of the As(III) oxidizing biofilms revealed the occurrence of several OTUs affiliated with families known to oxidize As(III) (e.g., Burkholderiaceae, Comamonadaceae, Rhodobacteraceae, Xanthomonadaceae). Furthermore, As-related functional genes increased in biofilter systems in line with the observed oxidative performances.

Suggested Reviewers: Chris Parsons
University of Waterloo
chris.parsons@uwaterloo.ca

Franco Baldi
Cà Foscari University
baldi@unive.it

Helena Guasch
University of Girona
helena.guasch@udg.edu

Ana Isabel Pelaez
Universidad de Oviedo Mieres
pelaezana@uniovi.es

Marta Vignola
University of Glasgow
marta.vignola@glasgow.ac.uk

Opposed Reviewers:

Cover Letter

ISTITUTO DI RICERCA SULLE ACQUE

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Dear Editor,

I have the pleasure to submit to your attention the manuscript titled ”Biological As(III) oxidation in biofilters by using native groundwater bacteria” by Simona Crognale, Barbara Casentini, Stefano Amalfitano, Stefano Fazi, Maurizio Petruccioli and Simona Rossetti from Water Research Institute, IRSA-CNR, and University of Tuscia for possible publication on "Science of The Total Environment”.

I state that the content and authorship of the submitted manuscript have been approved by all authors.

Thanking you in advance for your kind cooperation, I am looking forward to hearing from you.

Your sincerely,

Simona Rossetti

Dr. Simona Rossetti
Water Research Institute, CNR,
Via Salaria km 29,300
00015 Monterotondo (RM)
Tel.: International + 39 06 90672697
Fax: International +39 06 90672787
e-mail : rossetti@irsa.cnr.it

*Title Page

Biological As(III) oxidation in biofilters by using native groundwater bacteria

Simona Crognale [a], Barbara Casentini [a], Stefano Amalfitano [a], Stefano Fazi [a], Maurizio Petruccioli [b], Simona Rossetti [a*]

[a] Water Research Institute, National Research Council of Italy (IRSA - CNR), Via Salaria, km 29.300, Monterotondo, Rome, 00015, Italy

[b] Department for Innovation in Agroforestry and Biological systems (DIBAF), University of Tuscia, Viterbo, Italy

*Corresponding author

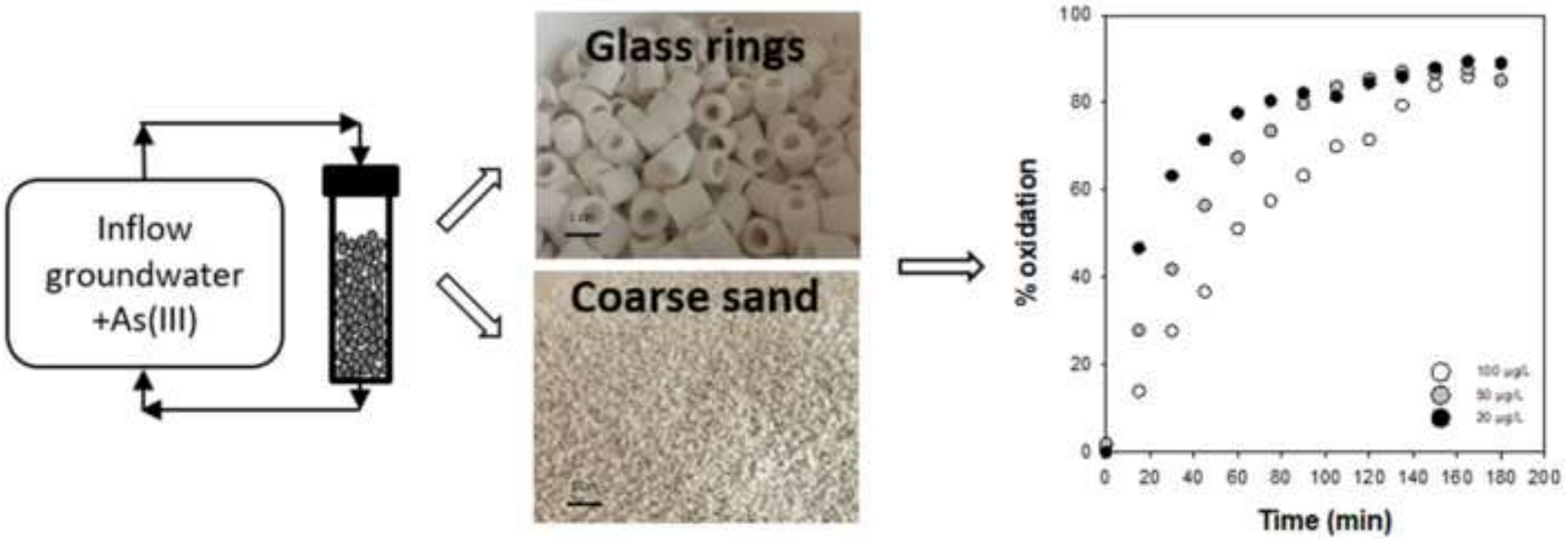
Dr. Simona Rossetti

Water Research Institute (IRSA) - National Research Council of Italy (CNR)

Via Salaria km 29.300, Monterotondo, Rome, 00015, Italy

rossetti@irsa.cnr.it

*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

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- Evaluation of the process efficiency in biofilters under varying operation conditions
- Development of highly performing As(III) oxidizing biofilms in biofilter reactors
- Enrichment of *aioA* genes in biofilters treating contaminated groundwater

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[a] Water Research Institute, National Research Council of Italy (IRSA - CNR), Via Salaria, km 29.300, Monterotondo, Rome, 00015, Italy

[b] Department for Innovation in Agroforestry and Biological systems (DIBAF), University of Tuscia, Viterbo, Italy

*Corresponding author

Dr. Simona Rossetti

Water Research Institute (IRSA) - National Research Council of Italy (CNR)

Via Salaria km 29.300, Monterotondo, Rome, 00015, Italy

rossetti@irsa.cnr.it

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Abstract

Arsenic (As) contamination in drinking water represents a worldwide threat to human health. During last decades, the exploitation of microbial As-transformations has been proposed for bioremediation applications. Among biological methods for As-contaminated water treatment, microbial As(III)-oxidation is one of the most promising approaches since it can be coupled to commonly used adsorption removal technologies, without requiring the addition of chemicals and producing toxic by-products. Despite the As(III) oxidation capability has been described in several bacterial pure or enrichment cultures, very little is known about the real potentialities of this process when mixed microbial communities, naturally occurring in As contaminated waters, are used. This study highlighted the contribution of native groundwater bacteria to As(III)-oxidation in biofilters, under conditions suitable for a household-scale treatment system. This work elucidated the influence of a variety of experimental conditions (i.e., various filling materials, flow rates, As(III) inflow concentration, As(III):As(V) ratio, filter volumes) on the microbially-mediated As(III)-oxidation process in terms of oxidation efficiency and rate. The highest oxidation efficiencies (up to 90% in three hours) were found on coarse sand biofilters treating total initial As concentration of 100 $\mu g\ L^{-1}$. The detailed microbial characterization of the As(III) oxidizing biofilms revealed the occurrence of several OTUs affiliated with families known to oxidize As(III) (e.g., *Burkholderiaceae*, *Comamonadaceae*, *Rhodobacteraceae*, *Xanthomonadaceae*). Furthermore, As-related functional genes increased in biofilter systems in line with the observed oxidative performances.

1. Introduction

Arsenic (As) is a well-known carcinogenic element widely distributed in natural aquatic environments representing a serious threat to human health worldwide (Nordstrom, 2002). Several physical-chemical methods are used for arsenic removal, such as coagulation/filtration, ion exchange, enhanced lime softening, adsorption and reverse osmosis (Ng et al., 2004; Nicomel et al., 2016).

Nevertheless, in recent years the research interest is moving towards the adoption of biotechnological solutions to be used in combination with traditional chemical As treatment processes to enhance the sustainability and cost-effectiveness of the process (Plewniak et al., 2018). Indeed, despite the high toxicity, some microorganisms are able to withstand high As levels and use it for energetic metabolism (Huang, 2014). Among the possible microbial As-transformations, the redox reactions involving As(III) oxidation and As(V) reduction are the most investigated for bioremediation purposes (Kumari and Jagadevan, 2016). In particular, microbiological As(III)-oxidation is one of the most promising application as a precursor step in As removal from contaminated groundwater, since conventional iron-based treatments are more effective in removing As(V) rather than As(III) (Fazi et al., 2016a). Pre-oxidation process is commonly performed trough the addition of chemical reagents such as potassium permanganate, chlorine, ozone, hydrogen peroxide or manganese oxide that can cause secondary problems arisen by the presence of residuals or from by-products formation, inducing a significant increase in operational costs (Driehaus et al., 1995; Katsoyiannis and Zouboulis, 2004; Kim and Nriagu, 2000).

