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PROCESSI DI RIMOZIONE BIOLOGICA DI ARSENICO DA ACQUE SOTTERRANEE: CARATTERIZZAZIONE E POTENZIALITÀ METABOLICHE DELLE COMUNITÀ MICROBICHE AUTOCTONE

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"We are not yet what we shall be,
but we are growing toward it,
the process is not yet finished…"

Martin Luther

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Short abstract

Arsenic (As) is a toxic element released in aquatic environments by geogenic processes or anthropic activities. To counteract its toxicity, several microorganisms have developed mechanisms to tolerate and utilize it for respiratory metabolism. However, still little is known about identity and physiological properties of microorganisms exposed to natural high levels of As and the role they play in As transformation and mobilization processes. 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 work aimed to explore the phylogenetic composition and functional properties of microbial communities in As-rich freshwater environments of geothermal origin and to elucidate the key microbial functional groups that directly or indirectly may influence As-transformations across a natural range of geogenic arsenic contamination. Mixed microbial communities associated with biofilm were also analysed in an As-rich geothermal environment along natural temperature, pH and oxygen gradients. Furthermore, this study investigated the biological pre-treatment process of arsenic-contaminated waters in laboratory-scale systems by evaluating the main operational parameters affecting the process performances.

The study provided the detailed description of structural and metabolic traits of native microbial communities in arsenic-rich freshwaters and associated with biofilm. The As detoxification

processes prevailed over As metabolic processes in such environments, concomitantly with the intriguing occurrence of novel thermophiles able to tolerate high levels of As.

This work also highlighted the contribution of native groundwater bacteria to As(III)-oxidation in laboratory-scale biofilters, under conditions suitable for a household-scale treatment system. The native groundwater bacteria were able to withstand high As concentration and easily formed highly As(III) oxidizing biofilms. 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 was also elucidated.

Keywords

arsenic, arsenite oxidation, groundwater, thermal waters, biofilter, As-related functional genes, microbiome

Extended abstract

The following section has been modified and adapted from the summary report of the PhD activities (oral communication) "Microorganisms in arsenic-rich freshwaters: distribution, metabolic potentialities and exploitation in water treatment systems" presented to the Doctoral Examining Board - La Tuscia University on May 2018.

This PhD thesis dealt with the structural and metabolic characterization of microbial communities exposed to natural high arsenic concentrations in aquatic environments. This project aimed also to optimize the biological pre-treatment process of arsenic-contaminated waters in laboratory-scale systems. The study provided the detailed description of structural and metabolic traits of native microbial communities in arsenic-rich freshwaters and associated with biofilm as well as the evaluation of the performance of microbial As(III)-oxidation in column systems by testing different filling materials (i.e., sintered glass rings, coarse sand) and operational parameters.

Arsenic (As) is a major toxic contaminant that represents a human health concern by impairing water quality on a global scale (Nordstrom, 2002). Despite the high toxicity of this element, microorganisms are able to resist to high As concentration and/or metabolize it. Previous research efforts aimed to elucidate the microorganisms and mechanisms involved in the biogeochemical cycling of this element, displaying potential for biotechnological applications (Crognale et al., 2017a). Over the last years, indeed, in a search for sustainable and cost-effective methods for water treatment, arsenic remediation turned to the potentialities of biological approaches (Plewniak et al., 2018). 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).

The main results obtained in the framework of the PhD thesis are related to the following tasks:

- characterization of arsenic-polluted natural environments through physical-chemical properties analysis, biomolecular characterization of i) bacteria involved in arsenic-resistance and metabolism in aquatic environments and ii) microbial communities associated with biofilm;
- evaluation of the metabolic potentialities and biological activity of As-related microbial communities in natural environments;
- implementation of innovative strategies for the removal of arsenic from drinking water at lab-scale, analysing microbial biofilm in biofilters.

In order to characterize microbial communities in natural As-rich freshwaters, water and biofilm samples were collected during two different sampling campaigns carried out on the Cimino-Vico volcanic area (Viterbo, VT). Water samples, collected on November 2015, included: thermal waters from hot springs (named as PAL, SSC, CAR), groundwaters (named as OLI, BEL, ANG, FON) and one lake water (Vico Lake). Hot spring "CAR" ("Carletti thermal pools") was also analysed in a second sampling survey on March 2016 in order to describe the microbial dynamics under variable physical-chemical conditions. This hydrothermal environment consists in a hot spring from which a channel of around 112 m is generated and is characterized by a natural temperature, pH and dissolved oxygen gradient in the presence of a constant high total As content. Eight water and fourteen biofilm (superficial and subsuperficial layers) samples were collected along the channel. Physical-chemical properties (pH, EC, DO, T, Fe, FeII, sulfides, anions, cations and arsenic concentration) were measured *in situ* by probes or in lab using spectrophotometric methods, ion chromatography, ICP-MS and atomic absorption spectrometry. Prokaryotic abundance and microbial communities composition were described by Next Generation Sequencing (NGS), flow cytometry and CARD-FISH using specific rRNA-targeted HRP-labelled probes (for *Bacteria*;

Alpha-, *Beta-*, *Gamma-*, *Delta-Proteobacteria*; *Chloroflexi*, *Firmicutes*, *Flavobacteria*, *Planctomycetes*, TM7 division, *Actinobacteria*, *Archaea*). Furthermore, during the first sampling campaign, Most Probable Number (MPN) technique (10-fold dilution series in triplicate) was used to quantify the cultivable fraction of some of the main microbial functional groups (general aerobic heterotrophs, autotrophic and heterotrophic As(III)-oxidizers, autotrophic and heterotrophic As(V)-reducers, sulfide-oxidizers, sulphate-reducers, nitrate-reducers, iron-oxidizers, iron-reducers and manganese-reducers). PCR amplification and qPCR were carried out using different primer sets in order to explore and quantify the distribution of several genes involved in the arsenic cycle in water samples. In detail, the arsenite oxidase (*aioA*), respiratory arsenate reductase (*arrA*), cytoplasmic arsenate reductase (*arsC*) and membranous As(III) efflux pump (*arsB*) genes were analysed. During the second campaign, clean microscopy slides were placed in the central section of the channel (corresponding to sampling site C4) and collected overtime (2-7-12 days) to monitor biofilm development, biomass increments in the earlier stages and changes in the three-dimensional structure during biofilm maturation. The bacterial community successional changes and the three-dimensional structure were described by applying *in situ* hybridization techniques in combination with Confocal Laser Scanning Microscopy (CLSM).

In laboratory-scale experiments, columns with an internal diameter of 30 mm and 65 mm and a height of 135 mm and 200 mm, respectively, were used for the construction of biofilters, with different bed-volume. Coarse sand and sintered glass rings were used separately as filling materials for the formation of biofilm. Initially, a natural groundwater without arsenic circulated throughout the biofilters for around 20 days for promoting biofilm generation. Then, continuous spikes of As(III) were added for the microbial communities acclimation for 16 or 43 days facilitating two different maturation stages of biofilm. The microbial As(III)-oxidizing

efficiency of the biofilm reactors was evaluated using a naturally not-contaminated groundwater amended with As(III). 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%. As_{tot} was analyzed by HG-AAS prior reduction to As(III) by 5% KI/Acid Ascorbic solution. As(V) concentration was obtained by the difference. Firstly, different flow rates were tested (70, 140, 250 ml/min) on biofilters with a bed volume of around 0.1L with two different maturation stages of the biofilm. The capability to oxidize three different As(III) concentrations (20, 50, 100 ppb) were also assessed in biofilters with two different bed volumes (around 0.1L and 0.7L) filled with sintered glass rings or coarse sand. The kinetic tests were followed every 15 minutes for three hours and every 10 minutes for 1 hour for the bioreactors with a bed volume of 0.1L and 0.7L, respectively. Filling materials were sampled at the end of the experiments for subsequent microbiological analysis. CARD-FISH technique in combination with CLSM was used to describe biofilm colonization and the three-dimensional structure of biofilm. Microbial community composition developed on filling materials was described by using high-throughput 16S rRNA gene sequencing. Arsenic related functional genes, involved in As(III)-oxidation (*aioA*), As(V) respiratory reduction (*arrA*) and arsenic resistance (*arsB* and *arsC*) were quantified by qPCR.

The characterization of As-rich aquatic environments revealed that the As concentrations reached up to 182 μg/L in groundwater and 362 μg/L in thermal waters, in line with previous studies (Fazi et al., 2016b). Prokaryotic abundance was higher in lake waters ($2.3x10^6 \pm 1.9x10^4$ cell/ml) and thermal waters (between $5.1x10^4 \pm 9.3x10^1$ cell/ml and $6x10^5 \pm 2.5x10^3$ cell/ml) than in groundwaters (between $2.2x10^4 \pm 2.6x10^2$ cell/ml and $3.3x10^5 \pm 4.7x10^3$ cell/ml). As revealed by CARD-FISH, *Proteobacteria* resulted to be the main microbial component; in

detail *Alpha-* and *Betaproteobacteria* were the most abundant groups retrieved in groundwater and surface water, whereas *Gammaproteobacteria* dominated in thermal waters. Moreover, *Archaea*, *Gammaproteobacteria* and High Nucleic Acids content cells (HNA) were relatively more abundant in thermal waters, whereas *Betaproteobacteria*, *Planctomycetes*, TM7, *Alphaproteobacteria* and Low Nucleic Acids content cells (LNA) were more represented in non-thermal waters. The microbial profiling obtained by NGS analysis has revealed the occurrence of *Nitrospirae*, *Bacteroidetes* and *Proteobacteria* phyla in groundwaters, and *Cyanobacteria* in Vico Lake. *Proteobacteria* was the predominant phylum in thermal waters but a number of sequence reads did not exhibit homology to known microbial taxa, which implies the presence in thermal waters of numerous so far unidentified microorganisms. As assessed by cultivation-dependent approach, nitrate and sulphate reduction and heterotrophic As(III)-oxidization were the main metabolisms occurring in geothermal area. No growth of autotrophic As(III)-oxidizers, autotrophic and heterotrophic As(V)-reducers, Fe-reducers and oxidizers, Mn-reducers and sulfide oxidizers was observed. The detection of *arsBC* genes in all screened samples suggested that As detoxification processes prevailed in groundwater and geothermal waters. Although As(III) oxidation was revealed by MPN analysis, arsenite oxidase gene (*aioA*) was not detected by PCR using different primer pairs. This could be most likely due to the high microbial diversity in our sample and to the primer coverage that may not be sufficient to capture high gene diversity.

Additionaly, the dynamics of microbial communities in water and associated with biofilms were evaluated along a channel receiving As contaminated thermal spring water (Carletti thermal pools) typically characterised by natural temperature, pH and dissolved oxygen gradient. Overall, the percentage of HNA cells in water samples decreased along the channel with the decrease of temperature (from 87.8% to 71.9%). No marked differences were found in terms of

prokaryotic abundance, with an average value of 7.4 x 10^5 cells/ml. As revealed by CARD-FISH, *Archaea* represented among 5.4% and 24.5% of total prokaryotic cells. Within the *Bacteria* domain, *Gammaproteobacteria* resulted to be the predominant group along the channel showing abundance ranging between 1.2 x 10^5 ± 5.1 x 10^3 and 5.3 x 10^5 ± 2.0 x 10^4 cells/ml. *Alphaproteobacteria*, *Betaproteobacteria* and *Deltaproteobacteria* represented on average 7% of total prokaryotic abundance. The remaining portion of bacterial groups represented less than 2.2% of total cells. These findings were in line with those observed by NGS revealing the predominance of genus *Thiofaba* along the channel (on average ~ 85% of total OTUs). *Epsilonproteobacteria* represented around 9.2% of total OTUs, while *Archaea* on average less than 1% of total OTUs. Prokaryotic abundance was higher in superficial biofilm samples (on average 5.3 x 10^9 cells/g) than in sub-superficial samples (on average 1.2 x 10^8 cells/g). Total abundance tends to decrease in superficial biofilm samples along the channel and increase in sub-superficial samples. The microbial communities were dominated by *Bacteria* in both superficial and sub-superficial layers representing on average the 90% of total prokaryotes. *Cyanobacteria* was the most abundant group in the superficial layer revealed by CARD-FISH technique, while *Alpha-* and *Deltaproteobacteria* were the main bacterial groups in sub-superficial layer. The microbial profiling obtained by NGS analysis has revealed that *Cyanobacteria* were mainly represented by *Spirulina*, *Leptolyngbya* and *Fischerella* genera. Moreover, OTUs affiliated to *Thaumarchaeota*, *Chloroflexi* (*Anaerolineaceae*, *Roseiflexaceae* families), *Bacteroidetes* and *Proteobacteria* (mainly *Alpha-* and *Beta-*) were retrieved. Significant changes in biofilm development, biomass increments and three-dimensional structure were observed in 12 days using hybridization technique in combination with CLSM. In detail, at 2 and 7 days the microbial community seemed to be equally represented by

Cyanobacteria, *Bacteria* and other prokaryotes. After 12 days, instead, *Cyanobacteria*, most probably affiliated to genus *Spirulina*, dominated the microbial community.

Furthermore, the biological As(III)-oxidation process was evaluated in laboratory-scale biofilters treating As-contaminated groundwater through the selection and the establishment of biofilms composed by native water microbial communities. The different maturation stages of the biofilm strongly influenced the performance of biofilter systems. Overall the highest As(III) oxidation was observed in the biofilters that were acclimated to As(III) for 43 days (LTA biofilters). In detail, the column system with sintered glass rings as filling materials with an acclimation stage of 43 days showed a final oxidation of 69.6% 100 ppb As(III) within three hours. Whereas, after 16 days of acclimation (STA biofilters), only 23.6% of As(III) was oxidized after 2.5 hours. Similar trend was observed in coarse sand biofilter, in which the system acclimated to As(III) for 43 days was able to oxidize up to 89% of initial As(III) within three hours. Markedly lower As(III) oxidation efficiencies were obtained in the biofilter acclimated to As(III) for 16 days, showing no more than 23.1% of As(III) oxidation within 2.5 hours. Sintered glass rings and coarse sand biofilter systems with a bed volume of 0.1 L and operating at a flow rate of 140ml/min were used to evaluate the influence of initial As speciation on As(III) oxidation efficiencies. Sintered glass rings biofilter was able to oxidize up to 67.6 - 69.6% without significant differences under all different speciation conditions analyzed. No remarkable differences in As(III) oxidation were observed using coarse sand biofilter (range between 85.8% and 90.0%), but the oxidation was slower using only As(III) rather than As(III):As(V) ratios of 2:3 and 3:2. The initial As(III) concentration affected biofilter oxidation performance mainly in terms of oxidation rate. Both in sintered glass ring and coarse sand biofilters, the highest initial As(III) concentration reflected the lowest oxidation rate and viceversa. On the contrary, no marked differences were observed in terms of oxidation

efficiency within three hours of experiment (up to 81.6% and 89.0% in sintered glass rings and coarse sand biofilters, respectively). The impact of the use of different initial As(III) concentrations was also assessed on biofilters with a bed volume of 0.7 L. The percentages of As(III)-oxidation in these biofilters didn't show remarkable differences within 60 minutes (up to 50.2% and 82.7% in sintered glass rings and coarse sand biofilters, respectively), but the oxidation rate was considerably higher than in biofilters with smaller bed volume. The oxidation efficiencies were higher in these biofilters rather than in smaller ones, probably due to the large amount of filling material (seven times more) and, consequently, of biofilm.

The prokaryotic abundance in sintered glass rings biofilters ranged between $1.7x10^7$ cells/g in STA biofilm and $2.0x10^7$ cells/g in LTA biofilm. A more marked difference was observed between STA and LTA biofilms established on coarse sand with $7.4x10^6$ cell/g and $3.5x10^7$ cells/g, respectively. Different microbial composition was observed in glass rings and coarse sand biofilms. *Proteobacteria* was the most abundant phylum showing values up to 40.7% and 45.8% of total OTUs in sintered glass rings biofilms and up to 33.5 % and 39.2% of total OTUs in coarse sand biofilms. This phylum was represented mainly by *Alphaproteobacteria*, affiliated to orders *Caulobacterales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Sphingomonadales*, and *Betaproteobacteria*, mostly belonging to orders *Burkholderiales*, *Nitrosomonadales* and *Rhodocyclales*. *Delta-* and *Gammaproteobacteria* were mainly represented by *Bradymonadales*, *Acidithiobacillales* and *Xanthomonadales*. Additionally, the occurrence of OTUs belonging to *Actinobacteria* (7.3 – 18.8 % of total OTUs), *Bacteroidetes* (9.2-12.5 % of total OTUs) and *Acidobacteria* (up to 10%) was revealed. Other phyla were found at minor extent with some exceptions as for example *Nitrospirae* in sintered glass rings biofilm with a bed volume of 0.7 L (9% of total OTUs), *Ignavibacteriae* in coarse sand biofilm with bed volume of 0.7 L (19.3% of total OTUs), *Planctomycetes* and *Armatimonadetes* in

sintered glass rings biofilm with a bed volume of 0.1 L (10.2 and 7.2% of total OTUs), and *Cyanobacteria* in coarse sand biofilm with bed volume of 0.1 L (27.3% of total OTUs). The quantification of As-related functional genes revealed the high abundance of genes involved in As transformations in biofilm samples. 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 $x10^8$ ± 8.9 x 10^7 gene copies/gWW and 1.7 x 10^{10} ± 3.6 x 10^9 gene copies/gWW. The genes for the As(III) membrane efflux pump (*arsB*) showed lower values on average around 2.2 x 10^6 ± 3.9 x 10^5 gene copies/g. The gene *aioA*, involved in As(III) oxidation was reported in all biofilm samples with values between 5.7 x 10^5 ± 2.3 x 10^5 and 1.8 x 10^6 ± 3.0 x 10^5 gene copies/g in sintered glass rings biofilms, and between 1.8 x 10^6 ± 1.2 x 10^5 gene copies/g and 6.0 x 10^6 ± 5.5. x 10^4 gene copies/g in coarse sand biofilters. Also gene involved in respiratory As(V) reduction (*arrA*) was found with values overall higher in sintered glass rings (on average 1.7 x 10^6 ± 4.1 x 10^4 gene copies/g) than in coarse sand biofilms (on average 1.2 x 10^6 ± 3.6 x 10^5 gene copies/g).

In conclusion, the study has described the structure and composition of microbiomes exposed to natural As contamination in aquatic environments, highlighting the occurrence of novel thermophiles able to tolerate high As concentrations. The main metabolic traits and potentialities of microorganisms involved in As transformation were also elucidated. Treatability studies highlighted the high potentialities of microbially-mediated As(III)-oxidation processes in biofilters with low-cost and easily available filling materials under experimental conditions very close to those used in household-scale treatment system and evaluated the impact of inflow water quality and hydraulic conditions to efficiently scale-up the process for field application.

1. Introduction

Elevated arsenic (As) concentrations represent a major water quality and health problem for millions of people worldwide (Huang, 2014). Arsenic can be found in aquatic environments primarily due to the natural interactions between groundwater and aquifer sediments, though sometimes As contamination can be related to anthropogenic activities (Herath et al., 2016; Oremland and Stolz, 2005). Since many groundwaters exceed the law limit concentration of 10 μg/L indicated by the World Health Organization (WHO), As contamination in aquatic environments has received great attention worldwide. Currently several technologies for As removal from groundwater are commonly used, such as precipitation with alum, iron, Fe/Mn, lime softening, reverse osmosis, electro dialysis, ion exchanges, adsorption on activated alumina/carbon (Mondal et al., 2006). In recent years, a range of inexpensive, iron-based, water clean-up technologies have been developed (Mohan and Pittman, 2007). Overall, most iron-based treatment methods are more effective in removing arsenic as As(V) rather than as the more toxic As(III) species, therefore an oxidation step as a pre-treatment may improve removal process efficiency (Cundy et al., 2008). Usually, this oxidation procedure is realized by chlorine, ozone, pure oxygen with half-lives of As(III) ranging from seconds to hours. These reagents are effective in oxidizing arsenite, but they may cause secondary problems due to the presence of residuals or by-products formation, inducing also a significant increase in operational costs (Katsoyiannis et al., 2004). Recently, the knowledge of microbially-mediated As-transformation processes has stimulated the interest in using biological removal technologies. In particular, the possible use of As(III)-oxidizing bacteria has been proposed as alternative pre-oxidation step overcoming drawbacks of chemical oxidation. The main added value is related to the lack of reagent addition and toxic by-products production. Coupling this biological process to arsenic removal by commonly used iron adsorbent materials may enhance

arsenic removal efficiency, extend treatment duration and trap As on adsorbent media in a more stable species. Due to the relevance of this topic, the ecology of arsenic impacted environments has been widely studied to better understanding the processes governing As mobilization into aquifers. Many autotrophic and heterotrophic As-resistant and As-metabolizing bacteria were isolated in pure culture and their main metabolic properties were described (Yamamura and Amachi, 2014). Despite the high potentialities of biological As-transformation processes, little is known about the presence and distribution of As(III)-oxidizing microbial communities in natural environments.

Information regarding As(III)-oxidation processes are scatterly reported in literature, and scanty experimental conditions for full-scale application of the process were tested so far. The elucidation of the identity and metabolic potentialities of As-related microorganisms in contaminated environments may sustain the exploitation of biological processes in remediation applications. Several additional aspects (such as, for example, the use of experimental conditions mimicking real situations) deserve future investigation for the evaluation of efficiency and suitability of microbial As-transformations in As bioremediation.

Since the paucity of information on biological As remediation processes, this PhD thesis is aimed to have more information about either the distribution of As-related biological processes in contaminated environments and the use of biological As-treatment processes to promote a reliable, cost effective and sustainable As removal from water.

In particular, this study provides the detailed description of structural and metabolic traits of microbiomes exposed to natural As contamination in freshwaters and associated with biofilm by coupling cultivation-dependent (Most Probable Number technique) and independent (flow cytometry, CARD-FISH, high throughput sequencing, qPCR) approaches. Furthermore, the main parameters affecting the overall performance of microbial As(III)-oxidation have been

tightly evaluated in column systems (biofilters) by testing different filling materials (i.e., sintered glass rings and coarse sand), operational parameters (e.g., flow rates, use of natural groundwater amended with As(III), effect of initial As(III) concentration, biofilm maturation, column volume). The As(III)-oxidizing biofilms in the bioreactors were carefully analysed by means of advanced microbial community characterization approaches.

2. State of the art

The following chapter has been modified and adapted from the original publication "Crognale, S., Amalfitano, S., Casentini, B., Fazi, S., Petruccioli, M., Rossetti, S. 2017. Arsenic-related microorganisms in groundwater: a review on distribution, metabolic activities and potential use in arsenic removal processes. *Reviews in Environmental Science and Bio/Technology*, 16(4):647-655"
Available at: https://link.springer.com/article/10.1007/s11157-017-9448-8

Arsenic is a major toxic contaminant that represents a human health concern by impairing water quality on a global scale (Nordstrom, 2002; Smedley and Kinniburgh, 2002). The inorganic arsenic species are predominant in aqueous environments, and their variation dynamics rely mainly on pH and redox potential. Under oxidizing conditions at pH less than 6.9, dihydrogen arsenate ($H_2AsO_4^-$) is the dominant species, whereas hydrogen arsenate ($HAsO_4^{2-}$) predominates at higher pH. Under reducing conditions at pH less than 9.2, the uncharged arsenite (H_3AsO_3) is the dominant species (Smedley and Kinniburgh, 2002).

Although both arsenate and arsenite are toxic, As(III) is 25-60 times more toxic than As(V) and several hundred times more toxic than most of the methylated arsenicals (Korte and Fernando, 1991). The As(V) toxicity is due to similar structure and properties with phosphate (Dixon, 1997). When As(V) replaces inorganic phosphate in the glycolytic pathway, the reaction proceeds forming unstable esters of arsenic and less ATP molecules are generated (Hughes, 2002). The toxicity of As(III) is related to the bonds of arsenite to sulfhydryl groups of amino acids that may inhibit the pyruvate dehydrogenase and the alpha-ketoglutarate dehydrogenase which plays an important role in biochemical reactions (Hughes, 2002).

Unless related to a pollution source point and anthropogenic activities, arsenic contamination occurs naturally within the subsurface aquifers (Oremland and Stolz, 2005). Biogeochemical processes in geothermal systems and water–rock interactions lead to arsenic mobilization into groundwater, either in reducing or oxidizing environments, under specific geochemical and

stratigraphical conditions (Smedley and Kinniburgh, 2002; Webster and Nordstrom, 2003). The induced mobilization from parent rocks, reductive dissolution, sulfide oxidation and alkaline desorption are the most common natural processes of arsenic release into groundwater (Ravenscroft et al., 2009). Natural arsenic contamination can be conveyed to other waterbodies owing to the hydrological interactions between groundwater and other aquatic environments. Therefore, the analysis of arsenic-related chemical and microbiological processes is of pivotal interest to understand the origin and fate of the contamination.

The arsenic contamination in groundwater has been widely studied from the geochemical point of view but less is known about the microbiology of arsenic in such environments. So far the presence of a variety of arsenic-resistant and transforming bacterial species has been highlighted in groundwater (Bahar et al., 2016; Corsini et al., 2014; Crognale et al., 2017b; Das et al., 2017; Davolos and Pietrangeli, 2013, 2011; Fazi et al., 2016b; Li et al., 2015; Liao et al., 2011; Paul et al., 2015b, 2015a; Sarkar et al., 2013; Wang et al., 2016). However, the biodiversity and metabolic potentialities of the microbial communities of such arsenic-rich aquatic environments are still poorly understood.

In recent years, bacteria associated with plants were also shown to play an important role in arsenic mobilization. As recently reviewed by Ma et al. (2016), the interaction between plants and microbes, especially rhizosphere microorganisms and endophytic bacteria, may enhance phytoremediation of arsenic-contaminated soil and shallow groundwater. Several microorganisms cause beneficial effects on their host plants by alleviating arsenic toxicity through their own metal resistance system and by facilitating plant growth (Ma *et al*., 2016).

Since the arsenic-related microbial activities have the potential to be exploited for biotechnological applications, several studies evaluated the effectiveness of biological treatments as alternative to physical-chemical treatment methods commonly applied for arsenic

removal from groundwater. However, most of the studies were performed at laboratory-scale under well-defined experimental conditions and the biological treatments are still lacking the robustness related to the scale-up of operation.

This review summarizes the current knowledge about arsenic contamination in groundwater, specifically highlighting the microbiological aspects including structural composition of microbial populations, metabolic potentialities and the possible use in arsenic-removal processes as an alternative to the established chemical treatments.

2.1 Arsenic-related microbial metabolic traits

In different arsenic-rich aquatic environments, many microorganisms have developed mechanisms to tolerate high arsenic concentration and/or utilize it for respiratory metabolism (Huang, 2014) (Fig. 1).

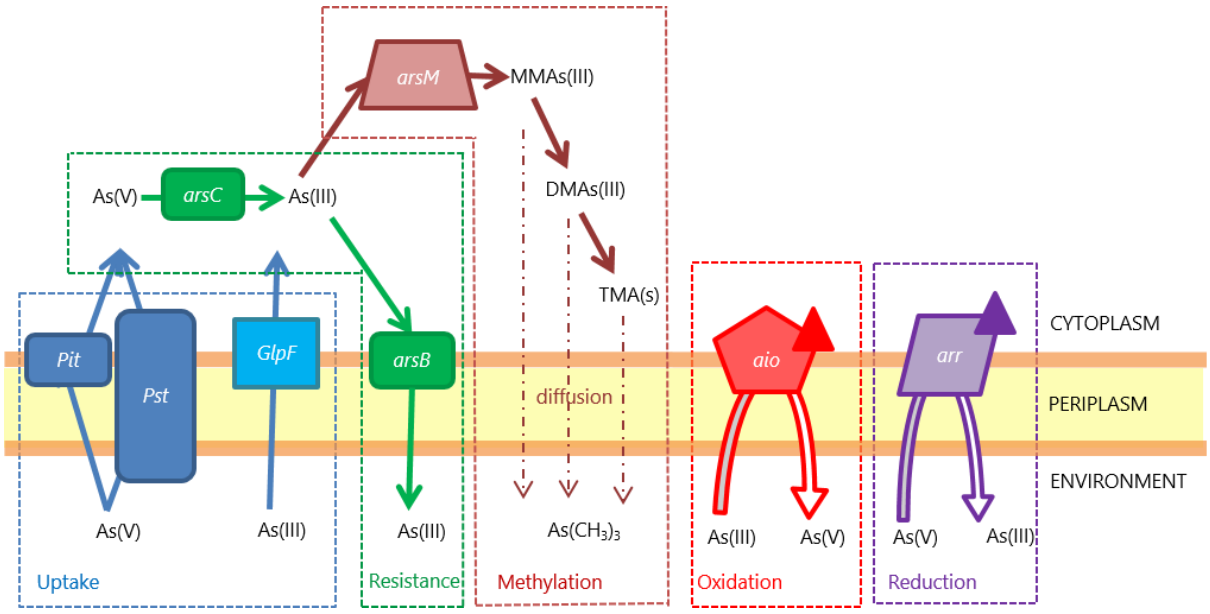

Figure 1. Overview of microbial processes involved in arsenic resistance and metabolism in the environment. Arsenic is taken up through phosphate transporters (arsenate) or aquaglyceroporins (arsenite). Inside the cell, As(V) is reduced to As(III) by *arsC*, which may be then extruded out of the cell by *arsB*. As an alternative, As(III) can be methylated by *arsM* forming the volatile intermediates monomethyl arsenite (MMAs(III)), dimethyl arsenite (DMAs(III)) and trimethyl arsine (TMAs). Arsenite and arsenate outside the cytoplasm can be oxidize and reduce by *aioA* and *arrA*, respectively.

In particular, they can mediate arsenic reduction, oxidation, methylation and demethylation reactions (Oremland and Stolz, 2003). Over the last years, descriptive and comparative genomics have helped to elucidate the genetic bases and the regulatory mechanisms involved in both arsenic resistance and metabolism (Appendix 1) (Andres and Bertin, 2016; Kumari and Jagadevan, 2016).

2.1.1 Arsenic uptake

It is primary to emphasize that no specific arsenic uptake system exists in prokaryotic cells. As(III) and As(V) enter into cells through transporter proteins due to their structural similarity to the substrates of the specific transporters (Kruger et al., 2013). As(V) enters bacterial cells through unspecific and specific phosphate transporters such as Pit (Phosphate inorganic transport) and Pst (Phosphate specific transport) respectively (Willsky & Malamy 1980; Rosen and Liu, 2009) (Fig. 1). The Pit system is a fully constitutive transmembrane protein, which catalyzes an exchange of intracellular phosphate for extracellular phosphate or arsenate (Kumari and Jagadevan, 2016). Evidences of As(V) uptake through phosphate transport systems resembling those used by prokaryotes were also found in Eukaryotes and Archaea (Bini, 2010; Dick et al., 2014).

The Pst system is an ATP-binding cassette transporter protein (ABC transporters) responsible for influx of ions such as phosphate and arsenate (Kumari and Jagadevan, 2016). Microbial communities that are constantly exposed to high amounts of arsenate express only Pst in order to regulate As(V) uptake (Bertin et al., 2011).

As(III) enters into bacterial cells through aquaglyceroporins like the glycerol facilitator GlpF that is primarily involved in transport of organic acids and urea. GlpF was shown to facilitate the transport of As(III) and Sb(III) in *E. coli* (Meng et al., 2004). GlpF homologues have been

identified in *Sinorhizobium meliloti* and *Pseudomonas putida* and are likely involved in As(III) transport across the cell membrane in these species (Páez-Espino *et al.*, 2009; Rosen and Liu, 2009). However, there are also examples of microorganisms isolated from highly arsenic-contaminated sites, such as *Thiomonas sp.* and *Herminiimonas arsenicoxydans*, that though lacking of GlpF homologues in their genomes are able to oxidize As(III) to As(V). The latter evidence suggests that As(III) uptake may occur via an unknown component (Arsène-Ploetze et al., 2010; Muller et al., 2007). Therefore, it is likely that other unidentified mechanisms regulate arsenic uptake in prokaryotes, as reported for hexose permeases in eukaryotes (Liu et al., 2004a).

As discussed by Zhu et al. (2014), the cellular conversion of As(V) into the more toxic As(III) could be related to the strategies adopted by microorganisms in the absence of oxygen in the Earth's atmosphere and with the availability of a large amount of trivalent inorganic As(III) species supplied by volcanism. These stringent environmental conditions forced the first microorganisms to develop mechanisms to face arsenic toxicity. Once the atmosphere became oxidizing, the majority of arsenite would have oxidized to arsenate and this strongly impacted on microorganisms able only to detoxify As(III). It is probable that any original As(III) elimination system was retained in bacteria and simply adapted to the new environmental conditions. Therefore, the simplest solution was to reduce the intracellular As(V) to As(III) for which the cells already had mechanisms for cytosolic removal. The involvement of arsenic compounds in important life processes on Earth represents an intriguing topic also for the implications it may have on the full comprehension of arsenic biotransformation in the environment (Zhu *et al*., 2014).

2.1.2 Arsenite oxidation

Microbial As(III) oxidation has been described as either a detoxification mechanism (Muller et al., 2003) or an energy source for chemolithoauthotrophic microbes (Garcia-Dominguez et al., 2008; Hoeft et al., 2007; Santini et al., 2000). The process is performed by various groups of bacteria and archaea which include both heterotrophic As(III) oxidizers and chemolithoautotrophic As(III) oxidizers (Oremland and Stolz, 2003). The heterotrophic oxidation of As(III) (equation 1) is considered primarily as a detoxification reaction that converts As(III) encountered on the cell's outer membrane into the less toxic form, As(V), without obtaining energy from the exergonic reaction (Santini et al., 2000).

$2H_3AsO_3 + O_2 \rightarrow HAsO_4^{2-} + H_2AsO_4^- + 3H^+$ (ΔG^0 = -256 kJ/Rx) (1)

So far, only a small number of bacteria (i.e., *Pseudomonas arsenitoxidans*; strain NT-26) have been described for the capability to use the energy gained from this reaction for cell growth (equation 2)

$H_3AsO_3 + ½\ O_2 \rightarrow H_2AsO_4^- + H^+$ (ΔG^0 = -113.34 kJ/mol) (2)

These microorganisms were found to grow chemolithoautotrophically with arsenite as electron donor, oxygen as electron acceptor and CO_2 as the carbon source for fixation (Battaglia-Brunet et al., 2002; Santini et al., 2000).

Within heterotrophic As(III) oxidizers, some facultative anaerobic bacteria capable of either aerobic As(III) oxidation or anaerobic As(V) reduction were reported (Gihring and Banfield, 2001; Handley et al., 2009).

Bacteria can oxidize As(III) to As(V) through the enzyme arsenite-oxidase. This enzyme, first purified and characterized from *Alcaligenes faecalis* (Anderson et al., 1992) (Fig. 1), 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 enzyme is involved either in arsenic detoxification by heterotrophic bacteria (Muller et al., 2003) or in energy generation by chemolithoautotrophic bacteria (Oremland et al., 2002; Santini et al., 2000). The genes encoding these two subunits were first identified and sequenced in the heterotrophic bacterium *H. arsenicoxydans* strain ULPAs1 (Muller et al., 2003).

Despite various denominations were used to indicate the genes involved in arsenite oxidation (e.g., *aoxB-aoxA*; *aroA-aroB*; *asoA-asoB*), the nomenclature was recently unified and the two genes encoding for large and small subunits of the arsenite oxidase were named as *aioA* and *aioB*, respectively (Lett et al., 2012). The *aioA* gene is similar to the molybdenum-containing subunits in the DMSO reductase family and distantly related to the catalytic subunit of respiratory As(V) reductase (*arrA* gene) (Oremland and Stolz, 2003; Silver and Phung, 2005; Stolz et al., 2010). *aioA* and *aioB* genes are found within a single operon together, with *aoxR* and *aoxS* encoding for a putative transcriptional regulator and a putative sensor histidine kinase, respectively (Kashyap et al., 2006). Koechler et al. (2010) demonstrated that arsenite oxidation relied exclusively on the AoxRS two-component signal transduction system in the genome of *H. arsenicoxydans*. In addition, to AoxRS system, a quorum-sensing mechanism was identified to contribute to As(III) oxidation in *Agrobacterium tumefaciens* (Sardiwal et al., 2010). Homologs of genes encoding for *aioA* were found in phylogenetically diverse strains including members of A*lpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Aquificae*, *Deinococcus-Thermus*, *Chlorobi*, *Chloroflexi*, *Nitrospira*, and *Crenarchaeota* (Yamamura and Amachi, 2014). *aioA*-like genes were amplified in a variety of arsenic-rich environments including mine, arsenical pesticide- 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).

Rarely, As(III) oxidation is combined with nitrate respiration or integrated into electron transport chain of anoxygenic photosynthesis (Zargar et al., 2012). This anaerobic pathway is catalyzed by the arsenite oxidase ArxAB discovered in *Alkalilimnicola ehrlichii* strain MLHE-1 (Hoeft et al., 2007; Zargar et al., 2010). ArxA is a novel type of As(III) oxidase forming a distinct phylogenetic clade within the dimethyl sulfoxide (DMSO) reductase family (Yamamura and Amachi, 2014) and seems to have more similarity to the dissimilatory reductase enzyme (Arr) than to arsenite oxidase enzyme (Aio) (Richey et al., 2009). The presence of arx homologs was reported in uncultured candidate division OP1 (Takami et al., 2012), reconstructed complete genome of the dominant organism (RBG-1) from deep sediment of the Colorado River, CO, USA (Castelle et al., 2013), As(III) oxidizers *Ectothiorhodospira* sp. PHS-1 and *Halorhodospira halophila* SL1 (Kulp et al., 2008; Zargar et al., 2012).

2.1.3 Arsenate reduction

2.1.3.1 As(V) reduction as a detoxification system

Arsenic resistant microorganisms are able to cope with high arsenic concentrations (Nriagu et al., 2007). As(V) is reduced to As(III) within the cell membrane by a cytoplasmic As(V) reductase (ArsC) through a detoxification process (Páez-Espino et al., 2009) found widely in microbes (Silver and Phung, 2005) (Fig. 1). Arsenic resistance in prokaryotes is conferred by the ArsRBC operon (Silver and Phung, 2005) whereas *arsR* and *arsC* genes encode for the regulator and cytoplasmic As(V) reductase respectively (Kruger et al., 2013). After As(V) reduction, As(III) is finally excreted out of the cells via a membrane efflux pump encoded by *arsB* gene (Rosen, 2002). This gene operates as a uniporter using the membrane potential to extrude arsenite and antimonite oxyanions (Meng et al., 2004). Supplementary genes may also be associated with ars operon; *arsA* codes for an ATPase which binds to *arsB* and makes the

As(III) efflux more efficient whereas *arsD* encodes for a protein which acts as an As(III) chaperone (Kruger et al., 2013; Páez-Espino et al., 2009; Silver and Phung, 2005). This enlarged ArsRDABC operon has been found in fewer bacterial genomes than *arsRBC* ones (Bhattacharjee and Rosen, 2007). The presence of at least one of *arsH*, *arsN*, *arsP*, *arsTX*, *arsO* and *glo* genes was recorded in some microorganisms (e.g., *Campylobacter jejuni*, *Microbacterium* spp.). These genes seem to be involved in arsenic resistance with accessory functions such as the encoding for a putative membrane permease, a thioredoxine system and an acetyltransferase-like protein (Kruger et al., 2013; Li et al., 2014; Páez-Espino et al., 2009). To date, the role of these genes in arsenic resistance has been not yet completely elucidated, since other unknown resistance processes might be involved (Kruger et al., 2013).

Less is known about the second family of As(III) carriers, acr3pA (also called arr3p), that contains *acr3* gene which has the same function of *arsB*, a component of the more common detoxifying operon (Achour et al., 2007). Although members of the family acr3pA were found in bacteria, archaea and fungi (Rosen, 2002; Wysocky et al., 2003), they were functionally characterized only in few species such as *Bacillus subtilis*, *Synechocystis* sp., *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* (Lopez-Maury et al., 2003; Ordonez et al., 2005; Sato and Kobayashi, 1998; Wysocki et al., 1997). It is worth noting that *arsB* is restricted to prokaryotes, whereas acr3 homologs are present in eukaryotes (Rosen, 2002).

2.1.3.2 Dissimilatory As(V) reduction

Many microorganisms are able to reduce arsenate by using As(V) as a terminal electron acceptor for a heterotrophic respiratory chain (Malasarn et al., 2004; Oremland et al., 2002; Oremland and Stolz, 2003; Stolz and Oremland, 1999). Dissimilatory arsenate respiring prokaryotes can use a variety of electron donors ranging from inorganic compounds like H_2

(Newman et al., 1997), over small organic acids (Niggemyer et al., 2001), sugars (Macy et al., 1993) and complex aromatic substrates like benzoate and toluene (Liu *et al.*, 2004b). So far, most known dissimilatory arsenate respiring prokaryotes can also use other terminal electron acceptors such as nitrate, various sulfur compounds, selenate, Fe(III), or fumarate (Laverman et al., 1995; Newman *et al.*, 1997; Stolz and Oremland, 1999). One exception is strain MLMS-1, an anaerobic deltaproteobacterium isolated from a soda lake. This strain is the first reported obligate arsenate-respiring chemoautotroph growing by coupling arsenate reduction with the oxidation of sulfide to sulfate (Planer-Friedrich et al., 2015).

Dissimilatory As(V) reduction takes place due to a membranous respiratory arsenate reductase encoded in the Arr operon which include *arrA* and *arrB* genes (Macy et al., 2000) (Fig. 1). ArrAB is a member of the DMSO reductase family as shown in *Chrysiogenes arsenatis* (Krafft and Macy 1998), *Bacillus selenitireducens* (Afkar et al., 2003), and *Shewanella* strain ANA-3 (Malasarn et al., 2008). Genes related to As(V) respiration were identified in the genomes of a large proportion of bacteria such as *Beta-*, *Gamma-*, *Delta-*, *Epsilonproteobacteria*, *Firmicutes*, *Chrysiogenetes* and *Deferribacteres* (Van Lis et al., 2013).

2.1.4 Arsenic methylation and demethylation

Arsenic methylation and demethylation are retained as detoxification processes since conferring higher arsenic tolerance (Yoshinaga and Rosen, 2014). Arsenic biomethylation is a multistep process that alternates reduction and methylation, producing methylated arsenicals such as methylarsenate (MMA(V)), dimethylarsenate (DMA(V)), and trimethylarsine oxide (TMAO(V)) (Bentley and Chasteen, 2002; Challenger 1945).

These processes are mediated by the products of the *arsM* and *arsI* genes, which encode an arsenite methyltransferase and a C·As lyase, respectively. *arsM* is responsible for the three

successive oxidative methylation and reduction steps starting from As(III) (Rahman and Hassler, 2014) (Fig. 1); *ArsI* is responsible for methylarsonous acid demethylation and organo-arsenical degradation (Yoshinaga and Rosen, 2014). *arsM* and *arsI* genes were detected in a large number of bacteria and archaea (Bhattacharjee and Rosen 2007; Ye et al. 2012; Yoshinaga and Rosen, 2014).

2.2 Microbial community characteristics and arsenic-related genes distribution in arsenic-rich groundwater

Recent studies reported the presence of many arsenic-related bacteria in contaminated groundwater worldwide (Bahar et al., 2016; Corsini et al., 2014; Das et al., 2017; Davolos and Pietrangeli, 2013, 2011; Fazi et al., 2016b; Li et al., 2015; Liao et al., 2011; Paul et al., 2015a, 2015b; Sarkar et al., 2013; Wang et al., 2016). The specific PCR primers, developed to amplify bacterial arsenic-related functional genes, are reported in Appendix 2. As detailed in the following, they were mainly evaluated on pure cultures with only sporadically applications for the analysis of real arsenic-contaminated waters.