Microorganisms involved in As(III)-oxidation were retrieved in a variety of As-rich environments including mine, arsenical pesticides or smelter-impacted sites, geothermal sites, geyser, soil and sediments (Engel et al., 2013; Heinrich-Salmeron et al., 2011; Lami et al., 2013; Quéméneur et al., 2010, 2008; Sultana et al., 2012). Microbial As(III) oxidation represents a detoxification process in heterotrophic microorganisms as *Herminiimonas arsenicoxydans*, *Hydrogenophaga* NT-14, *Stenotrophomonas* sp. MM-7 (Bahar et al., 2012; Muller et al., 2003; Vanden Hoven and Santini,

2004), or an energetic metabolism in chemolithoauthotrophic microbes, such as *Rhizobium* NT-26 and *Thiomonas arsenivorans* (Battaglia-Brunet et al., 2006; Garcia-Dominguez et al., 2008; Hoeft et al., 2007; Santini et al., 2000). Both oxidation mechanisms are carried out by the enzyme arsenite oxidase, firstly purified in *Alcaligenes faecalis* (Anderson et al., 1992). This enzyme is composed of a small subunit containing a Rieske [2Fe-2S] cluster and a large subunit harboring molybdopterin guanosine dinucleotide at the active site and a [3Fe-4S] cluster (Ellis et al., 2001). The two genes encoding for large and small subunits of the arsenite oxidase were named as *aioA* and *aioB*, respectively (Lett et al., 2012). Sometimes, the combination of As(III) oxidation with nitrate or chlorate reduction has been observed in microorganisms such as *Acidovorax* NO1 and *Azoarcus* DAO1 (Huang et al., 2012; Zargar et al., 2012).

During last decades, the potentialities of microbial As(III) oxidation were investigated in lab-scale experiments by using planktonic cells (Battaglia-Brunet et al., 2002), biofilms (Michel et al., 2007) and immobilized bacteria (Dastidar and Wang, 2012; Ito et al., 2012; Michon et al., 2010). Battaglia-Brunet et al. (2002) reported an oxidation rate of 166 mg $L^{-1}h^{-1}$ in a fixed bed column inoculated with a selected autotrophic As(III)-oxidizing population from an As-rich mine site. Furthermore, As(III)-oxidizing microorganisms, such as *T. arsenicoxydans,* were also used to operate biofilters coupled with arsenic removal treatment based on activated alumina and metallic Fe adsorbents (Ike et al., 2008; Wan et al., 2010). Other studies proposed the application of biotic As(III) and Fe(II)/Mn(II) oxidation in a fixed-bed upflow filtration unit for the oxidation and simultaneous removal of arsenic and dissolved Fe and Mn (Hassan et al., 2009; Katsoyiannis and Zouboulis, 2004; Katsoyiannis et al., 2004; Tani et al., 2004). Recently, the potentialities of biological As(III) oxidation were investigated by using mixed microbial communities in bioreactors filled with sand or perlites (Gude et al., 2018; Li et al., 2016). The laboratory-scale biofilter, inoculated with an enriched population of As(III)-oxidizing microorganisms from realgar mine sediments, showed the capability to oxidize 1100 μg As(III) L^{-1} within 10 min (Li et al., 2016). This process was also evaluated in sand filters by using a mixed microbial community from raw

groundwater. About 98% of As(III) at the initial concentration of 116 μg $L^{-1}$was oxidized in 38 days without acclimation to As(III) contaminated water and within three weeks when the biofilter was previously exposed to As-rich groundwater (Gude et al., 2018). Other investigations showed the ability of microorganisms grown on quartz sand to simultaneously remove arsenic (100-150 mg L^{-1}), iron (0.8-1.5 mg L^{-1}) and manganese (1-1.2 mg L^{-1}) from groundwater, with As removal up to 98.2% within 180 days (Yang et al., 2014). Although the high potentialities of the microbially-mediated As(III) oxidation, this process has received only scant attention (Crognale et al., 2017a). The majority of studies were performed by using As(III)-oxidizing microorganisms isolated from extreme environments (such as for example acid mine drainage, mine sediments and geothermal environments) and very little information is available on the process performances for the treatment of contaminated groundwater. The frequently reported long oxidation times (days or weeks) are not satisfactory to practically and efficiently couple this preliminary biological treatment to the fast As removal by adsorption. Overall, specific information about As-related functional genes and microorganisms involved in biological As(III)-oxidation was largely disregarded and only few studies reported the employment of mixed microbial communities (Crognale et al., 2017a).

This study aimed to evaluate the potentialities of biological As(III)-oxidation in laboratory scale biofilters treating As-contaminated groundwater through the selection and the establishment of biofilms composed by native water microbial communities. The oxidative performance of the biofilters was evaluated by using a variety of experimental conditions (e.g., various filling materials, flow rates, As(III) inflow concentration, As(III):As(V) ratio, filter volumes) in order to elucidate the best conditions to efficiently couple the proposed biological pre-oxidation to household-scale treatment units. The As(III)-oxidizing biofilms in the bioreactors were explored by applying an advanced microbial community characterization approach through flow cytometry, qPCR and high-throughput 16S rRNA gene sequencing.

2. Materials and methods

2.1 Biofilter set-up

Four polycarbonate columns (Ø 30 mm, height 135 mm) were used for the construction of biofilters with a bed volume (BV) of 0.1 L. Two biofilters with BV of 0.7 L (Ø 65 mm, height 200 mm) were also used in order to test As(III)-oxidation performance in systems with larger volumes (see Figure S1). Sintered glass rings (porosity 56.7%) and coarse sand (porosity 26.4%), chosen based on their easy availability and low cost, were separately used as filling materials for the construction of biofilters (herein named "glass" and "sand" respectively). Groundwater with As concentration ranging from 2.5 to 4.5 μg L^{-1} was used for biofilm growth. The water was let to circulate for 20 days throughout the biofilters (Fig. S1), afterwards inflowing water was continuously spiked with 100 μg As(III) L^{-1} and the water circulated in a closed system throughout the columns for a different amount of time. The biofilters exposed to short-term As(III) acclimation period (~ 15 days) were hereinafter named "STA biofilters". The biofilters operated with a long-term acclimation period (around 40 days) were named "LTA biofilters". Oxidation efficiency was periodically checked until the biofilm was able to oxidize > 60% of As(III) in 2 hours under the selected conditions.

Once this performance was achieved, biofilters with 0.1 L and 0.7 L BV were used in kinetic experiments, respectively, under different operation conditions (see Section 2.2). Water tanks and biofilters were kept in the dark at 25°C temperature for the entire duration of the experiments to prevent As(III) photo-oxidation. Possible As(III) oxidation within the tanks was absent within a period of 6 hours. Kinetic experiments were carried out by recirculating the same water from the inflow tank into biofilter a variable number of times (*number of recirculations*). This "recirculation" setup was chosen since preliminary tests demonstrated that As(III) was oxidized maximum up to 20% in both glass and sand biofilters by passing water only once through the biofilter. During kinetic tests, water samples were collected from the tanks every 15 minutes for three hours and every 10 minutes for 1 hour for the bioreactors with BV 0.1 L and 0.7 L, respectively. The total

operating period of biofilters with 0.1 L BV and 0.7 L BV was around 88 and 141 days, respectively.

Arsenic speciation during kinetic tests was assessed by hydride generation-absorption spectrometry (HG-AAS, Perkin Elmer AAnalyst 800). Arsenite determination was carried out using HCl 2% as carrier and reduction to arsine gas was performed with $NaBH_4$ 0.4%. Total As concentration (As_{tot}) was analyzed by HG-AAS following 30 min reduction to As(III) by 5% KI/Acid Ascorbic solution. As(V) concentration was obtained by difference.

Oxidation efficiency was calculated according to the equation:

$$oxidation\ efficiency\ (\%) = (1 - \frac{[\mathrm{As(III)}]}{[\mathrm{Astot}]}) * 100$$

The As(III)-oxidation rate (μg oxidized As(III) L^{-1} h^{-1}) was expressed as the maximum slope value of the kinetic curve obtained by plotting As(III) values measured at each sampling points during kinetic tests (by considering $R^2 > 0.9$).

2.2 The biofilters operating conditions adopted to evaluate the biological As(III) oxidation

A variety of kinetic experiments was carried out to highlight the influence of different chemical and hydraulic parameters on biological oxidation efficiency by biofilms established in the biofilters. The oxidative performance of glass and sand biofilters was evaluated to treat a volume of 1.6 L (BV 0.1 L) by testing the influence of:

i. flow rates (70-140-250 mL min^{-1}) at As(III) concentration of 100 μg L^{-1};

ii. initial As(III) concentration (20, 50, 100 μg L^{-1});

iii. As(III):As(V) ratio (100:0; 60:40, 40:60) at a total As concentration of 100 μg L^{-1}.

Furthermore, the scalability of the process at two different initial As(III) concentrations (50 and 100 μg L^{-1}) was tested by using glass and sand biofilters with larger bed volumes (0.7 L) to treat a volume of 11.1 L by keeping the linear velocity of 11.9 m h^{-1} (flow rate: 660 mL min^{-1})

corresponding to the value previously used in a household pilot unit for drinking water treatment of a 400 L day^{-1} (as described in Casentini et al., 2016).

2.3 Treatment of biofilm samples and DNA extraction

Filling materials were sampled at the end of the all experiments for microbiological analysis. An aliquot of sample (~ 1 g) was diluted (1:10 w/v) with sterilized buffer solution containing formaldehyde (2 % final concentration) in 15 mL Falcon tubes and further processed using Nycodenz density gradient centrifugation according to the protocol for cell purification of Amalfitano and Fazi (2008). Then the obtained aqueous solutions were used for cytometric analysis. Another aliquot of sample was immediately stored at -20°C until further processing. Approximately 1 g of filling material was used for DNA extraction by PowerSoil® DNA Isolation Kit (MoBio - Carlsbad, CA) by following the manufacturer's instructions. The quality of extracted DNA (1.6 < A260/280 < 1.8 and A260/230 > 2) was analyzed with a Nanodrop 3300 (Thermo Scientific, Italy). DNA was stored at − 20 °C in small aliquots.