Liao et al. (2011) isolated 11 arsenic transforming bacterial strains in Taiwan from groundwater with an arsenic concentration ranging between 0.3 and 0.78 mg/L. All isolates were able to resist at high level of arsenic with minimum inhibitory concentrations ranging from 0.15 to 15 g/L. The only one As(III)-oxidizing isolate which was belonging to the genus *Bosea*, was able to totally oxidize As(III) within 12 h, when cultured in a minimal salt medium containing 1.8 g/L glucose and 20 mg/L As(III) under aerobic conditions (Liao et al., 2011). Similar ability was also observed within 50 min in a real groundwater containing 780 μg/L As(III) (Liao et al., 2011). The other isolates were able to reduce As(V) to As(III) under both aerobic and anaerobic conditions. Among the isolates, nine were distributed into *Proteobacteria*, eight belonged to

Gammaproteobacteria and only one to *Alphaproteobacteria*, while two were affiliated to *Firmicutes* (Appendix 3). Moreover, the analysis of arsenic-related genes showed the absence of *arrA* gene in the isolates and the presence of *aioA* gene only in *Bosea* sp. Eight of the others isolates presented at least one of *arsB* and *arsC* genes. The isolates closely related to *Pseudomonas rhizosphaerae* did not show any PCR amplification. Others cultivable arsenic-resistant bacteria were found also in groundwater with an arsenic concentration of 5.6 μg/L (Davolos and Pietrangeli, 2011). In this study, the presence of the *arsB* gene was found in many of these isolates suggesting the intracellular reduction of As(V) and the efflux of As(III) from the cells. All of the bacterial strains used in this study were affiliated to the genera *Comamonas*, *Delftia*, *Acidovorax* and *Variovorax*. Two moderately oxidizing groundwater systems located in former oil refineries with an arsenic concentration between 58.3 and 61 μg/L were recently analysed (Davolos and Pietrangeli, 2013). In these samples, only culturable arsenic-resistant *Gammaproteobacteria* were found, and arsB genes were amplified in all isolates.

Recently, sixty-four arsenic resistant bacteria were isolated from arsenic contaminated groundwater in West Bengal (India) (Sarkar et al., 2013). These strains, mainly affiliated to *Proteobacteria* (*Alpha-*, *Beta-*, *Gammaproteobacteria*) and *Actinobacteria*, showed the ability to resist to different As(III) and As(V) concentrations in the range of 0.04-3 g/L and 3.7-37.4 g/L, respectively. Corsini et al. (2014) examined the arsenic transformation abilities of bacterial strains obtained by Italian groundwater with an arsenic concentration ranging between 0.7 and 171 μg/L. Among twenty-two bacterial isolates belonged to the genera *Achromobacter*, *Pseudomonas* and *Rhodococcus*, twenty of them were able to totally reduce 75 mg/L As(V) under aerobic conditions in 48 h and only two were able to oxidize 75 mg/L As(III) in 32-48h (Corsini et al., 2014). The presence of *aioA* gene was detected only in *Achromobacter* sp., while almost all arsenic-reducing isolates contains *arsC* or *arsB* genes. *Aliihoeflea* sp. was the most

efficient strain obtained in this study isolated from an arsenic contaminated groundwater in an aerobic biofilter able to oxidize 200 µg/L As(III) in 8h at 28°C and in 24h at 15°C. Recently, Paul et al. (2015a) isolated more than 170 strains from different groundwater collected in Barasat and Chakdaha of West Bengal, India (arsenic concentration range 0.01 - 1.37 mg/L). These strains belonged to *Proteobacteria*, *Actinobacteria* and *Firmicutes*. Under anaerobic conditions 30% and 50% of the isolates were able to grow with an high level of As(V) and As(III) (≥7.5 g/L and ≥0.75 g/L respectively). Under aerobic conditions, around 70% of strains grew at the same arsenic concentrations. Particularly in this contaminated environment, 60% showed an As(V) reduction ability, whereas As(III) oxidase activity was found to be less important. More than 50% of the isolates was endowed with cytosolic arsenate reductase gene (*arsC*) and As(III) transporter gene (*arsB*), while only 10% presented As(III) oxidase small subunit (*aioB*).

Few field studies were performed to describe the microbial community structure in groundwater ecosystems. Recently, Paul et al. (2015b) observed that *Betaproteobacteria* and *Gammaproteobacteria*, followed by *Bacteroidetes* and *Alphaproteobacteria*, mainly occurred in arsenic contaminated groundwater in India. At genus level, *Pseudomonas*, *Rheinheimera*, *Polaromonas*, *Methyloversatilis*, *Methylotenera*, *Hydrogenophaga* and *Flavobacterium* were mainly found, while members of *Aquincola*, *Brevundimonas*, *Parvibaculum*, uncultured *Actinobacteria* and *Aquabacterium* represented the less-frequent and less-abundant populations.

Li et al. (2015) showed large differences in microbial communities composition between wells water with low (<10 µg/L) and high (66-1088 µg/L) arsenic concentration in China. *Acinetobacter*, *Psychrobacter* and *Aliishewanella* were the predominant groups in high arsenic groundwater. The microbial community in low arsenic wells water showed a high diversity,

with the predominance of genera *Natronobacillus*, unclassified OP3, *Exiguobacterium*, *Sporosarcina*, and *Pseudomonas*.

Fazi et al. (2016) underlined that *Proteobacteria* were dominant in arsenic-rich groundwater. *Betaproteobacteria* and *Deltaproteobacteria* were the main groups retrieved, followed by *Chloroflexi*, *Firmicutes*, *Flavobacteria*, *Planctomycetes*, candidate division TM7 and *Actinobacteria* representing less than 5% of bacterial community.

The ability of the native microbial communities to oxidize As(III) was also analyzed in microcosms. Bacterial cells, after being collected on polycarbonate membranes, were re-suspended in a mineral base medium with an As(III) concentration of 100mg/L. After 28 days of incubation, groundwater samples showed different oxidation rate: only one was able to oxidize 95% of total As(III) and the others exhibited an oxidation rate between 5 and 36%. An arsenite-oxidizing bacterial strain was isolated from the microbial community that showed the higher arsenite oxidation. This isolate, affiliated to *Acinetobacter calcoaceticus*, was able to oxidize As(III) with a maximum efficiency of about 25% within 24 hour (Fazi et al., 2016b).

In line with the previous findings, the dominance of *Proteobacteria*, followed by *Firmicutes*, *Actinobacteria*, and *Nitrospirae*, was observed in arsenic groundwater aquifers from agricultural irrigation area (total arsenic concentration: 50-1000 μg/L; Wang et al. 2016). In particular, *Alishewanella*, *Psychrobacter*, *Methylotenera*, and *Crenothrix* were the predominant genera. More recently, Das et al. (2017) used a metagenomic approach to understand the structural and functional diversity present in arsenic contaminated waters in the aquifer system of the Ganges Brahmaputra delta (total arsenic concentration: 20-217 μg/L). The metagenomics analysis revealed the predominance of *Proteobacteria*, followed by *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, *Actinobacteria* and *Firmicutes*. *Nitrosomonas* was found to be the most predominant genus followed by *Pirellua*, *Verrucomicrobium*, *Methylobacterium*,

Rhodopirellula, *Burkholderia*, *Bradyrhizobium* and *Methylocystis.* Only 0.07% of proteins identified in this study were involved in arsenic metabolism, and mainly related to arsenic resistance metabolism. Only Ars complex and acr3 were identified, whereas genes related to As(III) oxidation were not detected.

The isolation of an arsenic hyper-tolerant diazotrophic bacterium was recently obtained from a polluted soil (Bahar et al., 2016). The Strain MM-17, identified as *Azospirillum* sp., was able to heterotrophically oxidize 92% of 7.5 mg/L As(III) within 8 h of incubation in real groundwater and complete oxidation was observed after 10 h. The cellular oxidation of As(III) was found to be catalyzed by the arsenite oxidase which is constitutively expressed.

Overall, the studies performed so far mainly revealed the composition of the cultivable fraction of the microbial populations and highlighted the ubiquitous presence of arsenic-resistant bacteria in groundwater. The use of cultivation-based approaches as primary strategy to describe the arsenic-related microbial processes has limited our knowledge on the actual structure and metabolic potential of the whole microbiome of arsenic-polluted groundwater. As shown in Appendix 3, the cultivable fraction of arsenic-resistant bacteria is chiefly represented by members of *Proteobacteria*, particularly *Betaproteobacteria* and *Gammaproteobacteria*. Species affiliated to *Alphaproteobacteria*, *Firmicutes* and *Actinobacteria* were found at a lower extent. The preponderance of arsenic-resistant bacteria belonging to *Proteobacteria* in groundwater is not unexpected since the majority of the bacteria isolated from other arsenic-polluted environments (i.e. gold and silver mines, soils) generally belong to the same phylum (Achour et al., 2007; Battaglia-Brunet et al., 2006; Chang et al., 2008; Santini et al., 2000; Weeger et al., 1999).

As(III)-oxidation capability was reported in almost half of the bacterial strains tested so far, while As(V)-reduction was shown in 73% of them. Unfortunately, the approaches utilized for

the screening of arsenic-transformation abilities in microorganisms were different and often not properly described. This lack of information strongly affects the full understanding of the real metabolic potential of the isolates. Moreover, 72% of the analyzed bacteria strains contained *arsB* or *arsC* genes, while only 18% and 15% carried *aioA* and *arrA* genes respectively (Appendix 3). The limited coverage observed so far with the available primer sets underlines the need to further explore the biodiversity of the functional genes which catalyze arsenic-transformation in the environment. The increasing application of high-throughput sequencing technologies and genomic approaches (e.g. genome sequencing, transcriptome, proteome and metabolome profiling) as well as further efforts in isolating microorganisms from arsenic contaminated environments will open up new frontiers in the field. The contribution of genomics may be of prime importance to a thorough understanding of the metabolisms and the interactions of microorganisms with arsenic at the level of both single species and microbial communities. Such approaches should pave the way for the utilization of microorganisms to design new, efficient and environmentally sound remediation strategies (Plewniak et al., 2018).

2.3 Biological processes for arsenic removal from contaminated groundwater

Typical technologies for arsenic removal mainly include chemical/physical treatments such as coagulation/filtration, ion exchange, enhanced lime softening, adsorption and reverse osmosis (Ng et al., 2004; Nicomel et al., 2016). In particular, adsorption technology becomes the most promising, owing to low management required, availability of economically sounding adsorbents (e.g., granular ferrous hydroxide (GFH), activated alumina, iron-coated sand, granular TiO_2 and MnO_2) and the treatment of a wide range of arsenic concentration (adsorption capacity up to ~ 50 mg As g^{-1} adsorbent material) (Mohan and Pittman, 2007; Nicomel et al., 2016). In most cases, since adsorption technologies are more efficient for As(V), a pre-

oxidation step to transform As(III) into As(V) is performed by using strong chemical oxidizing agents (Simeonova et al., 2005; Katsoyiannis et al., 2002).

Biological methods can be currently used for the remediation of arsenic contaminated environments, due to their environmental compatibility and possible cost-effectiveness. A number of potential applications of bacterial driven processes are available to remediate arsenic-contaminated groundwater (Kruger et al., 2013; Wang and Zhao, 2009). In particular, bioprecipitation, biosynthesis of adsorbent materials, biosorption and biovolatilization are the most interestingly reported (Table 1).

Table 1. Main biotechnological approaches for arsenic removal from waters

Technology	**Reference**
Precursor step	
As(III) microbial oxidation coupled to adsorption removal technology:	
Zero-valent iron	Wan et al. 2010
Granular activated carbon	Mondal et al.2008
Biogenic carbon oxide	Katsoyiannis and Zouboulis 2004
Manganese oxide	Katsoyiannis et al. 2004
Activated alumina and charcoal	Pal and Paknikar 2012
Bioelectrochemical As(III) microbial oxidation	Pous et al., 2015
Processes	
Bio-precipitation	Omoregie et al., 2013
Bio-synthesis of adsorbent materials	Mandal et al., 2006; Herlekar et al. 2014, Casentini et al. 2015
Bio-sorption	Mohan et al., 2007
Bio-volatilization	Jakob et al., 2010; Liu et al., 2011
Bio-filtration	Mondal et al. 2006
Fe–Mn oxidation coupled to As microbial removal	Chekalla et al. 1985; Katsoyiannis and Zouboulis 2004; Hassan et al. 2009
As and Fe–As sulfide precipitation driven by sulfate reducers	Newman et al. 1997

Arsenic bioprecipitation due to biogenic iron oxides and sulfides formation in microcosm studies highlighted that metal-reducing bacteria are responsible for the reductive transformations of As-, Fe-, Mn-bearing minerals and arsenic sorption onto freshly formed

hydrous ferric oxide (HFO). These processes were proved effective in lowering arsenic levels in groundwater (Omoregie et al., 2013). Katsoyiannis and Zouboulis (2004) reported that the biotic oxidation of iron by *Gallionella ferruginea* and *Leptothrix ochracea* contributed to increase arsenic removal (up to 95%) following the arsenic adsorption onto iron oxides. The ability of microorganisms to produce nanoparticles is also becoming relevant in the treatment of arsenic-rich drinking water (Mandal et al., 2006). Recently, Casentini *et al.*, (2015) reported the capability of *Klebsiella oxytoca* BAS-10 to synthetize FeOOH nanoparticles dispersed into extracellular polymeric substance hydrogel (Fe-EPS). This FeEPS, in 1:10 gel:water ratio, showed significant adsorption capacities for As(V) and As(III), with maximum values of 31.8 and 27.3 mg_{As}/g_{Fe}, respectively. Biosorption is an additional biotic process capable of removing heavy metals and other elements from diluted aqueous solutions (Mohan and Pittman, 2007). Biosorption processes were tested on industrial effluents, but a very limited number of studies report its implementation to treat drinking waters (Hasan et al., 2010; Prasad et al., 2013). Some studies reported that arsenic removal occurred mainly through direct absorption by microbial biomass and adsorption/coprecipitation with biogenic Fe or Mn hydroxides (Wang and Zhao, 2009; Hohmann et al., 2010; Hohmann et al., 2011).

Biovolatilization is an additional bioprocess able to remove arsenic from surface waters and soils (Jakob et al., 2010; S. Liu *et al.*, 2011), thus showing a very limited impact on aquifer contamination (Lloyd, 2010). Volatile arsenic is formed through a combination of inorganic species reduction and methylation reactions (Rahman et al., 2014). This process has been widely studied, but its application in arsenic remediation has not been fully exploited (Wang and Zhao, 2009). Both aerobic and anaerobic microorganisms (including bacteria and fungi) are responsible for the evolution of volatile arsenicals. However, in a previous study, the rate of arsenic volatilization induced by soil microbial communities was limited (0.0005-10% of

total arsenic content) (S. Liu et al., 2011). Yin et al. (2011) reported that three cyanobacterial species (*Microcystis sp*. PCC7806, *Nostoc sp*. PCC7120, and *Synechocystis sp*. PCC6803) were able to accumulate, methylate and volatilize As.

2.4 Microbial potential in arsenic remediation

A number of microbially-driven processes regulating the biogeochemical cycle of arsenic in aquifers are described in previous sections. In particular, biological As(III)-oxidation showed promising potential applications as a precursor step in arsenic-treatment, since most conventional iron-based treatment methods are more effective in removing As(V) rather than As(III) (Fazi et al., 2016a).

In arsenic treatment, an oxidation step to oxidize As(III) to As(V) is required to improve efficiency and it is realized by adding chemical reagents, such as potassium permanganate, chlorine, ozone, hydrogen peroxide or manganese oxide (Driehaus et al., 1995; Kim and Nriagu, 2000). These reagents are effective in oxidizing arsenite, but they may cause secondary problems arisen by the presence of residuals or from by-products formation, inducing a significant increase in operational costs (Katsoyiannis and Zouboulis, 2004). In any case, since As(III) is much more toxic than As(V), oxidative treatment of groundwater can also serve to reduce the toxicity of arsenic while facilitating its removal from the water supply (Kim and Nriagu, 2000). Furthermore, the bacterial As(III) oxidation was considered as an environment friendly alternative to traditional physical-chemical treatment methods, since it does not produce toxic by-products (Bahar et al., 2013).

Different operational conditions using either 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) were tested as feasible pre-treatments. Recently, many lab-scale

experiments were carried out achieving interesting results regarding microbial mediated As(III) oxidation. Corsini et al. (2014) studied arsenite adsorption with or without the presence of *Aliihoeflea sp*. 2WW in combination with goethite (4 g/L) in a model system with initial As(III) concentrations of 200 μg/L. Their results showed higher arsenic-removal efficiency (up to 95%) of the combined arsenite oxidizing biomass–goethite system than goethite alone (85%). Wan et al. (2010) obtained a high As(III) oxidation efficiency testing the use of *Thiomonas arsenivorans* in upflow fixed-bed reactors coupled to arsenic sequestration onto zero-valent iron-coated sand.

As(III) was totally oxidized in 15 days using an As(III) volume loading of 2.5 mg $L^{-1}h^{-1}$ in the presence of *T. arsenivorans.* The increase of the As(III) volume loadings to 10 mg $L^{-1}h^{-1}$ resulted in 80% of oxidation after 33 days (Wan et al., 2010). Battaglia-Brunet et al. (2002) tested the performance of a selected autotrophic As(III)-oxidizing population, named CASO1, in reactors using different growth conditions. Under aseptic conditions in a stirred glass reactor with a culture medium containing 100 mg/L As(III), the authors observed an As(III) oxidation rate of 12 mg $L^{-1}h^{-1}$. The oxidation was also tested in non-sterile conditions with tap water, using a fixed-bed column to simulate conditions with As(III) oxidation rate reaching 166 mg $L^{-1}h^{-1}$. Arsenite oxidizing biofilters were also realized using bacteria to perform pre-oxidation and coupled with arsenic removal treatment based on activated alumina and metallic Fe adsorbents (Ike et al., 2008; Wan et al., 2010). Microbiological As(III) oxidation can be also catalyzed by electrochemical processes with a polarized electrode serving as the sole terminal electron acceptor (Pous et al., 2015).

The use of As(III)-oxidizing bacteria in biofilms can be an excellent basis to design simple passive bio-processes (Battaglia-Brunet et al., 2005). The As(III) bio-oxidation step in water treatment can be developed using fixed-bed up-flow reactor systems inoculated with As(III)-

oxidizing bacteria and filled with pozzolana as a bacterial growth support (Battaglia-Brunet et al., 2005). According to Michel et al. (2007), the formation of a biofilm in a fixed-bed bioreactor is a physical barrier able to decrease As(III) access to sessile cells and influence the As(III) oxidation. Moreover, the presence of EPS matrix can act as a molecular sieve, sequestering cations, anions, apolar compounds and particles from the water phase (Flemming and Wingender, 2010). Interestingly, Casiot et al. (2006) reported that biofilm originating in treatments of As- and Fe-rich groundwater through biological Fe-oxidation processes increased the As(III) removal efficiency. The As(III) removal rate was around 0.33 μg L^{-1} h^{-1} and it was six times greater than in the presence of sterilized biofilm. By promoting biotic As(III) and Fe(II)/Mn(II) oxidation in a fixed-bed upflow filtration unit, simultaneous oxidation–removal processes were also tested (Hassan et al., 2009; Katsoyiannis and Zouboulis, 2004; Katsoyiannis et al., 2004). These processes were proposed as an efficient treatment technology for the oxidation and removal of dissolved Fe and Mn and for the simultaneous removal of arsenic (Katsoyiannis and Zouboulis, 2004; Katsoyiannis et al., 2004, 2007; 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 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 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), iron (0.8-1.5 mg/L) and manganese (1-1.2 mg/L) from groundwater, with As removal up to 98.2% within 180 days (Yang et al., 2014).

2.5 Remarks and challenges for future research

In conclusion, the research concerning abiotic and biotic factors influencing the arsenic behavior in groundwater is still challenging for health-related issues and bioremediation applications. The impact of arsenic-contamination on the environment was widely studied over the last decades and several studies focusing on arsenic distribution, geochemistry and biological transformations were performed. However, information specifically related to groundwater is scattered in the scientific literature and more explicative and clarifying studies are necessary to better elucidate arsenic-related microbial activities in this environment. Due to the unsatisfactory experimental conditions tested so far, the potentialities of microorganisms in arsenic removal processes in natural waters are not fully exploited as well as the diversity and distribution of functional genes controlling arsenic-transformation in such environments. Therefore, specific studies using experimental conditions mimicking real situations (e.g., long term studies performed at large scale and/or in water treatment plant) may help to evaluate the efficiency and the applicability of microbial arsenic remediation processes in combination with conventional methods. Among the different biological processes for arsenic removal from contaminated groundwater, microbiological As(III)-oxidation resulted to be the most promising application. The use of autotrophic As(III)-oxidizers may be preferred since this process is not dependent on organic carbon for their growth. Nevertheless, also the application of heterotrophic As(III)-oxidation could be exploited in bioremediation. Several aspects need to be further studied to efficiently implement oxidizing bio-filters in arsenic treatment units on a long-term basis. They may include the optimization of both biofilter geometry and hydraulic

parameters (e.g., influence of flow rate, monitoring of clogging after prolonged use). Moreover, additional aspects deserving further investigation concern the fine-tuning biofilm development (e.g. control to prevent the growth of pathogens in the filter, need of carbon supply to support fast reactions with time). Lastly, coupling of biological and chemical processes must be assessed in order to achieve a kinetic of biological oxidation compatible with chemical process for sufficient daily production of arsenic-free drinking water.

3. Aims and objectives

Arsenic contamination is a global concern for human health. Despite the high toxicity of this element, many microorganisms play a major role in As-transformation and mobilization processes. During last years, the contribution of genomics has been of prime importance to a thorough understanding of As-metabolism and the interactions of microorganisms with the contaminant at the level of both single species and microbial communities. However, still little is known about the identity and the physiological properties of microorganisms exposed to natural high levels of As in volcanic geothermal environments and only scant reports investigated the suitability of microbial As-transformation processes in the treatment of contaminated waters. The majority of the previous studies were performed with pure cultures obtained from extreme environments (such as for example acid mine drainage, mine sediments and geothermal environments) without a direct implication on the exploitation of biological processes for the treatment of water resources for human consumption. In particular, the As(III) oxidation capability has been described in several bacterial pure or enrichment cultures to date, but very little is known about the real potentialities of this process when mixed microbial communities, naturally occurring in As contaminated waters, are used.

The present PhD project is aimed to cover the gaps of knowledge in this field by investigating the As transformation processes mediated by mixed microbial communities occurring in a variety of aquatic environments including freshwaters to be treated for human consumption.

The main aim of this work is to evaluate the biotechnological potentialities of native arsenite-oxidizing bacteria for a sustainable and cost effective arsenic removal from naturally contaminated waters. In detail, the main objectives of the research activity carried out in this PhD project are:

- Characterization of arsenic-contaminated natural environments through physical-chemical properties analysis, biomolecular characterization of i) bacteria involved in As-resistance and metabolism in aquatic environments and ii) microbial communities associated with biofilm;
- Evaluation of the metabolic potentialities and biological activity of As-related microbial communities in natural environments;
- Implementation of innovative strategies for the removal of arsenic from drinking water in laboratory-scale column systems (biofilters);
- Characterization of As(III)-oxidizing microbial biofilm in biofilters.

4. Material and methods

4.1 As-rich aquatic environments: study sites and sampling

4.1.1 Freshwaters from Cimino-Vico volcanic area (Central Italy)

Water samples were collected from the Cimino-Vico volcanic area (Central Italy) (Fig. 2), a complex hydrogeological system characterized by several perched aquifers and a continuous basal aquifer flowing through volcanites (Baiocchi et al., 2013).

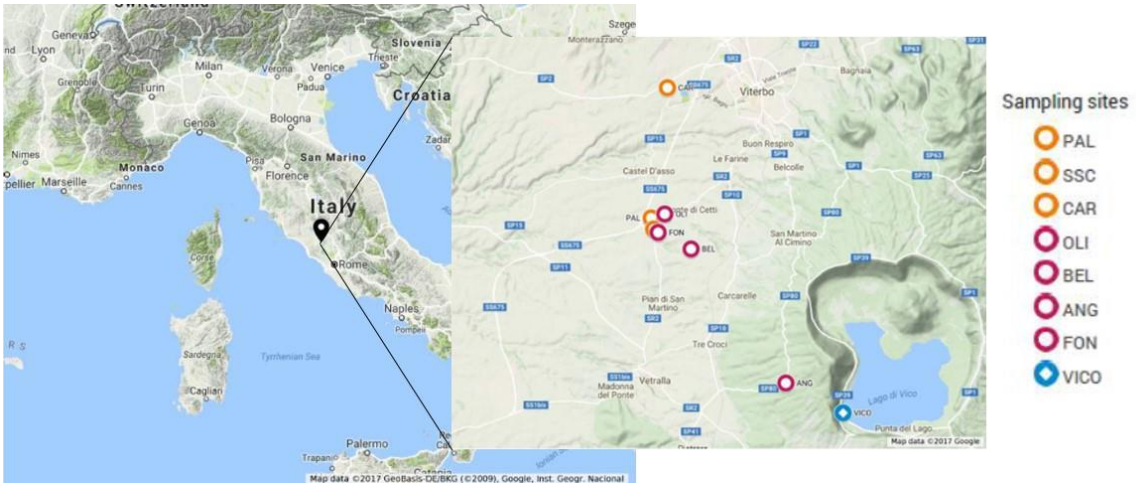

Figure 2. Study area and sampling sites.

In this volcanic region, widespread hydrothermal circulation was reported as underlined by the occurrence of geothermal fields (Casentini and Pettine, 2010; Dall'Aglio et al., 2001). The natural occurrence of arsenic is explained by the complexity of the hydrostratigraphy, the structural setting of the area and the related mixing occurring between water circulating in the basal volcanic aquifer and the fluids that rise from depth, all of which characterize the active geothermal system (Angelone et al., 2009; Armiento et al., 2015; Cinti et al., 2015). We selected eight different freshwater sources, differently influenced by the rising of As-rich thermal waters (50–64 °C) from a deep aquifer consisting of Mesozoic sedimentary rocks, locally uplifted,

fractured, and faulted (Baiocchi et al., 2013). The sampling was performed in autumn (November, 2015), and samples included (i) thermal waters: one hot spring (SSC) derived from a flowing well, 346 m deep, and two pools (CAR, PAL) collecting waters from natural hot springs; (ii) groundwaters from four wells (OLI, BEL, ANG, FON); (iii) surface waters sampled 2 m away from the west side shore of the Lake Vico (VICO), directly affected by hydrothermal upwelling. Different water aliquots were collected during the sampling for the chemical and microbiological analyses. In detail, 1 L of water was collected and kept at the same temperature of the original water sample for the DNA extraction. An aliquot of around 500 ml was collected for CARD-FISH and flow cytometry analyses, and 50 ml were used for the chemical analysis. Lastly, at each sample sites 1 ml of water in triplicate was immediately inoculated in liquid selective growth media used for the Most Probable Number analysis.

4.1.2 Biofilm from "Carletti thermal pools" (Cimino-Vico volcanic area)

The previous studied hot spring CAR was extensively analysed in a second sampling campaign, in 14th March 2016, in order to investigate microbial communities suspended in water column and associated with biofilm immersed in an As-rich geothermal environment characterized by natural temperature, pH and dissolved oxygen gradients. This site, known as "Carletti thermal pools", is a thermal area (42.422368N/12.063926E), located few km west from Viterbo (Central Italy) (Duchi et al., 1985; Pentecost, 1995; Minissale et al., 2002), artificially elevated over the ground few meters and it is canalized to form a thermal bath. This hydrothermal environment is characterized by a natural temperature and pH gradient in the presence of a constant high total As content. The thermal discharge is confined in a 3 m in diameter pool and is accompanied by a vigorously bubbling gas (CO_2-rich) phase. A relatively narrow (14 cm) channel allows the thermal water to gently flow for about 112 m to the end of the channel,

where a cooler pool is equilibrated with the environmental temperature (Di Benedetto et al., 2011). All artificial structures, channels and pools, made up by bricks, are covered by a 10-20 cm thick travertine deposit. The spring of Carletti, already studied in detail by Duchi et al. (1985) and Pentecost (1995), has a flow section of 150 cm^2 with a relatively constant water speed (7.7 cm/s) and flow rate (0.7 L/s).

Eight different sampling points were selected along the channel at a distance of approximately 14 meters from each other (Fig. 3). Around 1 L and 100 ml of water were separately collected starting from the end and going back up to the beginning of the channel for the DNA extraction and CARD-FISH/flow cytometry analyses, respectively. Around 50 ml of water was used for chemical analysis.

Figure 3. Study area and sampling sites.

Biofilm grown on travertine deposit along the channel under the water column was collected on the same day as water sampling. In detail, fourteen biofilm samples were collected at the same sampling points of water samples from the superficial (2 cm depth) and sub-superficial layer of travertine deposit (8 cm depth), with the sole exception of first (C1) and last sampling points (C8) where just superficial biofilm was collected. Core biofilm samples were obtained by drilling the travertine deposit with plastic cilinder with around 1 cm diameter in order to keep unaltered biofilm structure and stratification. These core samples were immediately stored at - 20°C and succcessively used for DNA extraction. Furthermore, around 1 g of superficial and sub-superficial biofilm was collected by using a plastic spoon, immediately fixed with a buffer solution containing formaldehyde and stored at + 4°C. These samples were used for CARD-FISH and flow cytometry analyses.

Moreover, clean microscopy slides were placed on the central point of the channel (corresponding to sampling site C4) with a Polyvinyl chloride (PVC) support and collected overtime (2-7-12 days) to monitor biofilm development, biomass increments in the earlier stages and changes in the three-dimensional structure during biofilm maturation.

4.1.3 Analytical procedure

Temperature (T), pH, electrical conductivity (EC), and dissolved oxygen (DO) were measured on-site by field probes (Hach HQ 40d). Sulfides and iron (total and Fe^{2+}) were determined by spectrophotometric methods (Casentini et al., 2016). Water samples (50 ml) were directly stored at 4°C for anion analysis by ion chromatography (Dionex DX-120); 50 ml of water were filtered in situ through 0.45 μm cellulose acetate membrane filters (Whatman), acidified with 2 % HNO_3, and stored at 4 °C for cation analysis by ICP-MS equipped with Octapole Reaction System (ORS) (Agilent 7500). Arsenic speciation 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 % in acetate buffered samples at pH 4-4.5. As_{tot} was analyzed by HG-AAS prior reduction to As(III) by 5% KI/Acid Ascorbic solution. As(V) concentration was obtained by the difference (details in Casentini et al., 2016).

4.2 Microbial As(III)-oxidation in lab-scale column systems

4.2.1 Biofilter system 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 (Fig. 4). 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). Biofilters, tested at IRSA-CNR laboratories, were fed by local groundwater with As concentration ranging from 2.5 to 4.5 μg/L. Inflow water quality, used for biofilm growth, showed features similar to As-rich groundwater in the nearby volcanic region (neutral pH, high sulfate and carbonate) and is characterized by a high oxygen content always close to saturation (>90%).

The water was let to circulate for 20 days throughout the biofilters (Fig. 4), afterwards inflowing water was continuously spiked with 100 μg As(III)/L 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.

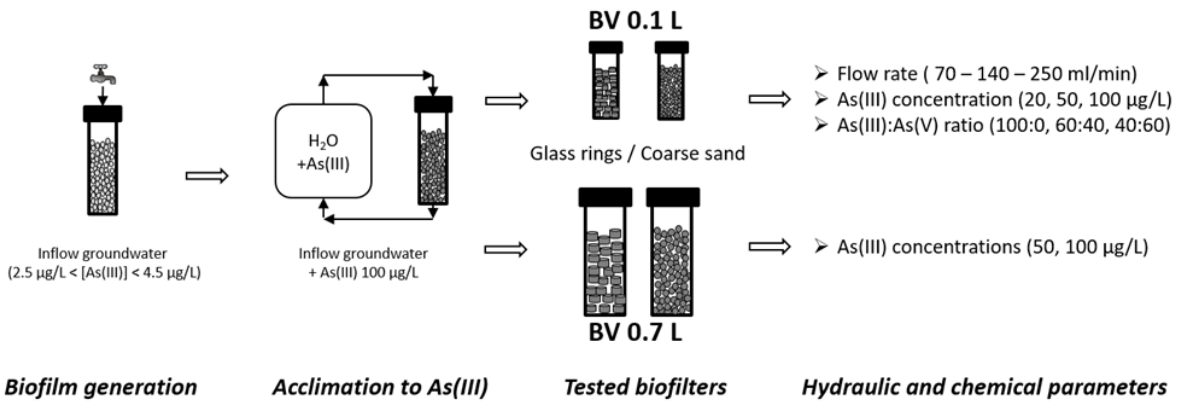

Figure 4. Schematic representation of biofilter set-up and tested experimental conditions.

Once this performance was achieved, biofilters with 0.1 L and 0.7 L BV were used in kinetic experiments under different operation conditions (see Section 4.2.2). 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.

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)/Lh) 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 $R^2 > 0.9$).

4.2.2 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) at As(III) concentration of 100 μg/L;

ii. initial As(III) concentration (20, 50, 100 μg/L);

iii. As(III):As(V) ratio (100:0; 60:40, 40:60) at a total As concentration of 100 μg/L.

Furthermore, the scalability of the process at two different initial As(III) concentrations (50 and 100 μg/L) 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 (flow rate: 660 mL/min) corresponding to the value previously used in a household pilot unit for drinking water treatment of a 400 L/day (as described in Casentini et al., 2016).

Filling materials were sampled at the end of the all experiments for microbiological analysis.

4.3 Microbiological characterization

4.3.1 Sample treatment

4.3.1.1 Water

At each sampling point, 500 ml of water were collected in a sterile plastic bottle and formaldehyde solution (FA, 1% vol/vol final concentration) was added for fixing microbial cells. All samples were kept at 4°C for a maximum of 24 h. A small aliquot of these fixed samples was subsequently used for flow cytometry analyses in order to estimate prokaryotic abundance. Further, the fixed samples (10–200 ml depending on total cell abundance) were filtered on polycarbonate membrane filters (pore size 0.2 mm, 47 mm diameter, Nuclepore) by gentle vacuum (<0.2 bar) then washed with 20 ml of Milli-Q water. The filters were stored in Petri dishes at -20°C until further processing.

About 750-1000 ml of water were filtered through polycarbonate membranes (pore size 0.2 mm, 47 mm diameter, Nuclepore) and immediately stored at -20°C until DNA extraction.

4.3.1.2 Biofilm

Core biofilm samples collected from Carletti channel and from lab-scale biofilter system were immediately stored in 50 ml Falcon tubes at -20°C for DNA extraction. Other aliquots (~1 g), immediately stored at + 4°C, were diluted (1:10, w/v) with sterilized buffer solution containing distilled water, NaCl, 0.1M $Na_4P_2O_7$, formaldehyde (2% final concentration), and Tween20 (0.5%) in 15 ml Falcon tubes and further processed using Nycodenz density gradient centrifugation according to the protocol of Amalfitano and Fazi (2008) for the detachment of microbial cells. In detail, samples were shaken for 15 min at 400 revolutions per minute (rpm) at room temperature and then sonicated in ice (20 W for 1 min; Microson XL2000 ultrasonic

liquid processor with 1.6-mm-diameter microtip probe, Misonix, NY, USA). Then, 1 ml of sonicated sample was put into a 2 ml tube and 1 ml of Nycodenz [Nycodenz; Nycomed, Oslo, Norvegia; densità 1.310 ±0.002 g/ml] was added at the bottom of the tube. After centrifugation at 14000 relative centrifugal force (rcf) for 90 minutes at 4°C, the supernatant aqueous solutions were filtered on polycarbonate membrane filters and used for CARD-FISH analysis. An aliquot of obtained aqueous solutions was used for cytometric analysis.

4.3.2 DNA extraction

DNA extraction was performed with PowerSoil® DNA Isolation Kit (MoBio – Carlsbad, CA, United States) by following the manufacturer's instructions. Briefly, the samples were added to a bead beating tube for rapid and thorough homogenization. Cell lysis occurred by mechanical and chemical methods. Total genomic DNA was captured on a silica membrane in a spin column format, then washed and eluted from the membrane. 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.

4.3.3 Prokaryotic abundance and communities composition

4.3.3.1 Flow cytometry

A small aliquot of fixed water samples was analyzed 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, to characterize microbial communities in fixed samples. 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). The forward scatter signals (FSC expressed in absolute units, AU) can be considered to be relatively proportional to cell size (Tzur et al., 2011), and related to the size variations of LNA and HNA cells.

4.3.3.2 Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization (CARD-FISH)

Fluorescence *in situ* hybridization technique was used to quantify the main microbial components belonged to bacteria and archaea domains. In particular, Catalyzed Reporter Deposition - Fluorescence *In Situ* Hybridization **(**CARD-FISH) analysis was performed following the protocol optimized by Fazi et al., (2013, 2007) using specific rRNA-target HRP-labeled probes (Biomers, Ulm, Germany): EUB338 I-III for *Bacteria*; ALF968 for *Alphaproteobacteria*; BET42a for *Betaproteobacteria*; GAM42a for *Gammaproteobacteria*; DELTA495 for *Deltaproteobacteria*; CFX and GNSB for *Chloroflexi*, LGC354mix for *Firmicutes*, CF319a for *Flavobacteria*, PLA46 for *Planctomycetes*, TM7905 for TM7, HGC69A for *Actinobacteria* and ARCH915 for Archaea. Details of probes are available at probeBase (Greuter et al., 2016) (Table 2).

Table 2. Probe sequences used in CARD-FISH.

TARGET	PROBE	PROBE SEQUENCE
Archaea	ARCH915	5'- GTGCTCCCCCGCCAATTCCT -3'
Bacteria	EUB338 I	5'- GCTGCCTCCCGTAGGAGT -3'
	EUB338 II	5'- GCAGCCACCCGTAGGTGT -3'
	EUB338 III	5'- GCTGCCACCCGTAGGTGT -3'
Alpha-proteobacteria	ALF968	5'- GGTAAGGTTCTGCGCGTT -3'
Beta-proteobacteria	BET42a	5'- GCCTTCCCACTTCGTTT -3'
Gamma-Proteobacteria	GAM42a	5'- GCCTTCCCACATCGTTT -3'
Delta-proteobacteria	DELTA495 a-c	5'- AGTTAGCCGGTGCTTCCT -3'
Firmicutes	LGC354a LGC354b LGC354c	5'- TGGAAGATTCCCTACTGC -3' 5'- CGGAAGATTCCCTACTGC -3' 5'- CCGAAGATTCCCTACTGC -3'
Chloroflexi	GNSB941 CFX1223	5'- AAACCACACGCTCCGCT -3' 5'- CCATTGTAGCGTGTGTGTMG -3'
Actinobacteria	HGC69a	5'- TATAGTTACCACCGCCGT -3'
Planctomycetes	PLA46	5'- GCCTTGCGACCATACTCCC -3'
Flavobacteria/Bacteroidetes	CF319a	5'- TGGTCCGTGTCTCAGTAC -3'
TM7	TM7905	5'- CCGTCAATTCCTTTATGTTTTA -3'

Briefly, filter sections were embedded in low gelling point agarose in order to avoid cell loss and subsequently dried at 35°C for 15 min. The filters were subsequently treated with lysozyme and proteinase K in order to allow the membrane permeabilization. After a pre-treatment with a lysozyme solution (37°C for 60 min), the filters were incubated in Proteinase K solution at 37°C for 25 min. After permeabilization, filters were incubated in 0.01 M HCl at room temperature for 10 min in order to inactivate the proteinase K and intracellular peroxidases. The filters were incubated overnight at 35°C under rotation to allow the hybridization with the specific rRNA-target HRP-labeled probes (Tab. 2). Lastly, the filters were rinsed with sterile MilliQ water and labeled with fluorescein labeled tyramides in order to allow the visualization of the hybridized cells.

The stained filter sections were inspected on a Leica DM LB30 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 1000X magnification. At least 300 cells were counted in at least 10 microscopic fields randomly selected across the filter sections. The

relative abundance of hybridized cells was estimated as the ratio of hybridized cells to total DAPI-stained (staining with 4,6-diamidino-2-phenylindole at a final concentration of 1 μg/ml).

4.3.3.3 Biofilm structure

CARD-FISH technique in combination with Confocal Laser Scanning Microscopy (CLSM) was used to observe microbial colonization and tridimensional structure of biofilm grown both on microscopy slides arranged in the central point of "Carletti channel" and on the filling materials of the biofilters. The protocol is detailed in Lupini et al. (2011). Chambered slides (10-well diagnostic microscope epoxy coated slides; well diameter: 6.7 mm – Thermo Scientific, Germany) were used to housing biofilm samples for CARD-FISH analysis. Washing, permeabilization and hybridization solutions were pipetted into the wells to avoid cell detachment. Slides were incubated in a humidified 50 ml tubes to keeping the sample hydrated during all the incubations and all the washing steps were done by dipping the slides in 50 ml of washing solutions and buffers. The specific probe EUB338 I-III mix was used for identifying bacterial cells (Tab. 2). Image elaborations were performed using Imaris 6.2 software (Bitplane AG, Zurich, Switzerland). Reflection and autofluorescence signals were used to visualize filling materials and phototrophic microorganisms in biofilms by using a FV1000 (Olympus Corp., Tokyo, Japan) confocal laser scanning microscope at the Center of Advanced Microscopy 'P. Albertano', Department of Biology, University of Rome 'Tor Vergata'.

4.3.3.4 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') or 341 F

(5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') targeting the regions V1-V3 and V3-V5 of bacterial and archaeal 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 QIIME1 and QIIME2 (2017.12 release) software tools (Caporaso et al., 2010). 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 Silva119 or Silva128 database release (Quast et al., 2013).

4.3.3.5 Phylogenetic analysis

By analyzing the freshwaters from Cimino-Vico volcanic area, the phylogeny of the majority of reads retrieved from thermal samples (SCC, CAR, PAL) was not successfully assigned with QIIME1. Therefore, these sequences were separately compared to the GenBank database with BLASTn. Together with the closest relatives, the sequences were aligned on the MEGA software version 6 (Tamura et al., 2013) using MUSCLE (Edgar, 2004) and the phylogenetic tree was calculated with the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

4.3.4 Metabolic potentialities

4.3.4.1 Most Probable Number technique

Most Probable Number (MPN) counts were performed on water samples from Cimino-Vico volcanic area and determined with triplicate 10-fold dilution series (to 10^{-9}) using liquid selective growth media, inoculated and incubated for 30 days at the same temperature of the original water sample. In particular, MPN was used to quantify aerobic heterotrophs, autotrophic and heterotrophic As(III)-oxidizers, As(V)-reducers, sulfide-oxidizers, sulfate-reducers, nitrate-reducers, iron-oxidizers, iron-reducers and manganese-reducers. For autotrophic and heterotrophic As(III)-oxidizers and As(V)-reducers, 1 ml of water sample was added to 9 ml of basal mineral medium (Corsini et al., 2014) supplemented with sodium arsenite (NaAsO2) or disodium hydrogen arsenate (Na2HAsO4) at a final concentration of 100 mg/L

with or without sodium lactate (1 g/L final concentration). Selective growth media for heterotrophs, sulfide-oxidizers and sulfate-reducers were prepared according to Cote and Gherna (1994). Growth media suggested by Lovley (2006) were used to quantify nitrate-, iron- and manganese-reducers with a mix of carbon sources at final concentration of 1g/L (sodium acetate, glucose and lactate in 2:1:1 ratio). The enumeration of iron-oxidizers was performed by using a $FeSO_4$-based mineral medium as described in Barron and Lueking (1990). The growth of As(V)-, Fe-, sulfate-, nitrate and Mn-reducing bacteria was estimated under anaerobic conditions carefully controlled during either the sampling and laboratory incubation. In particular, samples were dispensed into culture tubes, sealed under N_2 atmosphere with butyl rubber stoppers. The reducing growth media were autoclaved and, before inoculation, again stripped with nitrogen under axenic conditions. MPN (cell/mL) was estimated as described in Sutton (2010). Positive tubes were analyzed at the end of the incubation to confirm the screened metabolisms by evaluating the occurrence of the expected end-products.