2.4 Biofilm structure overview and prokaryotic abundance

The tridimensional structure and microbial colonization of filling materials were assessed using CARD-FISH technique in combination with Confocal Laser Scanning Microscopy, using specific rRNA-target HRP-labelled probes for Bacteria (EUB I-III) (Greuter et al., 2016), according to the protocol of Lupini et al. (2011). Image elaborations were performed using Imaris 6.2 software (Bitplane AG, Zurich, Switzerland).

Fixed samples were used to characterize microbial communities by using the Flow Cytometer A50-micro (Apogee Flow System, Hertfordshire, England) equipped with a solid state laser set at 20 mV and tuned to an excitation wave length of 488 nm. The volumetric absolute cell counting was carried out on samples stained with SYBR Green I (1:10,000 dilution; Molecular Probes, Invitrogen). Apogee Histogram Software (v89.0) was used to plot and analyze data; the light

scattering signals (forward and side scatters) and the green fluorescence (530/30 nm) were considered for the single cell characterization. Thresholding was set on the green channel and voltages were adjusted to place the background and instrumental noise below the first decade of green fluorescence. Samples were run at low flow rates to keep the number of events below 1000 events s^{-1}. The intensity of green fluorescence emitted by SYBR-positive cells allowed for the discrimination among cell groups exhibiting two different nucleic acid content (cells with Low Nucleic Acid content - LNA; cells with High Nucleic Acid content - HNA) (Amalfitano et al., 2014).

2.5 Real-time quantification of arsenic-related functional genes

The quantification of functional genes involved in arsenic transformations in biofilm and groundwater samples was performed by qPCR using Sso Advanced Universal SYBR Green Supermix (BIO-RAD, United States) according to the manufacturer's instructions on a CFX96 Touch Real-time PCR detection system. Different primer pairs were used according to the protocols reported in literature. In detail, arsenite oxidase gene (*aioA*) was targeted using aroA#2F - aroA#2R primer pair according to Inskeep et al. (2007); arrAf – arrAr primer set (Malasarn et al., 2004) was used for the amplification of respiratory reductase gene (*arrA*). Arsenate cytoplasmic reductase (*arsC*) was amplified using amlt-42-F/amlt-376-R primers according to Sun et al. (2004). Quantification of arsenite transporter (*arsB*) was carried out using arsB#1F - arsB#1R primers according to Achour et al. (2007). Melting curves were performed for each reaction to confirm the purity of amplified products.

2.6 High-throughput 16S rRNA gene sequencing and bioinformatics

Extracted DNA was amplified in a first PCR with the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3') targeting the region V1-V3 of bacterial 16S rRNA gene. Reactions were set up in 25 μL volumes containing 15

ng of DNA, 0.5 μM primers and 1X Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA). PCR settings: initial denaturation at 98°C for 10 s, 30 cycles of 98°C for 1 s, 60°C for 5 s, 72°C for 15 s and final elongation at 72°C for 1 min. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (50 μL) contained Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA), Nextera XT Index Primers and 5 μL of amplicon library template. PCR settings: initial denaturation at 98°C for 10 s, 8 cycles of 98°C for 1 s, 55°C for 5 s, 72°C for 15 s and final elongation at 72°C for 1 min. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). Library concentration was measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA). The purified libraries were pooled in equimolar concentrations and diluted to 4 nM. The samples were paired end sequenced (2x301bp) on a MiSeq platform (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples. 10% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

After checking read quality with fastqc, the sequences were processed and analyzed using QIIME2 software tools (2017.12 release). The reads were demultiplexed using demux plugin (https://github.com/qiime2/q2-demux), denoised, dereplicated and chimera-filtered using DADA2 algorithm (Callahan et al., 2016) and gathered in a feature table. The taxonomic analysis was based on a naïve-bayes classifier trained on 16S rRNA gene OTUs clustered at 99% similarities within the Silva128 database release (Quast et al., 2013).

2.7 Statistical analysis

The Principal Component Analysis (PCA), based on the correlation matrix, was run by comprising separately the quantification data of As-related functional genes, prokaryotic abundance and relative abundance of HNA and LNA cells. Additionally, PCA was performed separately by including the

As-related microbial composition as revealed by NGS. Only families (> 1% of total OTUs) generally known for their involvement in As-transformation processes were considered. All values were normalized by log(X+1). Shannon and Simpson indices for each sample were also generated using the software PAST v3.20 (Hammer et al., 2001).

3. Results

3.1 Microbial As(III)-oxidation in lab-scale biofilters

The oxidative performance of As(III)-oxidizing biofilm grown in biofilters (BV = 0.1 L) was evaluated using two different filling materials (coarse sand and sintered glass rings) to treat groundwater with initial [As(III)] of 100 μg L^{-1}. After about 2 weeks of acclimation period both biofilters were able to oxidize only 30% of As(III) within two hours (Fig. S2). The oxidation efficiency increased up to 60% and 80% in glass and sand, respectively, when the biofilms were acclimated for a period of 40 days (Fig. S2). Biofilters with biofilms at this maturation stage were selected for this study.

3.2 Influence of flow rate

The influence of flow rate on both materials was tested at 70, 140 and 250 mL min^{-1}, corresponding to a daily production of treated water of around 100, 200 and 360 L, with contact times of 1.4, 0.7 and 0.4 min, respectively. In a period of three hours, columns recirculated different volumes, corresponding to 8.4, 15.6 and 27.6 number of recirculations at flow rates of 70, 140 and 250 mL min^{-1} respectively. By increasing the flow rate, columns also treated a different number of Pore Volumes (PVs = BVs/porosity), corresponding to 222, 444, 794 in glass and 477, 955, 1705 in sand biofilters. As(III) oxidation efficiencies were always higher for sand (67.9-89.3%) compared to glass biofilters (≤ 69.6%). The oxidation increased proportionally with flow rate for both, with the exception of glass biofilter operating at 250 mL min^{-1} (Fig. 1 and Table S1). The overall oxidation was similar when the sand biofilter was operated at 140 and 250 mL min^{-1} (about 89%) while only

67.9% of As(III) was oxidized at 70 mL min^{-1} (Fig. 1a). After the same number of recirculations, the performances of the columns operating at 70 and 140 mL min^{-1} were identical, with an oxidation efficiency of about 40% and 65% in glass and sand, respectively (Fig. 1b). Remarkably lower efficiencies were measured at a flow rate of 250 mL min^{-1} for both materials. As shown in Figure 1c, the arsenite oxidation in biofilters was very similar in all columns when plotted versus treated pore volumes, with a value in the range 35-45% at 200-250 PVs for the columns operating at 70 and 140 mL min^{-1}. At 500 PVs, almost 70% of As(III) was oxidized in both biofilters operating at a flow rate of 140 mL min^{-1}. Also considering the PVs, the lowest performances were observed in the columns operating at highest flow rate. Since the highest oxidation efficiencies were obtained for both biofilters at 140 mL min^{-1}, this flow rate was chosen to evaluate the oxidation efficiencies of biofilm in the subsequent kinetic tests. Furthermore, the flow rate of 140 mL min^{-1} corresponds to a linear velocity of 11.9 m h^{-1}, the same value previously applied in a pilot unit for the daily production of 400 L drinking water (Casentini et al., 2016).

3.3 Influence of initial As concentration and speciation

Tests were performed in order to further evaluate the impact of different initial As(III) concentration and the presence of mixed system As(III)/As(V) on the overall efficiency of the biological process (Table 1). Oxidation (%) was reported according to elapsed time (min), number of tank recirculations, BVs and PVs for a better comparison. After 3 hours, sand biofilter kept the same oxidative performance under the different operating conditions (efficiency $\geq$ 85%), whereas As(III) oxidation efficiency of glass biofilter was lower and it clearly decreased with the increase of As(III) concentration (from 81.6% to 69.6%). No clear impact of the concomitant presence of As(V) was found in both systems. Oxidation efficiencies observed under all different speciation conditions were 67.6 - 69.6% in glass and between 85.8% and 90.0% in sand biofilters (Table 1 and Fig. S3). The oxidation rate was similar in both tests carried out with glass column, corresponding to 0.4 μg L^{-1} h^{-1}. Diversely, the oxidation rate in sand biofilter increased with the decrease of As(III)

concentration (0.8, 0.9 and 1.1 µg L^{-1} h^{-1} at 100, 50 and 20 µg As(III) L^{-1}, respectively). By comparing the performance of the two filling materials according to treated PVs, sand biofilters treated 955 PVs while glass only 455, due to higher porosity of this material. This means that considering the same period of time and number of recirculations, the water passed twice in the sand biofilters compared to the glass ones, thus contributing to the higher oxidation performance observed in these systems. By comparing similar treated PVs (Table 1), the difference in terms of oxidation efficiency was lower and the ability to oxidize As(III) at each water passage in the biofilter was similar.

3.4 Influence of biofilter dimensions

The arsenite biological oxidation efficiency was also tested using larger volume biofilters operating with similar linear velocity (11.9 m h^{-1}). By increasing 7 times the BV, from 0.1 to 0.7 L, a higher increase in overall oxidation performances was observed (Fig. S4) in both glass and sand columns. Larger biofilters showed the same ability in oxidizing an initial amount of 50 or 100 µg L^{-1} As(III) for both materials. After 1 hour glass biofilters oxidized about 45% and sand filters 80% of As(III). As shown in Figure S4, an overall increase of the oxidation efficiency up to 20 and 30% was obtained for glass and sand biofilter, respectively, compared to the smaller filters.