4.3.4.2 Real-time quantification of arsenic-related functional genes

The screening of functional genes known to be involved in the arsenic cycle was performed by PCR using the Taq98™ Hot Start 2X Master Mix (Lucigen, USA) according to the manufacturer's instructions. 10 different primer sets were used for the amplification of *aioA*, *arrA*, *arsB* and *arsC* genes according to the protocols reported in literature (Table 3).

In detail, arsenite oxidase gene (*aioA*) was targeted using aroA#1F - aroA#1R primers according to Karn and Pan (2016); arrAf - arrAr primer set (Malasarn et al., 2004) was used for the amplification of arsenate 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).

Table 3. Primer sets used for PCR amplification of As-related functional genes. In black bold primers used in qPCR.

Targeted gene	Primer set and name		Primer sequence (5'-3')	Amplicon length (bp)	Reference
Arsenite oxidase (*aioA*)	C	aoxBM1-2F	CCACTTCTGCATCGTGGGNTGYGGNTA	1085	Quéméneur et al., 2008
		aoxBM3-2R	TGTCGTTGCCCCAGATGADNCCYTTYTC		
		aoxBM2-1R	GGAGTTGTAGGCGGGCCKRTTRTGDAT		
	A	aroA #1F	GTSGGBTGYGGMTAYCABGYCTA	500	Inskeep et al., 2007
		aroA #1R	TTGTASGCBGGNCGRTTRTGRAT		
	B	aroA #2F	GTCGGYYGYGGMTAYCAYGYYTA	500	
		aroA #2R	YTCDGARTTGTAGGCYGGBCG		
	L	**aroA #1F**	GTSGGBTGYGGMTAYCABGYCTA	500	Karn and Pan 2016
		aroA #1R	TTGTASGCBGGNCGRTTRTGRAT		
	D	aroA95f	TGYCABTWCTGCAIYGYIGG	504	Hamamura et al., 2008
		aroA599r	TCDGARTTGTASGCIGGICKRTT		
Arsenate respiratory reductase (*arrA*)	**G**	**arrAf**	AAGGTGTATGGAATAAAGCGTTTGTBGGHGAYTT	160-200	Malasarn et al., 2004
		arrAr	CCTGTGATTTCAGGTGCCCAYTYVGGNGT		
	H	HAArrA-D1f	CCGCTACTACACCGAGGGCWWYTGGGRNTA	500	Kulp et al., 2006
		HAArrA-G2R	CGTGCGGTCCTTGAGCTCNWDRTTCCACC		
	I	AS1F	CGAAGTTCGTCCCGATHACNTGG	625	Song et al., 2009
		AS1R	GGGGTGCGGTCYTTNARYTC		
Arsenate cytoplasmic reductase (*arsC*)	**E**	**amlt-42-F**	TCGCGTAATACGCTGGAGAT	334	Sun et al., 2004
		amlt-376-R	ACTTTCTCGCCGTCTTCCTT		
Arsenite transporter (*arsB*)	**F**	**arsB#1F**	GGTGTGGAACATCGTCTGGAAYGCNAC	750	Achour et al., 2007
		arsB#1R	CAGGCCGTACACCACCAGRTACATNCC		

qPCR reactions were performed in triplicate for each sample by using quantification method based on SYBR Green chemistry. Each reaction was performed in 20 µL total volume including 10 µL Sso Advanced Universal SYBR Green Supermix (BIO-RAD, USA), 10 µM of each primer and 5 µL DNA as template. Standard curves were constructed by using the long amplicons method previously reported in Matturro et al. (2013). Standards for the absolute quantification were prepared from DNA extracted from pure cultures (e.g., *Acinetobacter calcoaceticus*, *Acinetobacter junii*, *Brevundimonas intermedia*, *Herminiimonas*

arsenicoxydans) and amplified by PCR with the primers listed in table 3. Purified and quantified PCR products, named as long amplicons, were serially diluted and the obtained standard curve showed a linearity range from 10^9 to 10 gene copies μL^{-1} (R^2 = 0.92 - 0.99) and a slope of around -3.4 corresponding to a 97-98.5% efficiency (E) of the qPCR reaction (E = 97-98.5%) calculated as E = 10(1/slope) - 1. qPCR reactions were performed on a CFX96 TouchTM Real-Time PCR Detection System (Biorad, Italy). Data was analyzed using Bio-Rad CFX Manager (Software version 3.1). Melting curves were performed for each reaction to confirm the purity of amplified products.

4.4 Statistical analysis

For the statistical elaboration of data regarding the microbial communities in As-rich freshwater in Vico volcanic district, the non-parametric Mann-Whitney U test was applied to verify the statistical difference between thermal and non-thermal waters for all physical, chemical and microbial parameters (Clarke et al., 1993). The chemical variables with $p < 0.05$ were incorporated into a Nonmetric MultiDimensional Scaling ordination plot (NMDS) in order to graphically synthesize the Euclidean dissimilarity between the two groups of water samples. Chemical and microbial abundance data were then projected onto the NMDS ordination using a vector-fitting procedure, in which the length of the arrow is proportional to the correlation between NMDS-axes and each variable. This method allowed determining the variation pattern of each projected variable discriminating the two groups of waters (Amalfitano et al., 2014; Foulquier et al., 2013). Chemical data were log-transformed, whereas values of cell abundance of the major prokaryotic subgroups, including flow cytometry (LNA and HNA cells) and CARD-FISH data (i.e. major phyla and classes of *Proteobacteria*) was normalized by log(X+1).

A multi-group SIMilarity PERcentage test (SIMPER), performed on NGS data using the Euclidean similarity measure, was performed to identify the microbial phylogenetic groups that were primarily responsible for observed differences between thermal waters and groundwaters. Non-parametric MANOVA (NPMANOVA) was used to test if microbial taxa obtained from 16S rRNA bar-coded pyrosequencing differed statistically among thermal waters and groundwaters.

The Principal Component Analysis (PCA), based on the correlation matrix, was performed by comprising separately the different physicochemical and microbial data obtained during the characterization of "Carletti thermal pools". Chemical data were log-transformed, whereas values of microbial groups revealed by NGS at phylum level (clusters > 1% of total OTUs were considered) were normalized by log(X+1).

In order to statistically evaluate the data obtained during biofilters experiments, the 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).

Statistical analysis and diversity indices (Shannon and Simpson) were generated using the software PAST v3.20 (Hammer et al., 2001).

5. Results and Discussion

5.1 Chemical and microbiological characterization of arsenic-rich aquatic environments of geothermal origin

The following chapter has been modified and adapted from the original publication "Crognale, S., Zecchin, S.., Amalfitano, S., Fazi, S., Casentini, B., Corsini, A., Cavalca, L., Rossetti, S. 2017. Phylogenetic structure and metabolic properties of microbial communities in arsenic-rich waters of geothermal origin. *Frontiers in Microbiology*, 8(DEC), p.2468."
Available at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.02468/full"

5.1.1 Water chemistry

Major physical and chemical parameters (T, pH, EC, DO, HCO_3^-, SO_4^{-2}) differed significantly based on water origin (Table 4). Thermal waters showed high EC (4610-5680 µS/cm), high concentrations of SO_4^{-2} (1264–1670 mg/L), typical of geothermal environments and highest temperature values (55.5-58°C).

Table 4. Main geochemical parameters of thermal waters, groundwaters and lake waters. The complete dataset is reported in appendix 4.

Samples	T (°C)	pH	EC µS/cm	DO mg/l	HCO_3^- mg/L	S^{-2} mg/L	F^- mg/L	Cl^- mg/L	NO_3^- mg/L	SO_4^{-2} mg/L	As_{tot} µg/L	As(III) µg/L
PAL	58.0	6.44	5680	0.2	1160	1.5	3.0	12.9	1.3	1554.8	362.1	107.4
SSC	57.8	6.41	5660	0.2	1121	2.1	2.8	12.6	<LOD	1670.4	351.9	12.0
CAR	55.5	6.49	4610	0.2	1026	0.1	3.2	13.5	<LOD	1263.9	329.2	172.5
OLI	24.9	6.65	724	1.33	708	0	3.0	15.3	6.4	20.0	22.9	0.0
BEL	21.7	7.04	356	3.16	232	0	4.0	19.6	5.9	33.6	152.1	5.4
ANG	18.7	6.28	250	7.21	195	0	1.5	9.1	3.2	15.8	182.4	8.9
FON	18.2	6.69	269	7.95	122	0	1.8	19.2	20.0	21.3	51.9	1.0
VICO	14.0	8.28	352	9.06	244	0	1.0	16.8	1.1	81.8	20.9	7.4

LOD = Limit of Detection

Groundwaters showed high values of V and U and low values of EC and sulfates. Nitrate concentrations reached the highest value in FON (20 mg/L). Nitrites and phosphates were below detection limits in all samples. Among other ions analyzed (Appendix 4), Sr and Li showed highest values in thermal waters (14270 and 176 µg/L respectively) whereas B varied between 73 (groundwater) and 1206 µg/L (thermal waters). The concentrations of Fe and Mn were

generally low and ranged between 0.15 - 0.84 mg Fe/L and 0.3 - 27 µg Mn/L. The highest As concentration was found in thermal waters (329.2-362.1 µg/L). Total arsenic concentrations showed relatively lower concentrations in VICO surface waters (20.9 µg/L) and groundwaters (22.9-182.4 µg/L). As(III) was more concentrated in PAL, CAR and VICO than in groundwaters; the geothermal sample SSC showed less than 4% of As(III). The NMDS ordination allowed visualizing the chemical dissimilarity between thermal and non-thermal waters, also showing the variation pattern of those variables that varied significantly between the two groups of waters (Fig. 5).

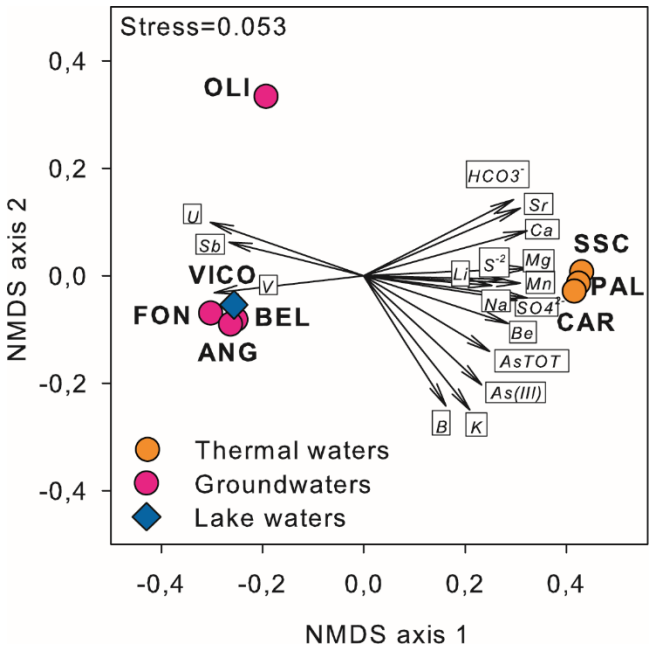

Figure 5. NMDS ordination plot, based on Euclidean distance matrix of log-transformed data, showing the variation patterns of chemical variables that varied significantly between thermal and non-thermal waters. The vector length is proportional to the correlation between the NMDS axes and each chemical variable. The stress value (i.e., < 0.2) suggests for an accurate representation of the dissimilarity among water samples.

5.1.2 Prokaryotic cell abundance and community structure

Prokaryotic abundance was higher in lake waters ($2.3x10^6 \pm 1.9x10^4$ cell/ml) and thermal waters (between $5.1x10^4 \pm 9.3x10^1$ cell/ml and $6x10^5 \pm 2.5x10^3$ cell/ml) than in groundwaters (between $2.2x10^4 \pm 2.6x10^2$ cell/ml and $3.3x10^5 \pm 4.7x10^3$ cell/ml) (Table 5). As assessed by

flow cytometry, the ratio between the mean green fluorescence and forward scatter signal intensity of prokaryotes was lower in thermal waters than in non-thermal waters. In groundwaters and surface waters, HNA cells showed lower percentages than those found in thermal waters (26% in lake water and 35.2% in groundwater except BEL with HNA = 87%). On average, the mean fluorescence intensity of the HNA cells was approximately four times higher than that of LNA cells.

Table 5. Main cytometric characteristics of thermal waters, groundwaters and lake waters. PAB: prokaryotic abundance. FSC: Forward side scatter. LNA: Low Nucleic Acid content cells. HNA: High Nucleic Acid content cells.

	Total Prokaryotes			**LNA cells (%)**	**HNA cells (%)**
	PAB 10^4 cells/ml	**Mean fluo Green FU**	**Mean FSC FU**		
PAL	5.1	239	93	6.1	93.9
SSC	11.3	101	34	50.0	50.0
CAR	60.0	315	194	14.7	85.3
OLI	6.3	78	29	70.5	29.5
BEL	33.3	180	44	13.0	87.0
ANG	3.4	96	29	53.9	46.1
FON	2.2	80	21	70.0	30.1
VICO	231.0	67	19	74.1	25.9

As shown in Figure 6, the main microbial components belonged to bacteria domain (from 15% up to 95% of total cells) and were mainly affiliated to *Proteobacteria*. *Alpha-* and *Betaproteobacteria* were the most abundant groups retrieved in groundwaters and surface waters, whereas *Gammaproteobacteria* dominated in thermal waters (reaching 81.5% of bacterial cells in CAR sample). TM7 division was mainly present in groundwaters reaching the highest percentage (8.5%) in FON sample. *Archaea* were found in all screened samples with abundance ranging between $1.9x10^2 \pm 0.2x10^1$ cell/mL and $6.0x10^4 \pm 1.1x10^3$ cell/mL (Fig. 6).

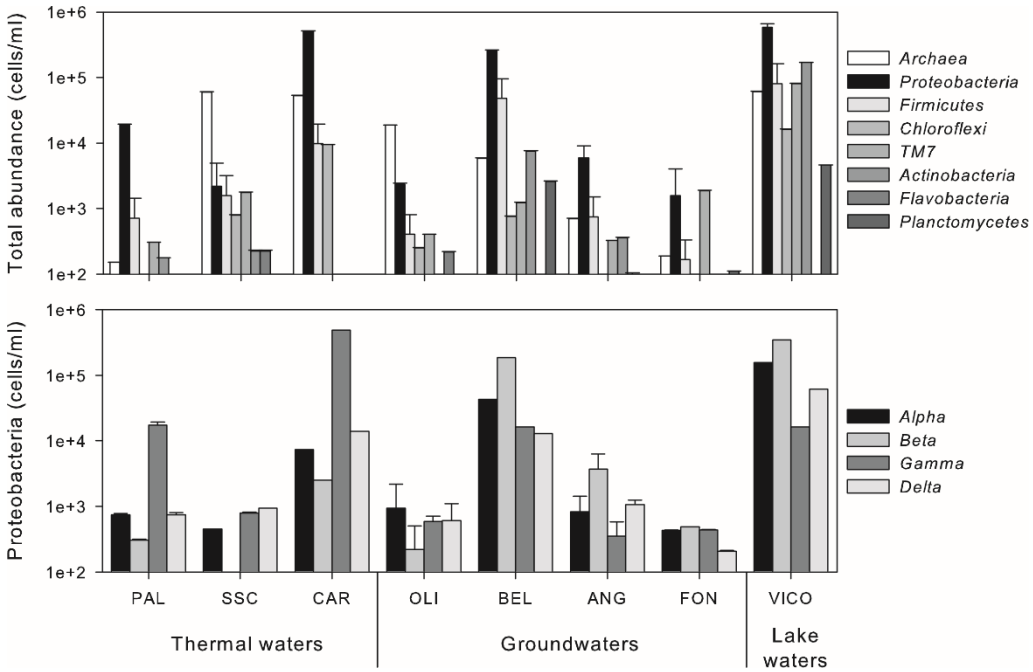

Figure 6. Abundance of phylogenetic clusters (*Archaea* and main phyla within *Bacteria*) (upper panel) and single classes within *Proteobacteria* (lower panel) in thermal waters, groundwaters and lake waters.

Following a vector-fitting procedure onto the NMDS ordination plot based on chemical dissimilarity, we showed that *Archaea*, *Gammaproteobacteria* and HNA cells were relatively more abundant in thermal waters, whereas *Betaproteobacteria*, *Planctomycetes*, TM7, *Alphaproteobacteria*, and LNA cells were more represented in non-thermal waters (Fig. 7).

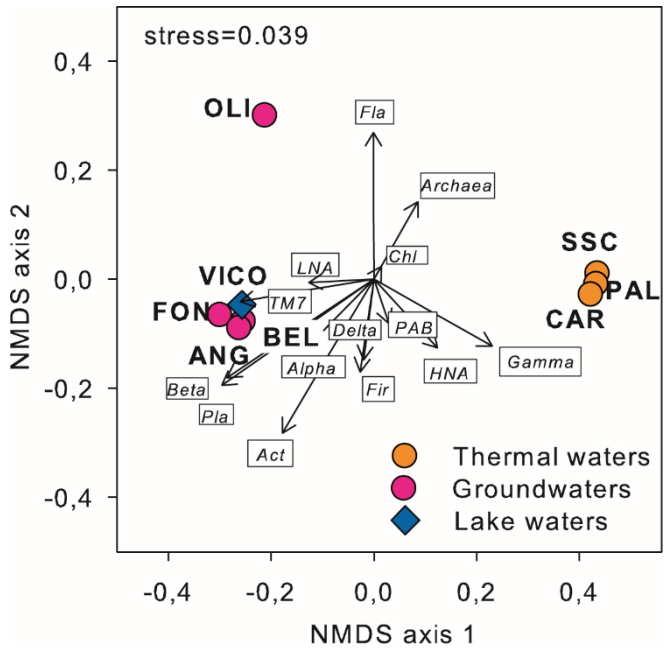

Figure 7. Cell abundance variation of the major prokaryotic subgroups, including flow cytometry (LNA and HNA cells) and CARD-FISH data (i.e. major phyla and classes of *Proteobacteria*), projected onto the NMDS ordination synthesizing the chemical dissimilarity between thermal and non-thermal waters (see Fig. 1). The vector length is proportional to the correlation between the NMDS axes and each microbial variable, upon normalization by log(X+1). *Act*, *Actinobacteria*; *Chl*, *Chloroflexi*; *Fir*, *Firmicutes*; *Fla*, *Flavobacteria*; *Pla*, *Planctomycetes*; *Alpha*, *Alphaproteobacteria*; *Beta*, *Betaproteobacteria*; *Gamma*, *Gammaproteobacteria*; *Delta*, *Deltaproteobacteria*; LNA, Low Nucleic Acid content cells; HNA, High Nucleic Acid content cells; PAB, Prokaryotic Abundance.

5.1.3 Next Generation Sequencing (NGS)

A total of 71401 ends reads were generated which were assigned into 2988 OTUs. All the resulting 16S rRNA gene fragments were then classified into 24 bacterial phyla, 55 classes, 118 orders, 188 families and 293 genera. Overall, *Proteobacteria* was the most abundant phylum (46.5% of total OTUs), followed by *Cyanobacteria* (12.1%), *Bacteroidetes* (7.6%), *Nitrospirae* (7.3%), *Firmicutes* (1.2%) and *Acidobacteria* (1.0%). The other 18 phyla represented less than 1.0% of total OTUs. On average, 22.5% of the OTUs showed a very low 16S rDNA identity with known bacterial taxa, with the highest percentage in thermal waters (up to 76.1%). The relative abundance of OTUs in water samples differed considerably (Fig. 8).

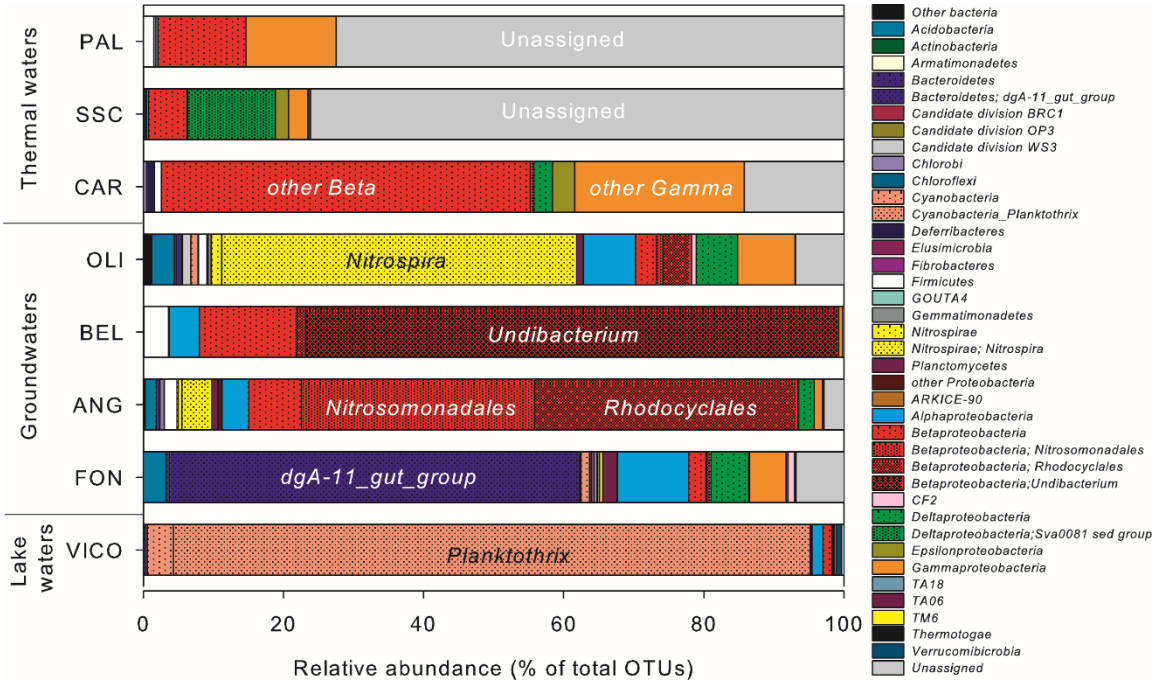

Figure 8. OTUs relative abundance in water samples estimated by NGS. Clusters making up less than 1% of total composition were classified as 'other bacteria' in thermal waters, groundwaters and lake waters.