3.5 Microbial cell abundance on filling materials.

The tridimensional structure reconstruction of the biofilms growing in the different biofilters was performed by combining CARD-FISH and CLSM. The analysis revealed the bacterial colonization on the surface of the filling materials (Fig. 2). The prokaryotic abundance was similar (16.4 ± 3.9 x 10^6 cells g^{-1} of filling material) in biofilms grown on glass rings in spite of the different acclimation period imposed (Table 2). Contrarily, a clear increase of the prokaryotic abundance was observed in LTA biofilm established on sand compared to STA biofilm (30.1 x 10^6± 7.6 x 10^6 cells g^{-1} and 7.4 x 10^6 cells g^{-1}, respectively). Marked cytometric differences were observed in microbial communities

grown on glass and sand. In particular, a high abundance of High Nucleic Acid content (HNA) cells was found in mature biofilm growing on coarse sand (up to 72.2%), while lower percentages were found in glass rings biofilms (on average 56%).

3.6 Arsenic related genes in biofilters

The quantification of As-related functional genes revealed the high abundance of genes involved in As transformations in biofilm samples (Fig. 3). Genes involved in resistance mechanisms to As (*arsBC*) are widespread and highly abundant. In particular, *arsC*, responsible of As(V) reduction within the cell membrane, showed highest values ranging between $3.8 \times 10^8 \pm 8.9 \times 10^7$ gene copies g^{-1} and $1.7 \times 10^{10} \pm 3.6 \times 10^9$ gene copies g^{-1}. The genes for the As(III) membrane efflux pump (*arsB*) showed lower values on average around $2.2 \times 10^6 \pm 3.9 \times 10^5$ gene copies g^{-1}. The gene *aioA*, involved in As(III) oxidation, was reported in all biofilm samples with values between $5.7 \times 10^5 \pm 2.3 \times 10^5$ and $1.8 \times 10^6 \pm 3.0 \times 10^5$ gene copies g^{-1} in glass biofilms. The abundance of *aioA* differed considerably between STA and LTA sand biofilms, ranging between $1.8 \times 10^6 \pm 1.2 \times 10^5$ and $6.0 \times 10^6 \pm 5.5 \times 10^4$ gene copies g^{-1}. Also gene involved in respiratory As(V) reduction (*arrA*) was found with values overall higher in glass biofilters mainly in STA biofilm (on average $1.7 \times 10^6 \pm 4.1 \times 10^4$ gene copies g^{-1}) than in sand biofilms (on average $1.2 \times 10^6 \pm 3.6 \times 10^5$ gene copies g^{-1}). The occurrence of As-related functional genes was reported also in groundwater used in the experiments (Fig. S5).

3.7 Microbial communities composition

NGS analysis performed on the biofilms and groundwater samples yielded a total of 292,496 sequence reads after quality control and bioinformatic processing. These reads resolved into 1042 OTUs. Overall, microbial communities colonizing biofilm grown on glass and sand showed similar structural characteristics, but differed considerably from groundwater used in the kinetic tests (Table S2). A high microbial diversity was observed in all biofilm samples as highlighted by the

359 Simpson index (range between 0.9 and 1). The Shannon index showed values higher in glass
360 biofilms (on average 4.0) than in sand ones (on average 3.4) and in particular these index values
361 were higher in biofilms grown in biofilters with a BV of 0.1 L (range between 3.4 and 4.3) rather
362 than in 0.7 L ones (range between 3.3 and 3.7). *Proteobacteria* was the most abundant phylum
363 showing values up to 40.7% and 45.8% of total OTUs in glass biofilms and up to 33.5% and 39.2%
364 in sand biofilms. This phylum was mainly represented by *Alphaproteobacteria*, affiliated with
365 orders *Caulobacterales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Sphingomonadales*, and
366 *Betaproteobacteria* mostly belonging to orders *Burkholderiales*, *Nitrosomonadales* and
367 *Rhodocyclales* (Fig. 4). Among these orders the occurrence of several OTUs affiliated with aerobic
368 and heterotrophic genera was reported (e.g. *Phenylobacterium*, *Hirschia*, *Woodsholea*,
369 *Bradyrhizobium*, *Rhodobacter*, *Roseomonas*, *Limnobacter*, *Leptothrix*, *Noviherbaspirillum*) as well
370 as to aerobic and autotrophic genera (i.e. *Hydrogenophaga*, *Variovorax).* Furthermore, OTUs
371 affiliated to anaerobic heterotrophic or mixotrophic genera, such as *Azoarcus* and *Acidovorax*, were
372 found. *Delta*- and *Gammaproteobacteria* were mainly represented by orders *Bradymonadales*,
373 *Acidithiobacillales* and *Xanthomonadales*; the latter order was mainly represented by members of
374 facultative anaerobic heterotrophic genus *Pseudoxanthomonas*. Additionally, the occurrence of
375 OTUs affiliated with *Actinobacteria* (7.3 - 18.8% of total OTUs), *Bacteroidetes* (9.2 - 12.5% of
376 total OTUs) and *Acidobacteria* (up to 10%) was revealed. *Acidobacteria* and *Bacteroidetes* were
377 mainly represented by the aerobic heterotrophic families *Blastocatellaceae*, *Cytophagaceae* and
378 *Chitinophagaceae*. Other phyla were overall found at minor extent with some exceptions as for
379 example *Nitrospirae* in glass biofilter with BV 0.7 L (9% of total OTUs), *Ignavibacteriae* in sand
380 biofiter with BV 0.7 L (19.3% of total OTUs), *Planctomycetes* and *Armatimonadetes* in glass
381 biofilter with BV 0.1 L (10.2 and 7.2% of total OTUs), and *Cyanobacteria* in sand biofiter with BV
382 0.1 L (27.3% of total OTUs). The relative abundance of OTUs in groundwater differed considerably
383 (Table S2 and Fig. S6). In this sample *Proteobacteria* and *Nitrospirae* represented up to 97% of

384 total OTUs, mainly affiliated with genera *Nitrosomonas* (on average 65.4% of total OTUs) and
385 *Nitrospira* (on average 28.5% of total OTUs).
386

387 ## 4. Discussion

388 The groundwater mixed microbial communities were able to promote a highly efficient and rapid
389 As(III)-oxidation through the development of biofilms enriched in arsenite oxidizers and specific
390 As-related functional genes.
391 A similar oxidation efficiency was observed between the two filling materials when the same
392 treated pore volumes are considered. However, the halved porosity of coarse sand compared to
393 glass rings allowed a higher number of recirculations through the biofilters within the same
394 operational time. This may explain the markedly different oxidative efficiencies observed between
395 glass and sand biofilters. The highest oxidation efficiencies of biofilm grown on sand can be also
396 related to the larger colonization surface available for microorganisms compared to glass rings. It is
397 worth to noting that arsenic was not retained during the operation of the biofilters, diversely from
398 previous studies (Li et al., 2016; Wan et al., 2010).
399 In line with the evidences reported in Gude et al. (2018), this study showed that a long exposure
400 time to As(III) promoted the formation of biofilms with marked capabilities to withstand As and to
401 oxidize As(III). The analysis performed on biofilm grown at different acclimation periods clearly
402 showed the increase of As(III) oxidation efficiencies with increase of the cell densities.
403 Interestingly, the highest oxidation efficiencies observed in this study occurred within few hours (2-
404 3 hours) and this finding sustains the feasibility of coupling this biological process with the As(V)
405 physical-chemical removal treatment.
406 Various hydraulic and chemical conditions were here tested for the first time to evaluate the
407 potentialities of microbial As(III) oxidation in biofilters by using native microbial population
408 occurring in As-contaminated groundwater.

409 The use of various flow rates strongly affected the oxidative performance of the biofilters, mainly
410 due to the different treated pore volumes rather than the contact time between water and filling
411 materials. Biotic oxidation was independent from contact time, most probably due to the imposed
412 very short contact times in selected filter columns. In particular, under the tested operation
413 conditions, a higher volume of the glass biofilter, a longer duration of the treatment or a higher
414 number of tank recirculations would be required to achieve the same oxidative efficiencies observed
415 within three hours by using sand biofilters.
416 Furthermore, the biological As(III) oxidation was found to be mostly affected by the initial As
417 concentration rather than As speciation, at natural groundwater concentrations range. The latter
418 suggests the process feasibility of treating natural groundwater containing both As(III) and As(V)
419 species common in volcanic regions (Crognale et al., 2017b; Fazi et al., 2016b). The initial
420 concentration of As(III) mostly impacted the oxidation rate rather than the efficiency. In particular,
421 the highest oxidation efficiency was observed at the lowest As(III) concentration, in line with
422 previous evidences on pure cultures (Bahar et al., 2016; Debiec et al., 2017).
423 Overall, the oxidation rates observed in this study were in line with those obtained by using pure or
424 enrichment cultures from low As-content groundwater (Casiot et al., 2006) but lower compared to
425 As(III)-oxidizing microorganisms from high As-content environments (Battaglia-Brunet et al.,
426 2002; Debiec et al., 2017; Li et al., 2016; Wan et al., 2010). The latter evidence suggests that the
427 overall performance of the biological oxidation may be further improved when As-richer
428 groundwaters are treated.
429 Moreover, a variety of different biological tools was employed to investigate the activity level and
430 the composition of the biofilms growing on glass rings and coarse sand. The relative abundance of
431 HNA and LNA cells was used as gross parameter to evaluate the portion of active cells in the mixed
432 microbial community, as previously observed in a large variety of microorganisms and aquatic
433 environments (Andreatta et al., 2004; Gasol and Del Giorgio, 2000; Lebaron et al., 2002; Sherr et
434 al., 2006; Thyssen et al., 2005; Troussellier et al., 1999). The analysis showed a higher relative

abundance of active cells (HNA) in LTA sand compared to the LTA glass biofilters in line with the observed oxidation performances. Even though the high abundance of HNA cells was already reported in As-rich aquatic environments (Crognale et al., 2017b; Fazi et al., 2016b), the evidence of a correlation between HNA content cells and As-transformation capabilities was not previously shown.

The analysis of As-related functional genes in LTA biofilters showed a higher abundance of *aioA*, *arrA*, and *arsC* genes in sand biofilters rather than in glass ones. As shown in Figure 5, the Principal Component Analysis (PCA) clearly distinguished the sand from the glass biofilters regardless of the columns BV.

The enrichment of As related functional genes in the all screened biofilters is consistent with the capability to resist high levels of As widely reported in a large variety of As-contaminated and As-free environments (Bertin et al., 2011; Escudero et al., 2013; Fazi et al., 2016b; Jackson et al., 2005). The *arsBC* system has been found in the genome of every bacterial species till now sequenced suggesting that arsenic resistance might not be confined to organisms inhabiting As-contaminated environments, but it is environmentally and phylogenetically widespread (Jackson et al., 2005; Jackson and Dugas, 2003; Takeuchi et al., 2007; Yang and Rosen, 2016). The *arsB* gene may be horizontally transferred and increasingly be present in a microbial population under conditions of long-term elevated arsenic stress (Cai et al., 2009). It has been also argued that bearing both aio and ars operons confers higher As resistance than bearing ars alone (Cai et al., 2009). The high occurrence of arsenite-oxidase gene (*aioA*) in sand biofilters, especially in LTA biofilms, was in line with the observed highest oxidation capabilities. Microbial As(III)-oxidation is considered as a detoxification mechanism in heterotrophic microorganisms or as energy source for chemolithoauthotrophic microbes (Battaglia-Brunet et al., 2002; Garcia-Dominguez et al., 2008; Hoeft et al., 2007; Muller et al., 2003; Santini et al., 2000). The wide occurrence of *aioA* gene was reported by Yamamura and Amachi (2014) in phylogenetically diverse strains including members

of *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Aquificae*, *Deinococcus-Thermus*, *Chlorobi*, *Chloroflexi*, *Nitrospira*, and *Crenarchaeota*.

Although As(V)-reductive transformations were not observed during the operation, the presence of *arrA* genes mainly in STA glass biofilters could be related to the occurrence of OTUs affiliated to microorganisms potentially able to perform As(V) reduction such as for example *Rhodococcus* and *Opitutus* (Corsini et al., 2014; Xiao et al., 2016).

Remarkably, the microbial community composition did not strongly differ between glass and sand biofilters. However, a relative similarity was observed between biofilters with the same BV regardless of filling material even though the analysis did not fully reveal a clear differentiation (Fig. 6).

The composition of microbial communities in bioreactors differed considerably from groundwater used for the experiments, suggesting an adaptation to high As(III) concentrations (Fig. 4 and Fig. S6). The performance of bioreactors was most likely related to the copious presence of microorganisms known for their As(III)-oxidation capabilities mostly affiliated with *Alpha-* and *Betaproteobacteria*, as previously observed (Gude et al., 2018; Li et al., 2016). The large occurrence of OTUs affiliated with *Burkholderiales*, at higher extent in sand biofilms, is most likely related to the observed oxidation capabilities. The mechanisms of arsenic resistance and arsenite oxidation, indeed, have been widely investigated in members of *Burkholderiales* revealing a high diversity and wide distribution of As-related genes in this order (Li et al., 2014). As observed in this study, the occurrence of *Rhizobiales*, *Nitrosomonadales*, *Rhodocyclales* and *Xanthomonadales*, together with *Burkholderiales*, was also previously reported as major component of microbial communities in household filter sand used for arsenic, iron and manganese removal from groundwater (Li et al., 2014). OTUs affiliated with As(III)-oxidizing families *Rhodocyclaceae*, *Chitinophagaceae*, *Nitrospiraceae*, *Comamonadaceae* and *Bradyrhizobiaceae* were relatively more abundant in both glass and sand biofilters with 0.7 L BV, whereas OTUs belonging to *Nocardiaceae*, *Oxalobacteraceae*, *Xanthomonadaceae*, *Acetobacteraceae* and *Rhodobacteraceae*

were relatively more abundant in biofilters with 0.1 L BV (Fig. 6). Within these families, the occurrence of OTUs affiliated with *Bradyrhizobium*, *Rhodobacter*, *Roseomonas*, *Limnobacter*, *Hydrogenophaga*, *Leptothrix* and *Variovorax* confirmed the active role of microbial communities in As(III) oxidation (Bagade et al., 2016; Bahar et al., 2013; Battaglia-Brunet et al., 2006; Li et al., 2016; Sultana et al., 2012; Vanden Hoven and Santini, 2004; Yamamura et al., 2014). NGS analysis revealed the occurrence at minor extent of OTUs affiliated with *Azoarcus* and *Acidovorax* genera which comprise species known for their capability to facultatively anaerobically oxidize As(III) coupling with chlorate or nitrate reduction (for examples Ac. sp strain NO1; Az. sp. strain DAO1) (Huang et al., 2012; Rhine et al., 2006; Sun et al., 2011).

In conclusions, this study highlighted the high potentialities of the biological As(III) oxidation process in biofilters with low-cost and easily available filling materials under experimental conditions very close to those used in household-scale treatment system. Our finding showed that a material with low porosity allows either a better biomass retention and biofilm growth due to the higher available surface and a high number of treated pore volumes. The start-up of the biofilter can be easily performed by using the As-contaminated groundwater within a reasonable period of time. The native groundwater bacteria were able to withstand high As concentration and easily formed highly As(III) oxidizing biofilms.

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Table 1. Arsenite oxidation in biofilters with BV 0.1 L fed at 140 mL min^{-1} at different initial As concentration (20, 50, 100 µg L^{-1}) and different As(III):As(V) ratio (100:0; 60:40, 40:60). As(III)-oxidation efficiencies (%) are reported according to elapsed time (min), n. tank recirculations, bed volumes (BVs) and pore volumes (PVs) for a better comparison of the performances.

					As(III) Oxidation (%)					
					As(III) Initial Concentraton			***As Initial Speciation***		
	time (min)	***n. tank recirculation***	***BVs***	***PVs***	**100 µg L^{-1}**	**50 µg L^{-1}**	**20 µg L^{-1}**	**100% As(III)**	**60% As(III)**	**40% As(III)**
GLASS RINGS	*0*	*0*	*0*	*0*	**0.0**	**0.0**	**2.6**	**0.0**	**0.0**	**0.0**
	15	*1.3*	*21*	*37*	**5.5**	**9.0**	**19.4**	**5.5**	**7.5**	**12.1**
	30	*2.6*	*42*	*74*	**21.3**	**25.8**	**41.0**	**21.3**	**15.0**	**17.7**
	45	*3.9*	*63*	*111*	**27.9**	**33.1**	**46.0**	**27.9**	**21.7**	**24.4**
	60	*5.2*	*84*	*148*	**31.7**	**39.5**	**52.2**	**31.7**	**29.3**	**34.4**
	75	*6.5*	*105*	*185*	**34.3**	**44.4**	**52.7**	**34.3**	**32.6**	**40.0**
	90	*7.8*	*126*	*222*	**40.9**	**50.8**	**58.8**	**40.9**	**44.0**	**43.5**
	105	*9.1*	*147*	*259*	**44.3**	**52.3**	**61.6**	**44.3**	**47.0**	**50.5**
	120	*10.4*	*168*	*296*	**48.3**	**56.0**	**64.9**	**48.3**	**50.4**	**56.3**
	135	*11.7*	*189*	*333*	**59.0**	**61.8**	**69.3**	**59.0**	**55.8**	**60.6**
	150	*13*	*210*	*370*	**56.7**	**64.7**	**76.3**	**56.7**	**60.2**	**63.7**
	165	*14.3*	*231*	*407*	**63.5**	**67.4**	**79.3**	**63.5**	**64.1**	**66.6**
	180	*15.6*	*252*	*444*	**69.6**	**69.6**	**81.6**	**69.6**	**67.7**	**69.6**
COARSE SAND	***time (min)***	***n. tank recirculation***	***BVs***	***PVs***	**100 µg L^{-1}**	**50 µg L^{-1}**	**20 µg L^{-1}**	**100% As(III)**	**60% As(III)**	**40% As(III)**
	0	*0*	*0*	*0*	**1.5**	**1.9**	**0.0**	**1.5**	**0.0**	**0.0**
	15	*1.3*	*21*	*80*	**13.9**	**27.9**	**46.7**	**13.9**	**30.4**	**32.4**
	30	*2.6*	*42*	*159*	**27.7**	**41.9**	**63.3**	**27.7**	**46.4**	**43.4**
	45	*3.9*	*63*	*239*	**36.7**	**56.5**	**71.5**	**36.7**	**57.8**	**56.6**
	60	*5.2*	*84*	*318*	**51.1**	**67.4**	**77.6**	**51.1**	**68.2**	**63.6**
	75	*6.5*	*105*	*398*	**57.5**	**73.5**	**80.4**	**57.5**	**74.9**	**71.6**
	90	*7.8*	*126*	*477*	**63.3**	**79.8**	**82.2**	**63.3**	**80.9**	**77.0**
	105	*9.1*	*147*	*557*	**70.0**	**83.6**	**81.4**	**70.0**	**84.5**	**80.7**
	120	*10.4*	*168*	*636*	**71.5**	**85.4**	**84.4**	**71.5**	**86.3**	**84.2**
	135	*11.7*	*189*	*716*	**79.4**	**87.2**	**86.0**	**79.4**	**87.0**	**85.5**
	150	*13*	*210*	*795*	**84.0**	**86.7**	**88.0**	**84.0**	**87.5**	**84.2**
	165	*14.3*	*231*	*875*	**86.0**	**87.6**	**89.4**	**86.0**	**89.5**	**84.9**
	180	*15.6*	*252*	955	**89.0**	**85.1**	**89.0**	**89.0**	**90.0**	**85.8**