In surface waters (VICO), *Cyanobacteria* represented the 94.6% of total OTUs and they were mainly affiliated to the *Planktothrix* genus (90.9%). *Nitrospirae*, *Bacteroidetes* and *Proteobacteria* dominated groundwater samples. *Nitrospirae* phylum was largely represented by the genus *Nitrospira* (up to 50.7% of total OTUs). The genus dgA-11 gut group, belonging to *Bacteroidetes*, was found only in groundwaters (up to 58.6% of OTUs in FON sample). *Betaproteobacteria* were mainly represented by orders *Burkholderiales*, *Nitrosomonadales*, and *Rhodocyclales*, affiliated to genera *Undibacterium* and *Azoarcus* and family *Gallionellaceae*. *Alpha-* and *Gammaproteobacteria* were mainly affiliated to orders *Rhizobiales*, *Sphingomonadales*, *Rickettsiales*, *Rhodospirillales* and to genus *Stenotrophomonas*.

Proteobacteria was the predominant phylum reported in thermal waters, mainly affiliated to *Betaproteobacteria* (from 5.3% up to 52.6% of total OTUs) and *Gammaproteobacteria* (from 2.7% up to 24.2% of total OTUs), but the taxonomic affiliation at lower level classification was

not identified. *Deltaproteobacteria* were mainly retrieved in one thermal sample (SSC) affiliated to the genus Sva0081_sediment_group, belonging to *Desulfobacterales*. As assessed by NPMANOVA, thermal waters differed considerably from non-thermal waters. Lake waters were not considered in this analysis, since NGS results highlighted a low diversity in this sample with the predominance of only one genus. According to SIMPER test (Appendix 5), the unassigned portion of OTUs, *Betaproteobacteria*, *Bacteroidia*, *Nitrospira*, *Gamma-* and *Alphaproteobacteria* were the first six classes that explained more of the overall dissimilarity between the thermal waters and groundwaters, contributing together > 99% of total variability (Fig. 9).

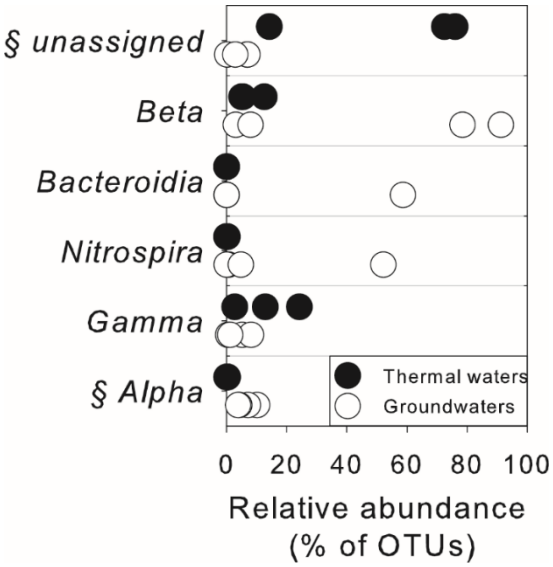

Figure 9. Microbiological variables that more significantly explained the overall dissimilarity (according to SIMPER test) between thermal waters and groundwaters at the class level. §=statistically different between two groups of water according to NPMANOVA analysis ($p<0.05$).

5.1.4 Phylogenetic analysis of unassigned bacterial 16S rRNA gene sequences from thermal waters

A number of sequence reads did not exhibit homology to known microbial taxa, which implies the presence in thermal waters of numerous so far unidentified microorganisms. In SSC and PAL, 76.1% and 72.5% of total OTUs, respectively, were not identified using known databases.

In CAR, the unassigned portion was around 14.2% of total OTUs. The phylogenetic analysis of thermal samples showed that most of the unassigned 16S rRNA gene sequences were related to *Epsilonproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Figure 10). Among *Epsilonproteobacteria*, the sequences were related to the members of *Nitratiruptor tergarcus* (~ 90% of similarity). Additionally, the sequences phylogenetically affiliated to the *Gammaproteobacteria* showed 95% of similarity with *Thiofaba tepidiphila*. The sequences affiliated with *Firmicutes* were only distantly related to *Caldicellulosiruptor saccharolyticus*, *Desulfotomaculum thermosapovorans*, *Thermoanaerobacterium thermosaccharolyticum* and *Symbiobacterium thermophilum* (~ 75-80% of similarity). Sequences phylogenetically related to *Actinobacteria*, *Nitrospirae* and *Betaproteobacteria* were also retrieved.

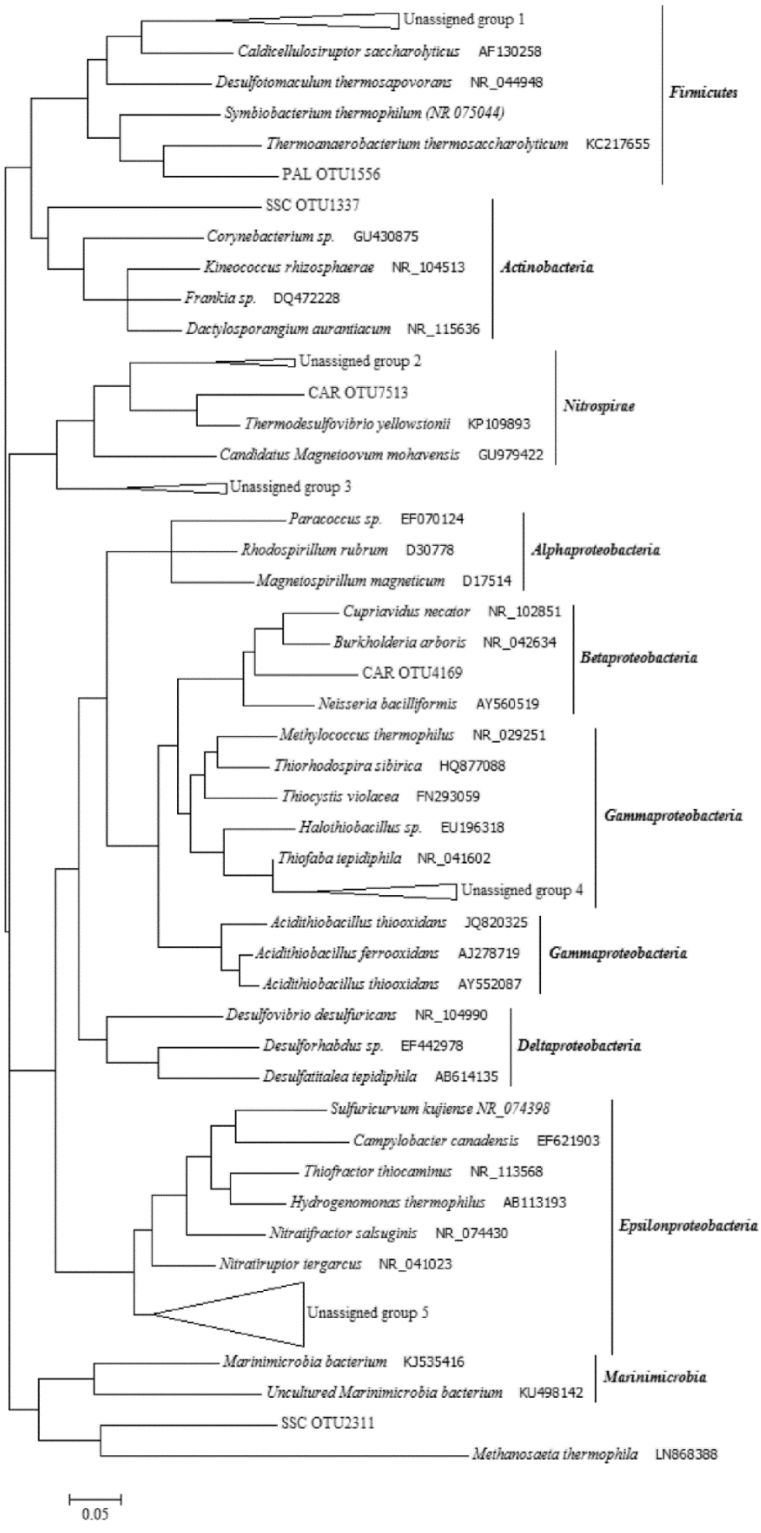

Figure 10. Phylogenetic tree constructed with unassigned 16S rRNA gene sequences obtained from the thermal samples. Unassigned group 1 (76 sequences): PAL OTUs 3865/3142/1624/5721/7240; SSC OTU 5116. Unassigned group 2 (17 sequences): CAR OTUs 1743/886/2900. Unassigned group 3 (13 sequences): SSC OTUs 676/3207/8283/6610; Unassigned group 4 (6 sequences): PAL OTUs 5277/848; SSC OTUs 3302/1374/2608; CAR OTU 5062. Unassigned group 5 (58 sequences): SSC OTUs 1776/2215/8205/6801/8038/363/3362/4634/7530/3704/6025/535/4726/3400/5198/6069/43/7956/1858/3460/6393/6056/2124; PAL OTUs 1583/1883.

5.1.5 Most Probable Number

Among the tested microbial functions, only nitrate and sulphate reduction, and heterotrophic As(III)-oxidization were found in all the samples (Fig. 11). In particular, nitrate and sulphate reduction were found as main driving metabolisms in groundwaters where these microbial functional groups were retrieved at high abundance (up to ~ 10^4 cells/mL). As expected, lower concentrations were found associated to surface waters in which aerobic heterotrophs were mainly observed. In line with the anoxic reaction environment, no aerobic heterotrophs were found in thermal waters. No growth of autotrophic As(III)-oxidizers, As(V)-reducers, Fe-reducers and oxidizers, Mn-reducers and sulfide oxidizers was observed by MPN. The sole As related metabolism found in the samples was related to heterotrophic As(III) oxidation. The abundance of the latter microbial functional group ranged between $0.1x10^1$ and $1x10^3$ cell/mL (Fig. 11).

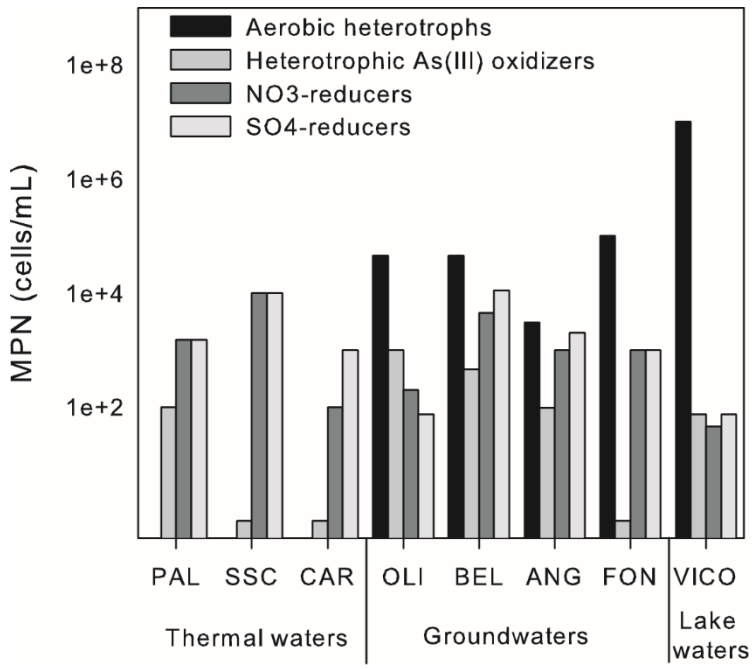

Figure 11. Most Probable Number (MPN) counts of the main microbial functional groups in thermal waters, groundwaters and lake waters.

5.1.6 Real-time quantification of arsenic-related functional genes

A preliminary screening by PCR showed the occurrence of *arsB*, for arsenite efflux pump, and of cytoplasmic arsenate reductase (*arsC*) genes in all screened samples, whereas arsenite oxidase (*aioA*) and arsenate respiratory reductase (*arrA*) were not amplified with any primer set under any tested condition. The abundance of *arsB* and *arsC* genes was estimated by real-time PCR. *arsB* and *arsC* genes were found in all samples at low concentration: *arsB* genes ranged between $0.3x10^2 \pm 0.01x10^2$ and $0.4x10^3 \pm 0.1x10^2$ gene copies/ml whereas *arsC* genes were found at concentrations up to $0.4x10^3 \pm 0.3x10^2$ gene copies/ml except in CAR (< d.l.) (Fig. 12).

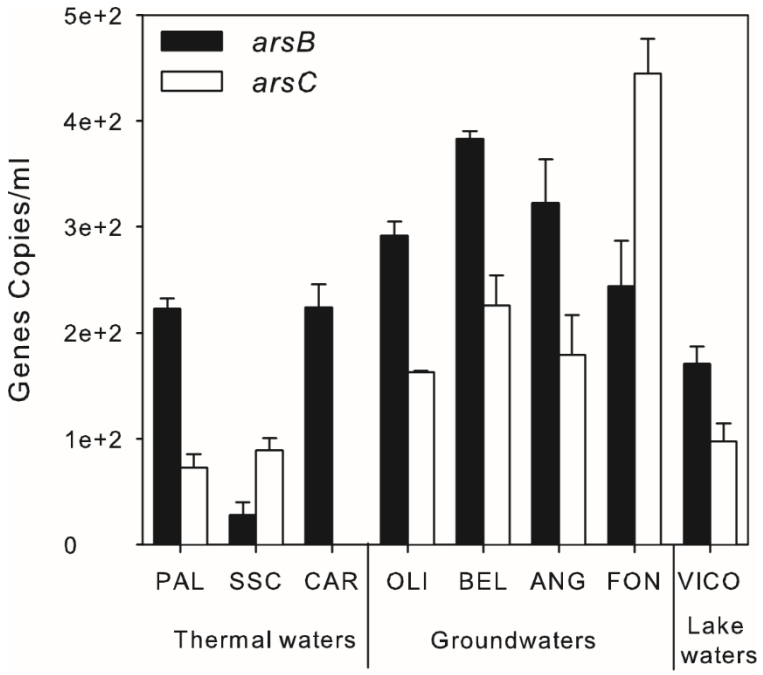

Figure 12. Arsenic-related genes abundance (gene copies/ml) estimated by qPCR in thermal waters, groundwaters and lake waters.

5.1.7 Discussion

5.1.7.1 Arsenic occurrence and microbial community composition

In this study, As concentrations were in line with previous studies which reported values up to 100 μg/L in groundwaters and 370 μg/L in thermal waters of Cimino-Vico volcanic area (Angelone et al., 2009; Armiento et al., 2015; Baiocchi et al., 2013; Fazi et al., 2016b).

In accordance with literature findings reporting a variety of arsenic-resistant and transforming bacterial species in similar environments (Bahar et al., 2016; Liao et al., 2011; Paul et al., 2015a; Sarkar et al., 2013), we found the predominance of *Proteobacteria* in groundwaters. In particular, the presence of *Undibacterium* and *Azoarcus* genera in groundwater was often observed in other As-contaminated waters (Ghosh and Sar, 2013; Griebler and Lueders, 2009). Some cultivable representatives of these genera were previously isolated from several environments, including drinking waters, and showed the ability to grow under anoxic condition using nitrate as final electron acceptor (i.e., *Azoarcus* spp.) (Reinhold-Hurek et al., 2015). The high nitrate concentration observed in FON groundwater indicated that the surficial local aquifer could be impacted by agricultural activities and affected by faecal contamination, as suggested by the high relative abundance of OTUs affiliated to dgA-11 gut group (58.6% of total OTUs), usually isolated from animal gastrointestinal tract and feces (Ozutsumi et al., 2005; Tsukinowa et al., 2008).

Consistently with redox conditions commonly found in geothermal environments (Ballantyne and Moore, 1988), thermal waters were generally characterized by low DO content and high As(III) concentration. The prevalence of oxidized As form, observed only in SSC sample, may indicate oxygen exposure during geothermal water uprising.

The high abundance of HNA cells found in thermal waters was in line with the highly selected microbiomes described by NGS. Indeed, LNA and HNA groups detected by flow cytometry

are generally considered as constitutive traits of microbial communities in a variety of environments. Their relative contribution was reported to vary according to ecosystem properties and to reflect different phylotype composition, lifestyles and growth potential of the small genome LNA vs. the large genome HNA cells (Bouvier et al., 2007). HNA are also believed as the most active fraction (Lebaron et al., 2002), even though different phylogenetic affiliations were reported (Vila-Costa et al., 2012; Romina Schiaffino et al., 2013). Detection of HNA and LNA has been reported over a wide range of aquatic ecosystems covering large environmental gradients in bacterial (Andreatta et al., 2004; Gasol and Del Giorgio, 2000; Sherr et al., 2006; Thyssen et al., 2005; Troussellier et al., 1999) and archaeal populations (Trigui et al., 2011).

In this study, archaea were found at high abundance in thermal waters, likely due to high temperature and reducing redox conditions (Rogers and Amend, 2005; Antranikian et al., 2017). Moreover, in thermal waters, a dominance of OTUs only distantly related to members affiliated to *Epsilonproteobacteria*, *Gammaproteobacteria* and *Firmicutes* was found, thus indicating the occurrence of bacterial biodiversity so far unexplored. Interestingly, the main cluster of unassigned OTUs (group 5 within *Epsilonprotebacteria* in Fig. 10) was found in SSC sample. Some of the closest relatives are known thermophilic chemolithoautotrophs able to utilize molecular hydrogen as an electron donor and oxygen or nitrate as electron acceptor. Overall, the microbial profiling obtained by NGS analysis revealed in thermal waters the occurrence of microorganisms involved in sulfur and nitrogen biogeochemical cycles (e.g. *Sulfuricurvum kujiense*, *Desulfotomaculum thermosapovorans*, *Thiofaba tepidiphila*, *Nitratiruptor tergarcus*, *Nitratifactor salsuginis, Nitrospira, Azoarcus, Stenotrophomonas*). It is worth to noting that, differently from previous studies performed on high-enthalpy geothermal systems (e.g. Yellowstone National Park), the bacterial phyla typically found in such environments (e.g.

Aquificae, *Chloroflexi*, *Deinococcus–Thermus*) were not retrieved. This is most likely related to the different geochemical conditions occurring at Cimino-Vico volcanic area (e.g., lower T, nearly neutral pH, slightly reducing redox conditions) which may strongly affect the microbial community structure.

Both As species were found in lake waters and they were likely related to redox processes mediated by high organic carbon availability (litter deposition, surface water runoff, 2.5 mg/L DOC) which represents, together with the light, one of the main driving forces of the complex interplay of biological activities occurring in such environment.

Even though the lake is directly affected by hydrothermal upwelling (ARPA 2012), the microbiological characteristics of Lake Vico were different from other waters analyzed. The abundance of *Betaproteobacteria* in this aquatic environment showed similar values reported at the same location in previous studies (Davolos and Pietrangeli, 2013; Fazi et al., 2016b). Furthermore, the presence of *Planktothrix* was recurrently observed in this area (Manganelli et al., 2016).

5.1.7.2 Linking microbial metabolic traits to arsenic biogeochemistry

As assessed by the cultivation-dependent approach, sulfate and nitrate reduction and heterotrophic As(III)-oxidation were the main metabolisms occurring in geothermal area. Despite the low concentrations of NO_3, the involvement of microbial communities in nitrate-reduction was observed in our samples. This suggested that microbial communities could activate this process as a result of an increase in nitrate concentration due to anthropic contamination (e.g. agricultural activity). Nitrate-linked microbial transformation of As was reported in As-contaminated aquatic environments, and some microorganisms were found to mediate anaerobic As(III) oxidation by coupling to nitrate reduction (Zhang et al., 2017).

Differently from alluvial environments (Islam et al., 2004), Fe- and Mn-related metabolisms were not found in our samples, since they were not likely to play an essential role in this geothermal area (Piscopo et al., 2006).

Remarkably, As detoxification processes prevail in groundwater and thermal waters. The detection of *arsBC* genes is consistent with previous evidences in a variety of environments (Davolos and Pietrangeli, 2013; Liao et al., 2011; Paul et al., 2015b). *arsB* and *arsC* genes were found simultaneously in most of the samples in line with the assumption generally accepted that their environmental distribution is similar (Davolos and Pietrangeli, 2013; Escudero et al., 2013).

Although heterotrophic As(III) oxidation was revealed by MPN analysis, *aioA* gene was not detected by PCR using different primer sets. This finding was probably due to the high microbial diversity in our samples and to the primer coverage that may not be sufficient to capture a high gene diversity. It is known that the investigation of As-related functional genes through PCR approaches as well as through metagenome sequencing is currently difficult in ecological studies (Fahy et al., 2015). A wide variety of *aioA*-like genes exists in natural environments and often these genes show low homology to currently known gene sequences (Hamamura et al., 2008; Jiang et al., 2015). Hence, new primer designs should integrate the most recent sequencing data as well as biochemical data and genetic context (Fahy et al., 2015). The primers in use so far were designed on few microbial strains and were mainly used only on isolates (Inskeep et al., 2007; Karn and Pan, 2016; Quéméneur et al., 2008). Few studies reported the detection of As-related genes in mixed microbial communities in natural environments (Engel et al., 2013; Escudero et al., 2013; Lami et al., 2013), in spite of their presence in different prokaryotic groups such as *Proteobacteria*, *Firmicutes*, *Chrysiogenetes*, *Deinococcus-Thermus*, *Deferribacteres* and *Chrenarchaeota* isolated from a variety of As-rich

environments (e.g. mine, arsenical pesticide- or smelter-impacted sites, geothermal sites, geyser, soil and sediments) (Cai et al., 2009; Heinrich-Salmeron et al., 2011; Inskeep et al., 2007; Sultana et al., 2012). Considering the paucity of information on As-related functional genes, further efforts will be devoted to the analysis of the gene diversity level associated with bacteria found in this geothermal environment.

5.2 Biofilms in As-rich hydrothermal environments: microbial composition and successional changes

The hot spring "CAR", analyzed during the first sampling campaign and described in section 5.1, was selected to further evaluate the biofilm structure and spatial dynamics under variable physical-chemical conditions. Due to the peculiar structural and geochemical characteristics of this study site, the biofilms were analysed along natural temperature, pH and oxygen gradients in the presence of high total As content.