Table 2. Prokaryotic abundance (cells g^{-1}) and HNA/LNA cells relative abundance (%) in biofilm grown on sintered glass rings and coarse sand. STA; biofilm exposed to As(III) for a short-term acclimation period (around two weeks) before the kinetic tests; LTA, biofilm exposed to As(III) for a long-term acclimation period (around 40 days) before the kinetic tests in a biofilter with BV 0.1 L or 0.7 L.

		10^6 cells g^{-1}	**LNA%**	**HNA%**
Glass rings	STA	17	29.6	70.4
	LTA 0.1 L	20	48.0	52.0
	LTA 0.7 L	12	39.9	60.1
Coarse sand	STA	7.4	50.2	49.8
	LTA 0.1 L	35	27.8	72.2
	LTA 0.7 L	25	28.5	71.5

Figure legends

Figure 1. Arsenite oxidation kinetics at different flow rates (70 mL min^{-1}, 140 mL min^{-1}, 250 mL min^{-1}) in glass rings ("glass") and coarse sand ("sand") biofilters. The oxidative efficiency (%) was plotted according to elapsed time (min) (panel a), n. tank recirculations (panel b), and pore volumes (PVs) (panel c).

Figure 2. CLSM combined images showing the spatial distribution (X−Y, X−Z, and Y−Z planes) of Bacteria (green) identified by CARD-FISH in biofilms grown on glass rings and coarse sand. The hybridized bacterial cells were excited with the 488 nm line of an Ar laser (excitation) and observed in the green channel from 500 to 530 nm (emission). Filling materials were visualized by their reflection signal (405 nm line of a diodo laser) and appear gray. The image is composed by 81 optical sections (step size: 0.40 μm).

Figure 3. Abundance of arsenic-related functional genes estimated by qPCR in biofilms (gene copies g^{-1}). *arsB*, arsenite transporter; *arsC*, arsenate cytoplasmic reductase; *aioA*, arsenite oxidase; *arrA*, respiratory arsenate reductase; STA "material", biofilm established on glass rings or coarse sand and exposed to As(III) for a short-term acclimation period (around two weeks) before the kinetic tests; LTA "material", biofilm established on glass rings or coarse sand in a biofilter with BV 0.1 L or 0.7 L and exposed to As(III) for a long-term acclimation period (around 40 days) before the kinetic tests.

Figure 4. Operational taxonomic units (OTUs) relative abundance (%) estimated by high-throughput sequencing in long-term acclimated (LTA) biofilms with BV 0.1 L and 0.7 L. Clusters at order level in proteobacterial classes *Alphaproteobacteria* (panel a), *Betaproteobacteria* (panel

831 b), *Delta*- and *Gammaproteobacteria* (panel c) are represented. The graphical representation of
832 order composition within phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Nitrospirae* is
833 reported in panel d.

834

835 **Figure 5.** Principal Components Analysis biplot performed using quantification data of As-related
836 functional genes (*aioA*, *arrA*, *arsB*, *arsC*), prokaryotic abundance (cells g^{-1}) and the relative
837 abundance of low and high nucleic acid content cells (HNA and LNA). The vector length is
838 proportional to the correlation between corresponding parameter and the PCA axis 1 and 2. The
839 symbol size indicating biofilm samples are proportionally related to the average oxidation rate.

840

841 **Figure 6.** Principal Components Analysis biplot representing the typifying As-related microbial
842 composition at family level. Length of arrows represents the correlation between corresponding
843 parameters and PCA axis 1 and 2. The symbol size indicating biofilm samples are proportionally
844 related to the average oxidation rate. Histogram plots show the contribution of each variable (vector
845 projection values) expressed as the correlation with the x- and y-axis. Families known for their
846 involvement in As-resistance and As(III) oxidation processes are reported in black and red,
847 respectively. Families not involved in As-transformations and with an occurrence <1% are not
848 considered in this analysis. Ace, *Acetobacteraceae*; Ana, *Anaerolineaceae*; Bra, *Bradyrhizobiaceae*;
849 Cau, *Caulobacteraceae*; Chi, *Chitinophagaceae*; Com, *Comamonadaceae*; Cyt, *Cytophagaceae*;
850 Gem, *Gemmatimonadaceae*; Hyphomi, *Hyphomicrobiaceae*; Hyphomo, *Hyphomonadaceae*; Mic,
851 *Microbacteriaceae*; Myc, *Mycobacteriaceae*; Nitroso, *Nitrosomonadaceae*; Nitrosp,
852 *Nitrospiraceae*; Noc, *Nocardiaceae*; Oxa, *Oxalobacteraceae*; Rhodob, *Rhodobacteraceae*; Rhodoc,
853 *Rhodocyclaceae*; Rhodos, *Rhodospirillaceae*; Sph, *Sphingomonadaceae*; Xan, *Xanthomonadaceae*.

854

Figure 1

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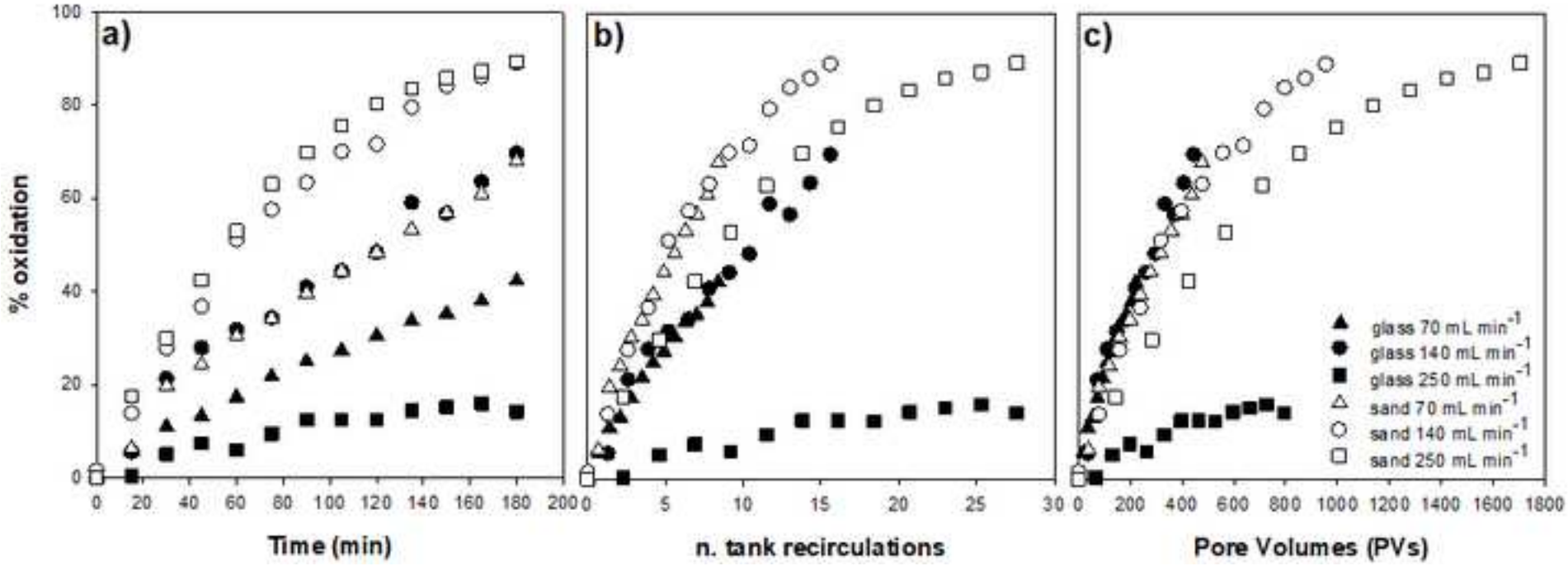