5.2.1 Water chemistry

A strong decrease of temperature was observed along 100 m distance with values passing from 52.4 °C to 16.4 °C from the first to the last sampling point (Appendix 6). In contrast, an increase in pH and DO values was observed along the channel. The pH varied from 7.25 to 8.61. The dissolved oxygen moved from 1.84 mg/L to 9.08 mg/L from the origin to the end of the channel. Total arsenic concentration is constant along the channel with an average value of 195.2 µg/L, with a slight increase between C2 (214.8 µg/L) and C4 (218.5 µg/L). As(III) concentration decreased along the channel with the highest value in C3 (161.4 µg/L) and the lowest one in C7 (79.3 µg/L). The principal component analysis allowed to recognize the variation patterns of major physicochemical determinants (Fig. 13).

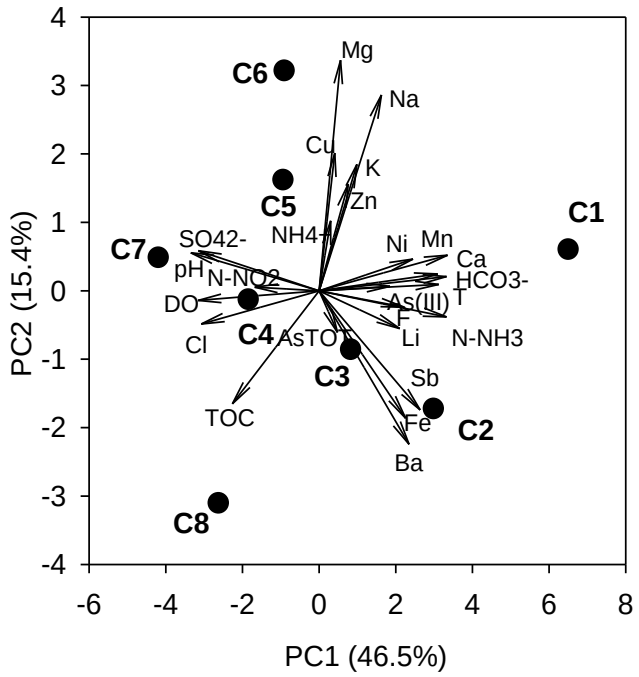

Figure 13. Principal Components Analysis biplot representing the typifying chemical composition. The vector length is proportional to the correlation between corresponding parameter and the PCA axis 1 and 2.

The first component explained most of the variance within the dataset (46.5 %), thus highlighting the difference between samples along the channel. In particular, the initial section of the channel was characterized by a higher content of HCO_3^-, $N\text{-}NH_3$ and Ca^{2+}, Mn, Sb, Fe, F^-, and Ni. In contrast, higher levels of SO_4^{2-}, NO_2^-, and total organic carbon were found in the final section of the channel.

5.2.2 Prokaryotic abundance and community structure in water

Total prokaryotic abundance in water samples increased from 6.8 x 10^5 cells/ml to 8.2 x 10^5 cells/ml along the channel (Table 6). A slight decrease of cells with High Nucleic Acid content (HNA) was observed from the origin to the end of the channel. Indeed, HNA cells were around 87.8% of total cells in C1 and 71.9% in C8.

Table 6. Main cytometric characteristics of water samples. PAB: prokaryotic abundance. LNA: Low Nucleic Acid content cells. HNA: High Nucleic Acid content cells.

	Distance (m)	PAB 10^5 cells/ml	HNA (%)	LNA (%)	Size au	Fluo au
C1	0	6.8	87.8	12.2	207	248
C2	14	7.3	85.2	14.8	196	256
C3	28	6.6	84.7	15.3	198	257
C4	42	7.2	84.6	15.4	194	254
C5	56	7.8	82.2	17.8	194	250
C6	70	7.3	81.4	18.6	195	249
C7	84	7.6	77.8	22.2	191	248
C8	100	8.2	71.9	28.1	187	241

Cytograms showed high cytometric similarity between water samples highlighting a highly similar microbial community along the channel. In line with this result, CARD-FISH analysis revealed a similar microbial community composition in water column along the channel (Fig. 14). The main microbial component in water samples belonged to bacteria domain (on average 79.3 % of total DAPI-stained cells) mainly affiliated with *Proteobacteria* (Fig. 14). *Gammaproteobacteria* was the predominant group along the channel showing abundance ranging between $1.2 \times 10^5 \pm 5.1 \times 10^3$ and $5.3 \times 10^5 \pm 2.0 \times 10^4$ cells/ml (range: 16.7 - 77.1 % of DAPI-stained cells). *Alpha-*, *Beta-* and *Deltaproteobacteria* represented on average 7% of total prokaryotic abundance. The other bacterial groups overall represented less than 2.2% of total cells. Meanwhile, on average 15.4 % of total prokaryotic cells belonged to *Archaea*, with a cell abundance ranging between $4.1 \times 10^4 \pm 1.2 \times 10^3$ cells/ml and $1.9 \times 10^5 \pm 6.4 \times 10^3$ cells/ml.

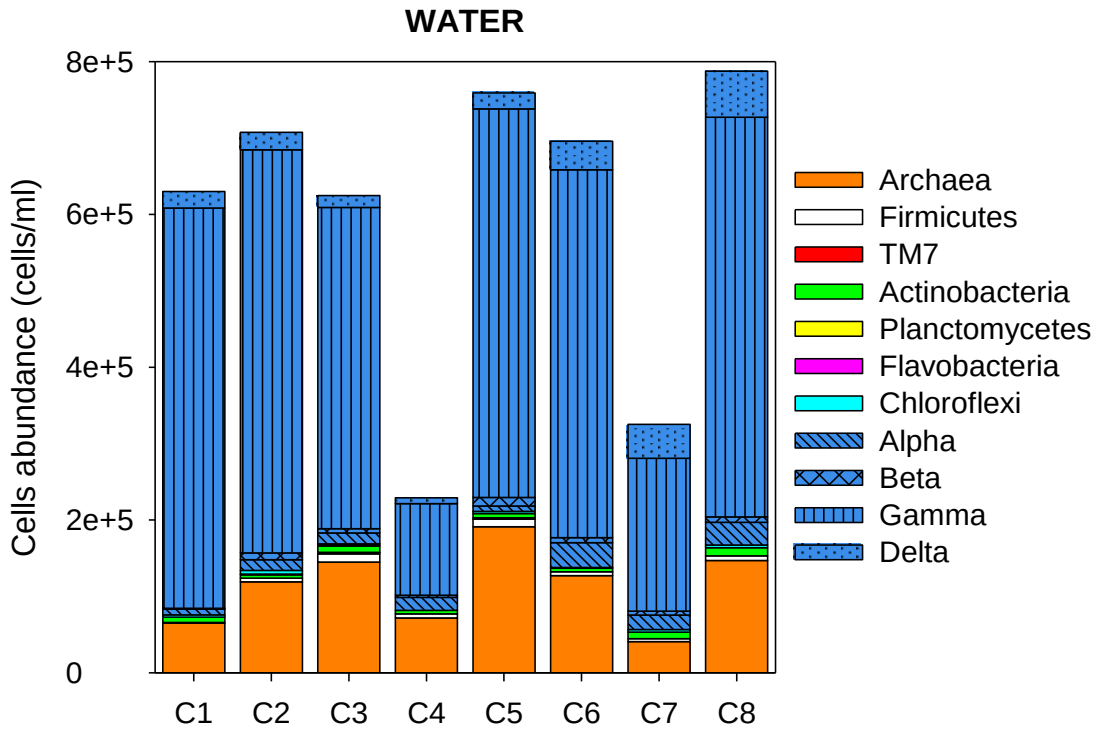

Figure 14. Abundance of phylogenetic clusters (*Archaea* and main phyla within *Bacteria*) and single classes within *Proteobacteria* (in blue) in the different water sampling points.

The outputs from high-throughput sequencing were in line with the results generated from CARD-FISH analysis. In particular, a total of 57,943 reads were generated by C1, C4, and C7 water samples. These reads resolved into 239 OTUs. Overall, *Proteobacteria* was the most abundant phylum, mainly represented by *Gammaproteobacteria* affiliated with genus *Thiofaba* (on average~85% of total OTUs) (Fig. 15). *Epsilonproteobacteria* represented around 9.2% of total OTUs. *Archaea* represented on average less than 1% of total OTUs. A low level of biodiversity was found in water samples with Shannon index values ranging between 0.6 and 0.8 and very similar Simpson index values (range: 0.26 - 0.30).

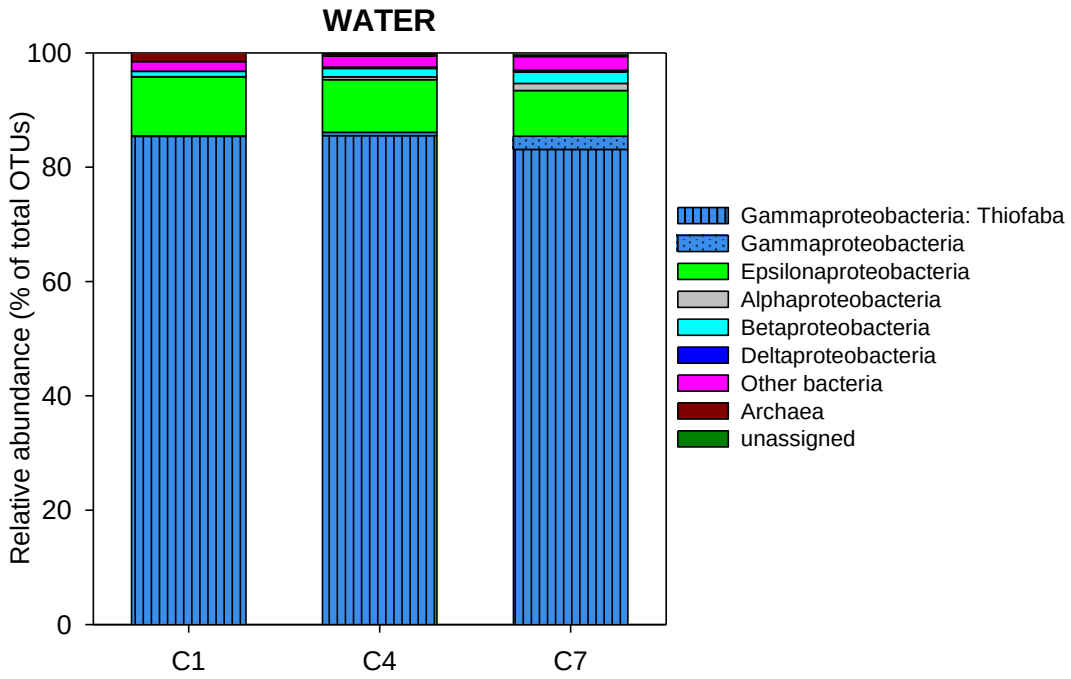

Figure 15. OTUs relative abundance in water samples estimated by NGS. Bacterial clusters making up less than 1% of total composition were classified as 'other bacteria'.

5.2.3 Prokaryotic abundance and community structure in biofilm

Overall, prokaryotic abundance was higher in superficial biofilm samples (on average $5.3 \times 10^9 \pm 1.2 \times 10^{10}$ cells/g) than in sub-superficial ones (on average $1.2 \times 10^8 \pm 6.7 \times 10^7$ cells/g) (Fig. 16). Along the channel, total abundance tends to decrease in superficial biofilm samples and increases in sub-superficial samples. In particular, values decreasing from 3.8×10^9 cells/g to 4.0×10^7 cells/g were observed in superficial biofilms from C2 to C8 samples. In contrast, increasing values from 5.2×10^7 cells/g to 8.8. $\times 10^7$ cells/g were observed in sub-superficial biofilms.

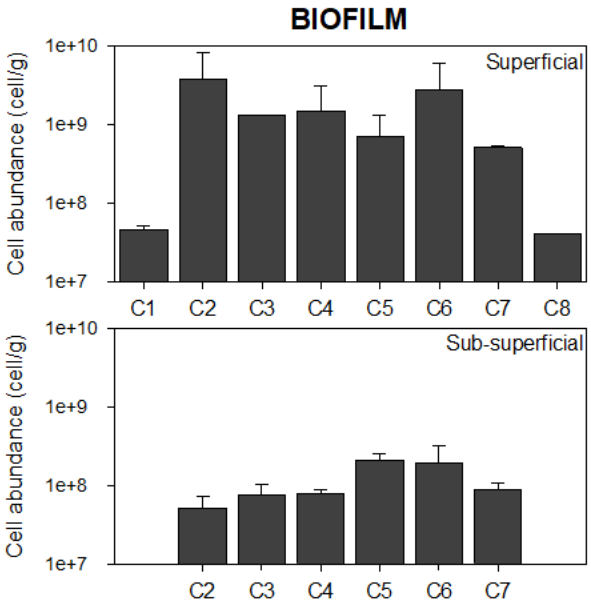

Figure 16. Total prokaryotic abundance in superficial and sub-superficial biofilm samples.

The microbial communities were dominated by *Bacteria* both in superficial and in sub-superficial layer representing on average the 90% of total prokaryotes estimated by CARD-FISH. Among bacterial cells, *Cyanobacteria* were highly abundant in superficial biofilm showing values moving from $2.7 \times 10^9 \pm 3.0 \times 10^7$ cells/g in C2 to $1.9 \times 10^7 \pm 3.5 \times 10^6$ cells/g in C8 (Fig. 17). Lower cyanobacterial abundance was observed in sub-superficial biofilm with an average value of $1.2 \times 10^7 \pm 7.1 \times 10^6$ cells/g. The various classes within *Proteobacteria* counted together on average $1.3 \times 10^8 \pm 1.4 \times 10^8$ cells/g and $3.1 \times 10^7 \pm 2.1 \times 10^7$ cells/g in superficial and sub-superficial layer, respectively.

High-throughput sequencing generated a total of 142,563 reads by C1, C4, C7 and C8 superficial and sub-superficial biofilm samples that resolved into 949 OTUs. The results showed a high microbial diversity among biofilm samples. Shannon and Simpson index values ranged between 2.4 and 2.7 and 0.8 and 0.9 respectively.

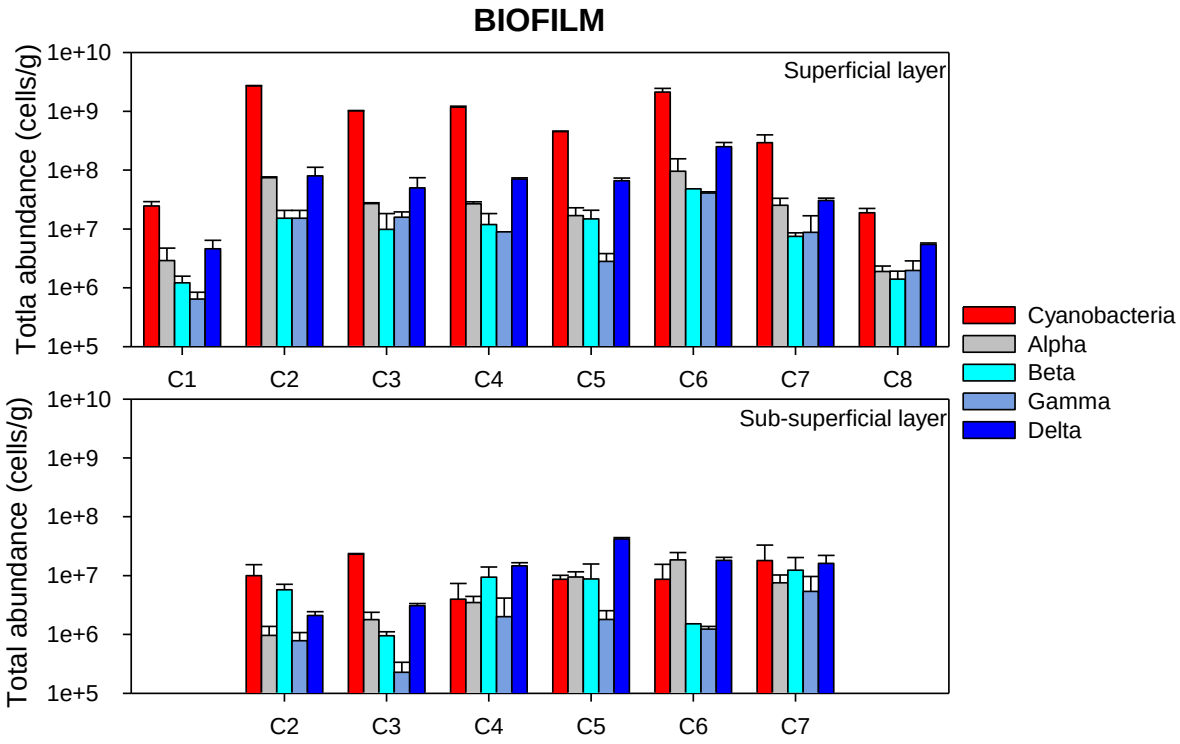

Figure 17. Total abundance of *Cyanobacteria* and single classes within *Proteobacteria* in superficial and sub-superficial biofilm samples.

OTUs affiliated with *Cyanobacteria* were more abundant in superficial layer (on average 37.3 %) than in sub-superficial one (on average 13.0 %) (Fig. 18). In particular, these OTUs mainly belonged to *Spirulina* (around 35 % in C4 and C7 superficial biofilm), *Leptolyngbya* (on average 8.4 % in superficial layer and 1.8 % in sub-superficial one), and *Fischerella* (up to 7.7 % in C4 sub-superficial biofilm) genera. Overall, members of *Proteobacteria* represented on average around 20% of total OTUs in biofilm samples. In particular, OTUs affiliated with *Alphaproteobacteria* were the most abundant both in superficial and sub-superficial biofilms. In particular, they mainly belonged to orders *Rhodobacterales*, *Rhodospirillales*, and *Sphingomonadales*. Furthermore, a high abundance of OTUs affiliated with *Rhodomicrobium*, a genus belonging to order *Rhizobiales* within *Alphaproteobacteria*, was mainly observed in C1 site (14.8 %). OTUs affiliated with *Gammaproteobacteria* represented on average 3.4 % and 1.7% of total OTUs in superficial and sub-superficial biofilms, respectively.

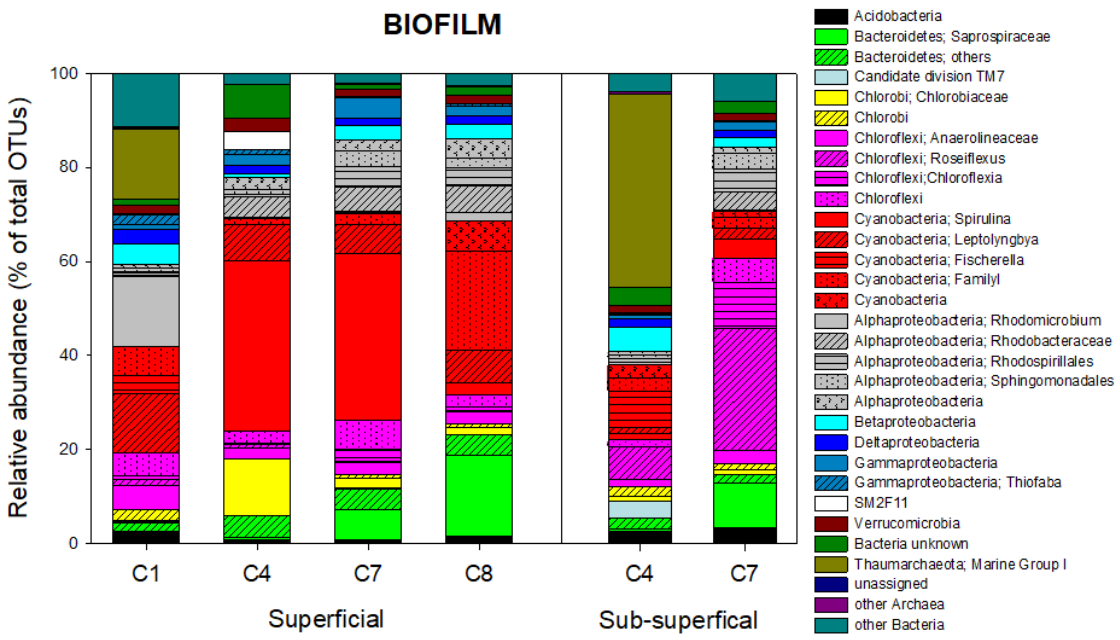

Figure 18. OTUs relative abundance in biofilm samples estimated by NGS. Clusters making up less than 1% of total composition were classified as 'other Bacteria' or 'other Archaea'.

The phylum *Chloroflexi* represented on average 8.8 % of total OTUs in superficial biofilms and up to 43.7 % in sub-superficial layers. In particular, these OTUs were mainly affiliated with family *Anaerolineaceae* in superficial biofilm and with genus *Roseiflexus* in sub-superficial one. Members of this phylum were highly abundant mainly in sub-superficial biofilm in C7 site.

OTUs affiliated with phylum *Bacteroidetes*, mostly belonging to family *Saprospiraceae*, increased along the channel up to 21.6 % of total OTUs in site C8.

The retrieved OTUs affiliated with phylum *Chlorobi* represented on average 2.6 % of total OTUs both in superficial and sub-superficial biofilms, with the sole exception of superficial biofilm in site C2 in which members of *Chlorobiaceae* represented up to 12.1 % of total OTUs.

As far as archaea domain is concerned, *Thaumarchaeota* represented between 14.9 % and 41.0 % of total OTUs in C1 and C4 sub-superficial biofilm, respectively. In the other biofilm samples the OTUs belonging to archaea domain represented less than 0.5 % of total OTUs.

5.2.4 Tridimensional structure and successional changes of biofilm

The development, biomass increments and three-dimensional structure of biofilm growing on microscopy slides placed on the central point of the channel (corresponding to sampling point C4) were observed by combining CARD-FISH and CLSM. After 2 and 7 days CLSM examination revealed biofilm assemblages with similar microbial community equally represented by filamentous *Cyanobacteria*, *Bacteria* and other prokaryotes (Fig. 19). After 12 days a highly complex and multi-stratified biofilm was observed, with a high amount of filamentous *Cyanobacteria*, similar to genus *Spirulina*, dominating the microbial community. Within the dense network of filamentous autotrophs, bacterial and prokaryotic cells were also visible.