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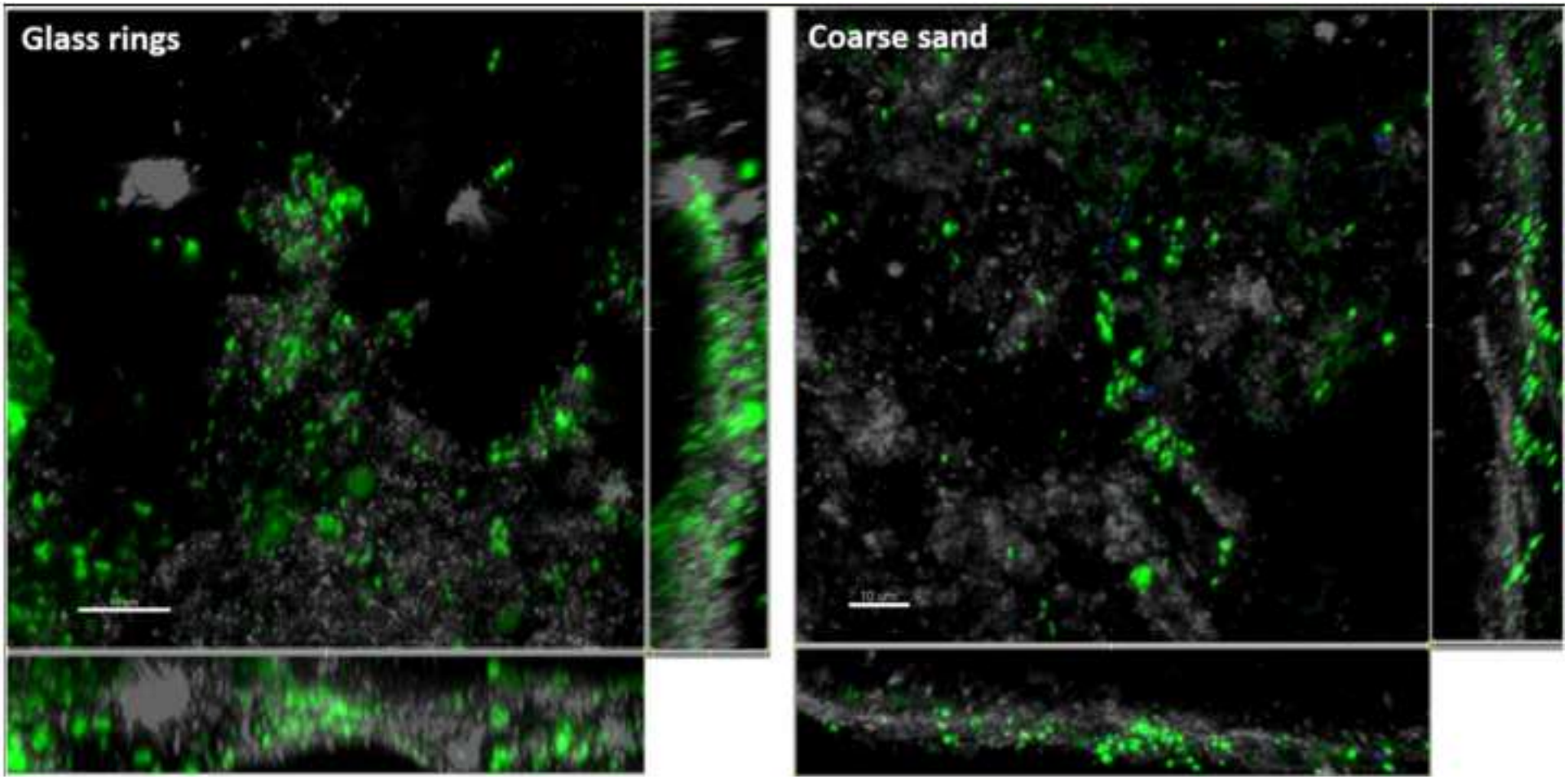

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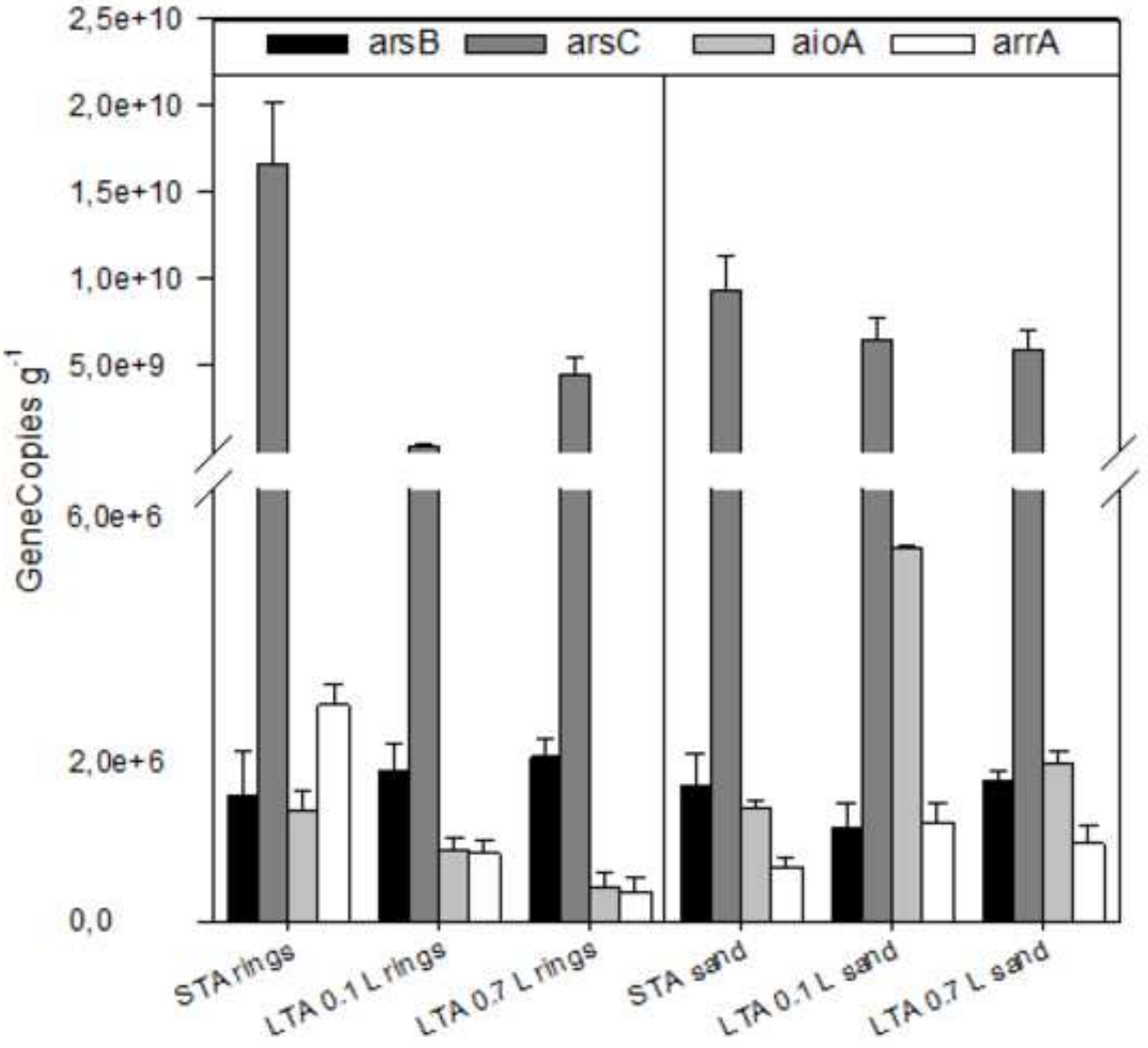

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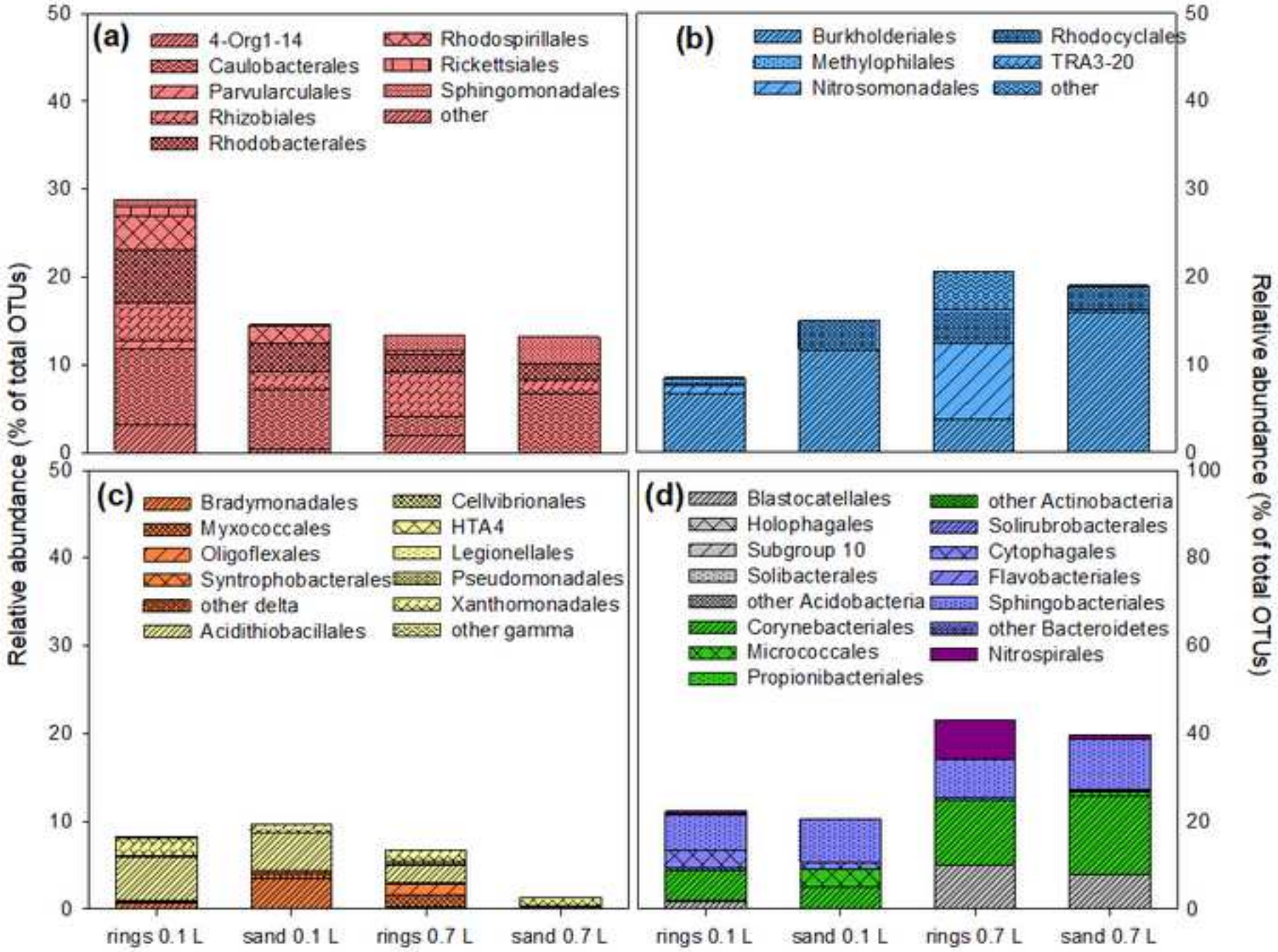

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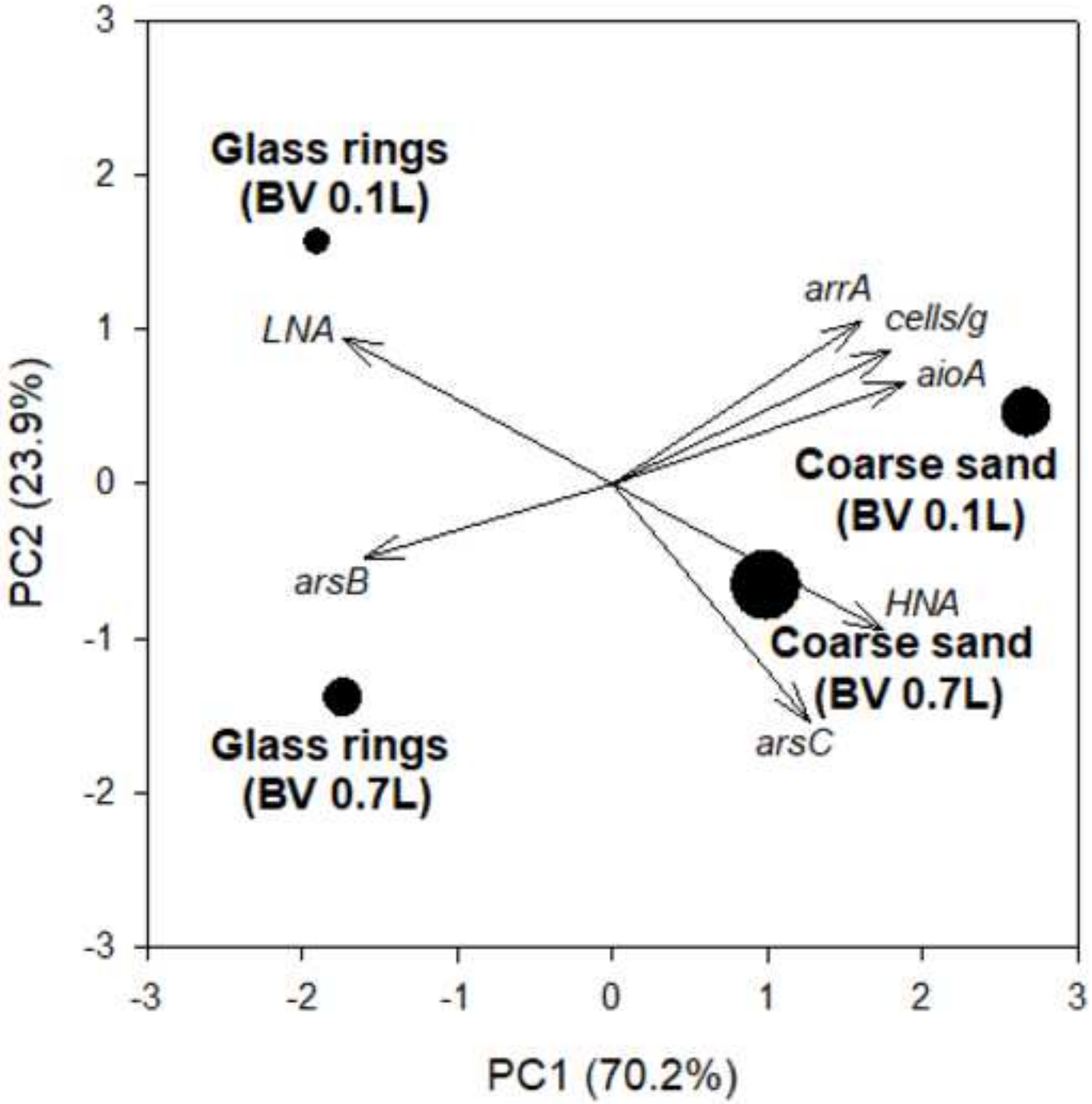

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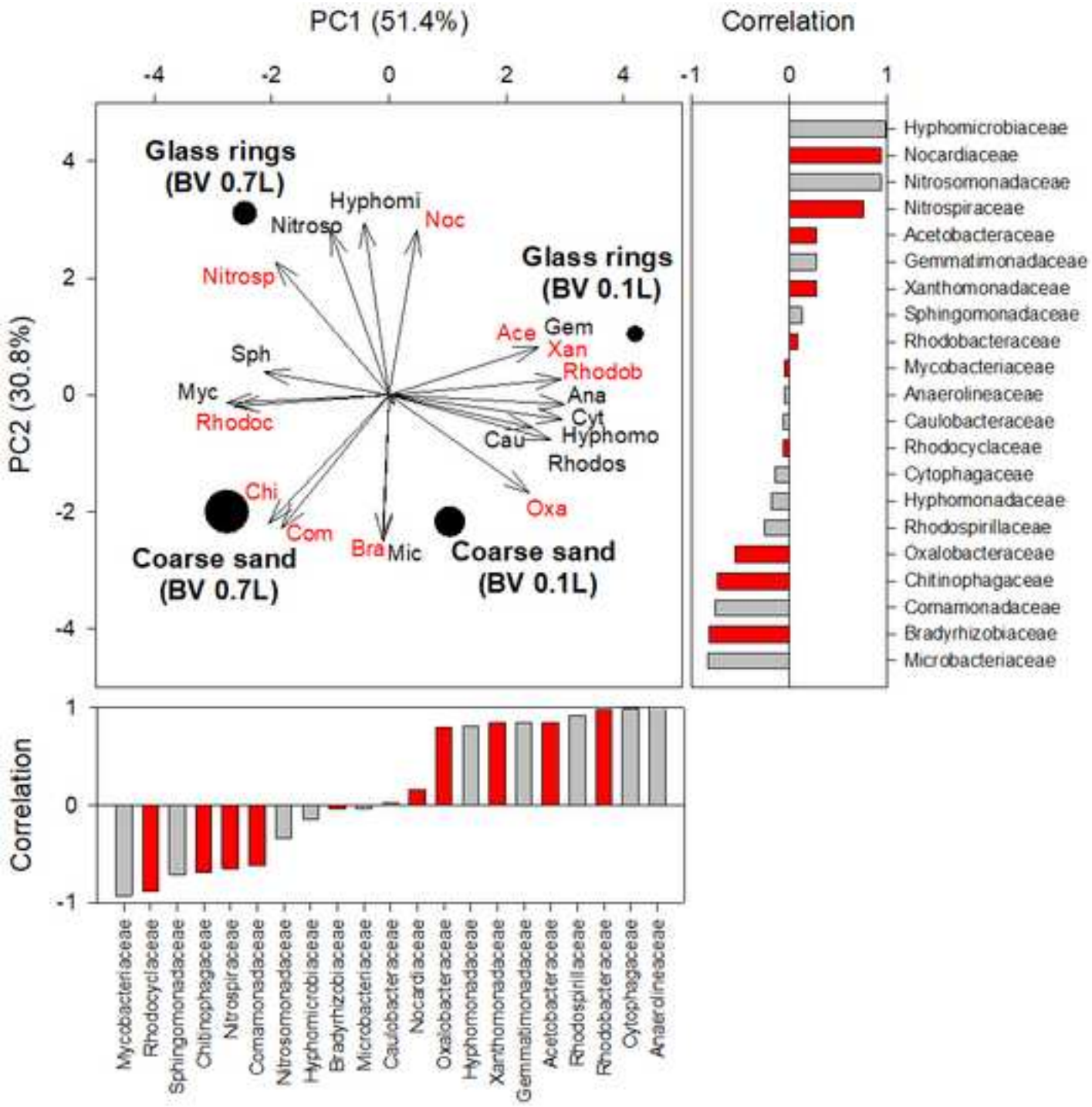

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