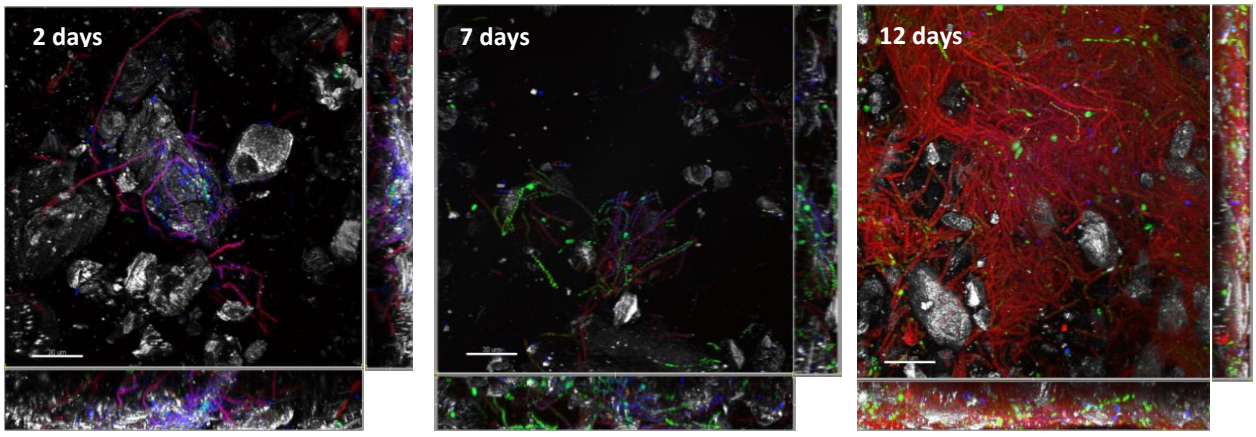

Figure 19. CLSM combined images showing the spatial distribution (X−Y, X−Z, and Y−Z planes) of Bacteria (green), Cyanobacteria (red) and other prokaryotes (blue) identified by CARD-FISH in biofilms. The hybridized bacterial cells were excited with the 488 nm line of an Ar laser (excitation) and observed in the green channel from 500 to 530 nm (emission). Calcite crystals were visualized by their reflection signal (405 nm line of a diodo laser) and appear of gray colour.

5.2.5 Discussion

Chemical characteristics of "Carletti thermal pools" were in line with those previously observed in similar aquatic geothermal environments in Central Italy (Fazi et al., 2016b; Valeriani et al., 2018). Total As concentration was lower than previously reported and the decrease of As(III)

along the channel was most likely dependent by the simultaneous increase of dissolved oxygen and light exposure (Fazi et al., 2016b).

Microbiological characteristics in water samples were in line with those observed in the previous sampling campaign; in particular, total prokaryotic abundance and HNA cells exhibited similar values previously found in the same hot spring (see section 5.1). The high abundance of HNA cells in the initial section of the channel suggested a higher microbial acivity compared to last section since these cells are often considered as the most active fraction (Lebaron et al., 2002). The decrease of HNA cells along the channel is most likely related to the transition from an anaerobic/anoxic high temperature aquatic environment to aerobic and low temperature conditions. Overall, hot springs are characterized by a low biodiversity due to their extreme conditions in terms of temperature and chemical characteristics (Kemp and Aller, 2004; Chiriac et al., 2017). In this study, low levels of biodiversity were observed in water columns probably due to high occurrence of hydrogen sulfide and other chemical compounds dissolved in volcanic hot spring waters (Giampaoli et al., 2013). As previously reported by Piscopo et al. (2006) and Valeriani et al. (2018), the geochemical conditions observed in volcanic hot springs in central Itay usually create an environment more suited to pioneer/resistant species reflecting low microbial diversity. In line with this report, the microbial community in the water column analyzed in this study was dominated by *Thiofaba* genus (*Gammaproteobacteria* class), able to grow under strict aerobic conditions with a chemolithoautotrophic metabolism. Members of this genus are well known for their capability to oxidize sulfur compound by utilizing H_2S as electron donor for CO_2 reduction (Van Gemerden, 1993) and are widely reported in hot springs worldwide (Gulecal-Pektas and Temel, 2017; Gumerov et al., 2011; Huang et al., 2011; Mori and Suzuki, 2008; Valeriani et al., 2018).

Furthermore, the occurrence of *Epsilonproteobacteria* in water column confirmed the involvement of microbial community in sulfur cycle along the channel. Members of this class, indeed, has been proposed to being actively involved in sulfur cycling, constituting one of the major sulfur-oxidizers microbial group in high temperature thermal springs (Campbell et al., 2006).

The occurrence of *Gamma*- and *Epsilonproteobacteria* and the absence of microorganisms actively involved in energetic As-metabolism were in line with the findings previously reported in the same hot spring (see section 5.1) confirming the capability of microbial community inhabiting such geothermal environment to resist high As concentration and to play a key role in sulfur cycling.

Altough *Thiofaba* genus and *Epsilonproteboacteria* were highly abundant in water, very low occurrence was found in biofilm samples. Remarkably, the complex microbial community inhabiting the biofilm along the channel was mainly composed by phototrophic microorganisms, in line with those previously observed in other hot springs (Bilyj et al., 2014; Coman et al., 2013; Potillo et al., 2009; Stal et al., 2017; Wang et al., 2013). In particular, the predominant metabolic pathway was the oxygenic and anoxygenic phototrophy driven by members of *Cyanobacteria*, *Chloroflexi*, *Chlorobi* and *Alphaproteobacteria* (Coman et al., 2013). A stratified microbial communy was retrived in biofilm along the channel as previously observed in the hot springs mirobial mats; in particular the aerobic photoautotrophic *Cyanobacteria* inhabited the superficial layer while the anoxic phototrophs dominated underneath (Fig. 20) (Konhauser 2007; Liu et al., 2011; Coman et al., 2013; Pagaling et al. 2012; Hanada, 2016).

The differences in cyanobacterial OTUs were most likely linked to the water temperature. In particular, it was previously documented that *Fischerella* species prevails at temperatures

around 40 °C (Konhauser 2007) corresponding to C4 site, while *Leptolyngbya* prefers temperatures around 55 °C (McGregor and Rasmussen 2008; Roeselers et al. 2007; Sompong et al. 2008) corresponding to C1 site. The high occurrence of filamentous *Cyanobacteria* involved in CO_2 fixation, such as *Leptolyngbya* and *Spirulina*, was in line with previous reports in hot springs wordlwide (Amarouche-Yala et al., 2014; Subudhi et al., 2018; Valeriani et al., 2018).

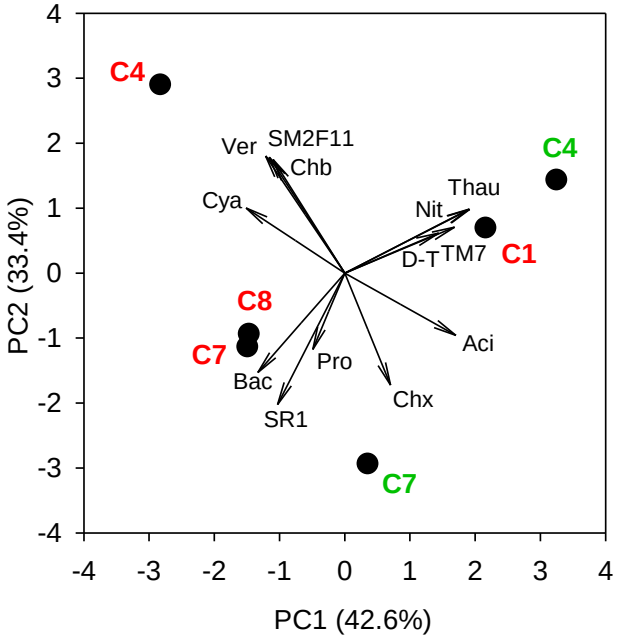

Figure 20. Principal Components Analysis biplot representing the typifying microbial biofilm composition at phylum level as revealed by NGS. Length of arrows represents the correlation between corresponding parameters and PCA axis 1 and 2. Biofilm samples from the superficial and sub-superficial layer are reported red and green, respectively. Aci, *Acidobacteria*; Bac, *Bacteroidetes*: Chb, *Chlorobi*; Chx, *Chloroflexi*; Cya, *Cyanobacteria*; D-T, *Deinococcus-Thermus*; Nit, *Nitrospirae*; Pro, *Proteobacteria*; SMF11; SR1, Candidate division SR1; Thau, *Thaumarchaeota*; TM7, Candidate division TM7, Ver, *Verrucomicrobia*.

Due to the different oxygen availability, anoxygenic phototrophic microorganisms dominated the sub-superficial layer and were mainly represented by green and purple sulfur/non-sulfur bacteria, such as members of *Chlorobi*, *Chloroflexi* and *Alphaproteobacteria*.

Green sulfur bacteria, mainly affiliated with family *Chlorobiaceae*, were found in biofilm along the channel in line with their anaerobic and autotrophic metabolism (Imhoff 2014). These

phototrophs are able to oxidize elemental sulfur and sulfide and to carry out photosynthesis only under anoxic conditions (Asao and Madigan 2010; Imhoff 2014; Madigan et al., 2017). Furthermore, the occurrence of *Chloroflexi*, affiliated with family *Anaerolinaceae* or genus *Roseiflexus* currently known as "Filamentous Anoxygenic Phototrophs", was previously reported in thermophilic cyanobacterial mats (Gaisin et al., 2016; Kambura et al., 2016; Tank et al., 2017; Valeriani et al., 2018).

Anaerobic and anoxygenic phototrophic purple non sulfur bacteria were frequently reported in suldific hot springs with temperature around 50°C (Ainon, Tan and Vikineswary, 2006; Bilyj et al., 2014; Imhoff, 2017). In line with this finding, members of *Alphaproteobacteria*, mainly affiliated with *Rhodomicrobium* genus, were encountered in biofilm samples along the channel. Members of this genus preferably grow photoheterotrophically under anoxic conditions in the light, but can also use hydrogen, sulfide or ferrous iron as electrons sources (Imhoff et al., 2005).

Remarkably, a high abundance of OTUs affiliated with *Thaumarchaeota* was observed in the initial and central section of the channel (Fig. 20). Members of this phylum represent the most abundant Archaea on Earth inhabiting moderate but also extreme environments, such as hot springs and acidic soils (Stieglmeier et al., 2014). The habitat temperatures range from 1°C to 97°C with pH values ranging from 2.5 to 9. This phylum includes all known autotrophic archaeal ammonia oxidizers as well as environmental sequences representing microorganisms with unknown metabolism (Pester et al., 2011; Stieglmeier et al., 2014). Lastly, OTUs affiliated with *Saprospiraceae* (mebers of *Bacteroidetes*) were more abundant in the final section of the channel (Fig. 20) likely due to their ability to live under aerobic and mesophilic conditions (McIlroy and Nielsen, 2014).

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

The following chapter has been modified and adapted from the original publication "Crognale, S., Casentini, B., Amalfitano, S., Fazi, S., Petruccioli, M., Rossetti, S. (2019). Biological As(III) oxidation in biofilters by using native groundwater microorganisms". *Science of the Total Environment*, 651(1),93-102".
Available at: https://www.sciencedirect.com/science/article/pii/S0048969718336209

5.3.1 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) concentration of 100 μg/L. After about 2 weeks of acclimation period both biofilters were able to oxidize only 30% of As(III) within two hours (Fig.21). 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. 21). Biofilters with biofilms at this maturation stage were selected for this study.

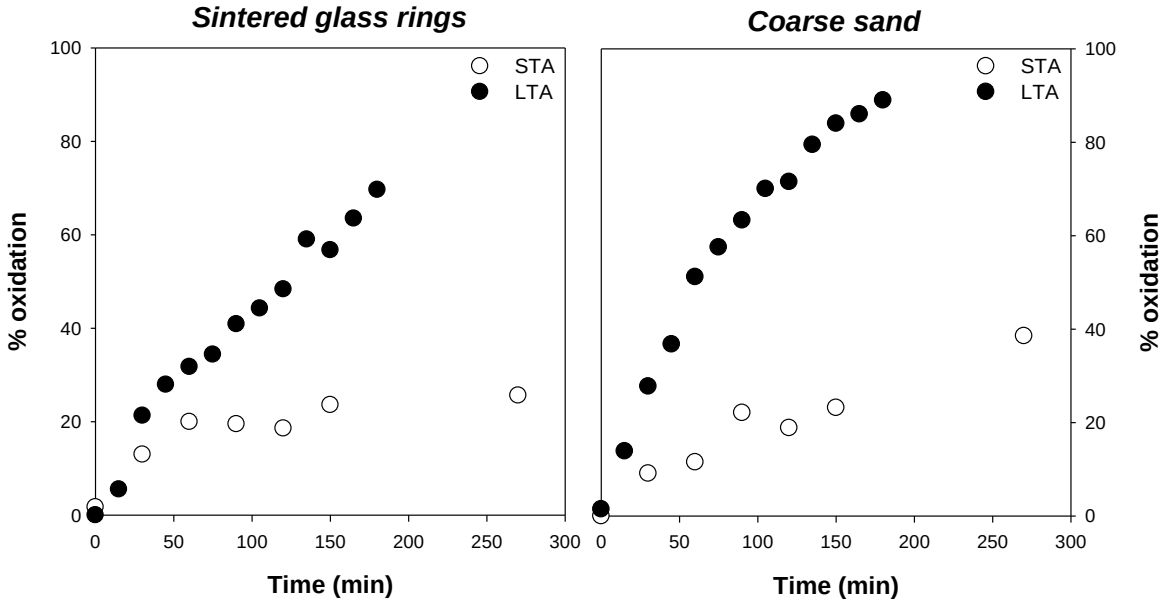

Figure 21. Arsenite oxidation kinetics in glass and sand biofilters with BV 0.1 L after different acclimation period to As(III). LTA: biofilm exposed to As(III) for a long-term acclimation period (around 40 days) before performing the kinetic tests. STA: biofilm exposed to As(III) for a short-term acclimation period (around two weeks) before the kinetic tests.

5.3.2 Influence of flow rate

The influence of flow rate on both materials was tested at 70, 140 and 250 mL/min, 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 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 (Fig. 22 and Appendix 7).

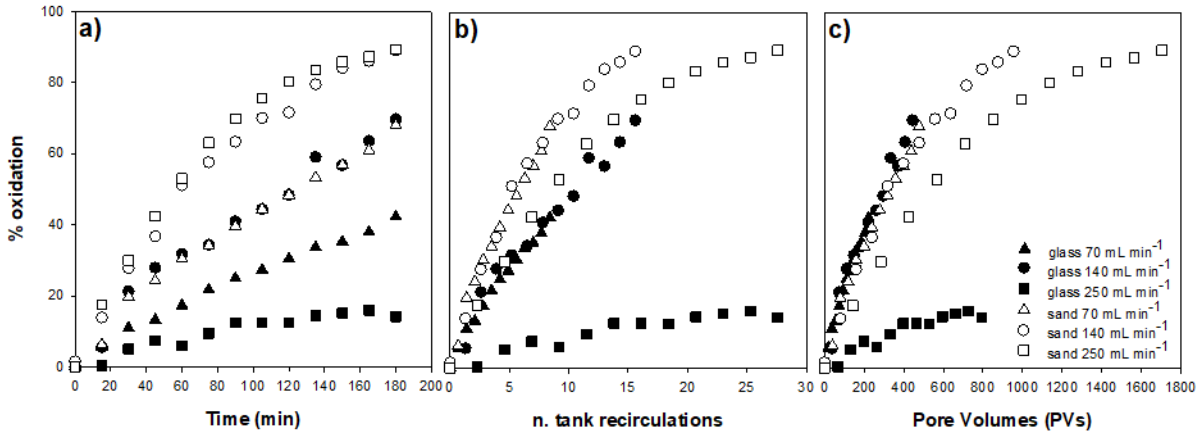

Figure 22. Arsenite oxidation kinetics at different flow rates (70 mL min^{-1}, 140 mL min^{-1}, 250 mL min^{-1}) in glass rings ("glass") and coarse sand ("sand") biofilters. The oxidative efficiency (%) was plotted according to elapsed time (min) (panel a), n. tank recirculations (panel b), and pore volumes (PVs) (panel c).

The overall oxidation was similar when the sand biofilter was operated at 140 and 250 mL/min (about 89%) while only 67.9% of As(III) was oxidized at 70 mL/min (Fig. 22a). After the same number of recirculations, the performances of the columns operating at 70 and 140 mL/min were identical, with an oxidation efficiency of about 40% and 65% in glass and sand,

respectively (Fig. 22b). Remarkably lower efficiencies were measured at a flow rate of 250 mL/min for both materials. As shown in Figure 22c, 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. At 500 PVs, almost 70% of As(III) was oxidized in both biofilters operating at a flow rate of 140 mL/min. 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, 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 corresponds to a linear velocity of 11.9 m/h, the same value previously applied in a pilot unit for the daily production of 400 L drinking water (Casentini et al., 2016).

5.3.3 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 7). 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 ≥ 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 7 and Fig. 23). The oxidation rate was similar in both tests carried out with glass column, corresponding to 0.4 μg/Lh. Diversely, the oxidation

rate in sand biofilter increased with the decrease of As(III) concentration (0.8, 0.9 and 1.1 µg/Lh at 100, 50 and 20 µg As(III)/L, respectively).

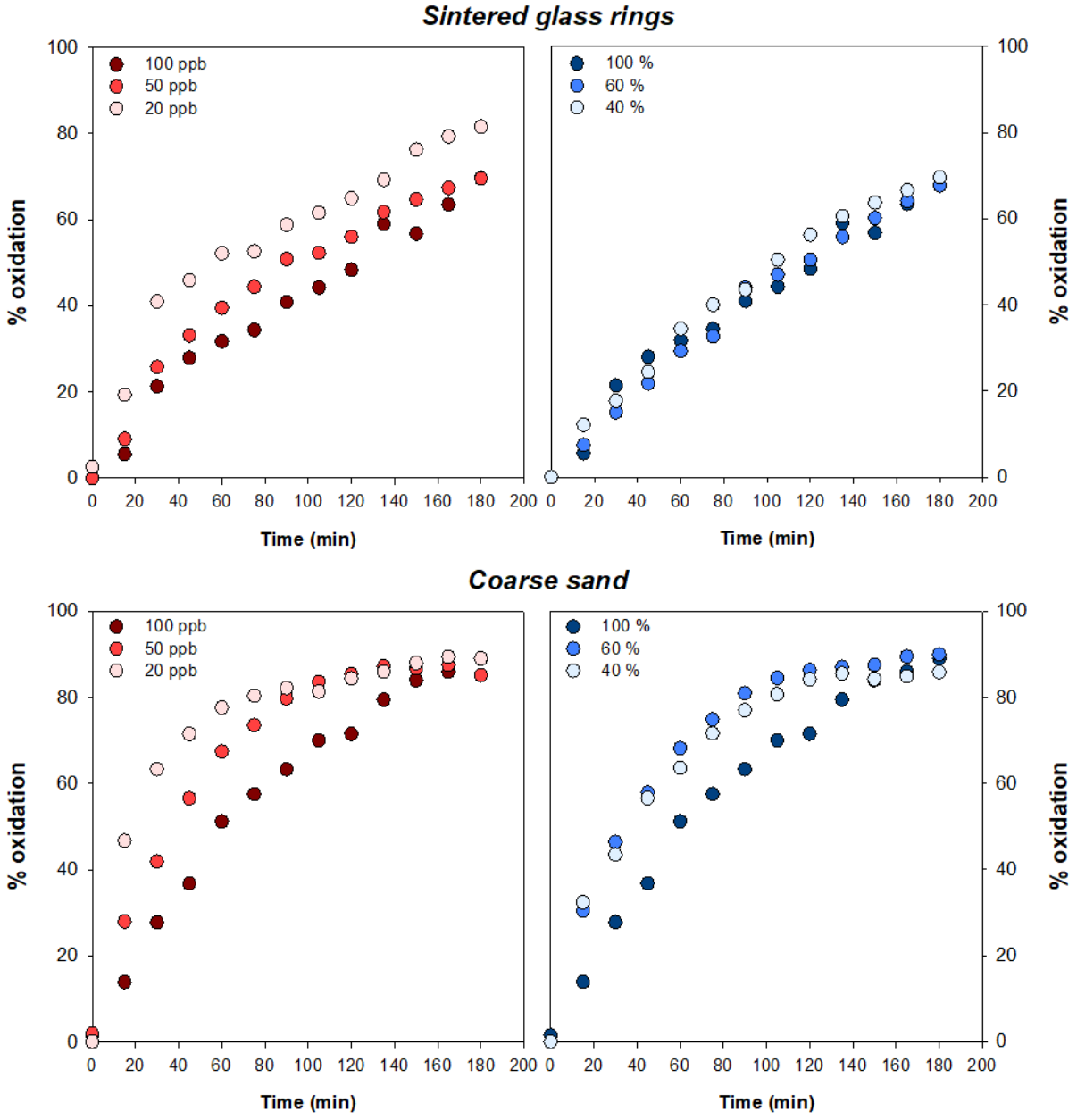

Figure 23. Arsenite oxidation in biofilters with BV 0.1 L fed at 140 ml/min at different initial As concentration (20, 50, 100 µg/L) and different As(III):As(V) ratio (100:0; 60:40, 40:60).

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.

Table 7. Arsenite oxidation in biofilters with BV 0.1 L fed at 140 mL/min at different initial As concentration (20, 50, 100 µg/L) 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**	**50 µg/L**	**20 µg/L**	**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**	**50 µg/L**	**20 µg/L**	**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**

By comparing similar treated PVs (Table 7), 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.

5.3.4 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). By increasing 7 times the BV, from 0.1 to 0.7 L, a higher increase in overall oxidation performances was observed (Fig. 24) in both glass and sand columns.

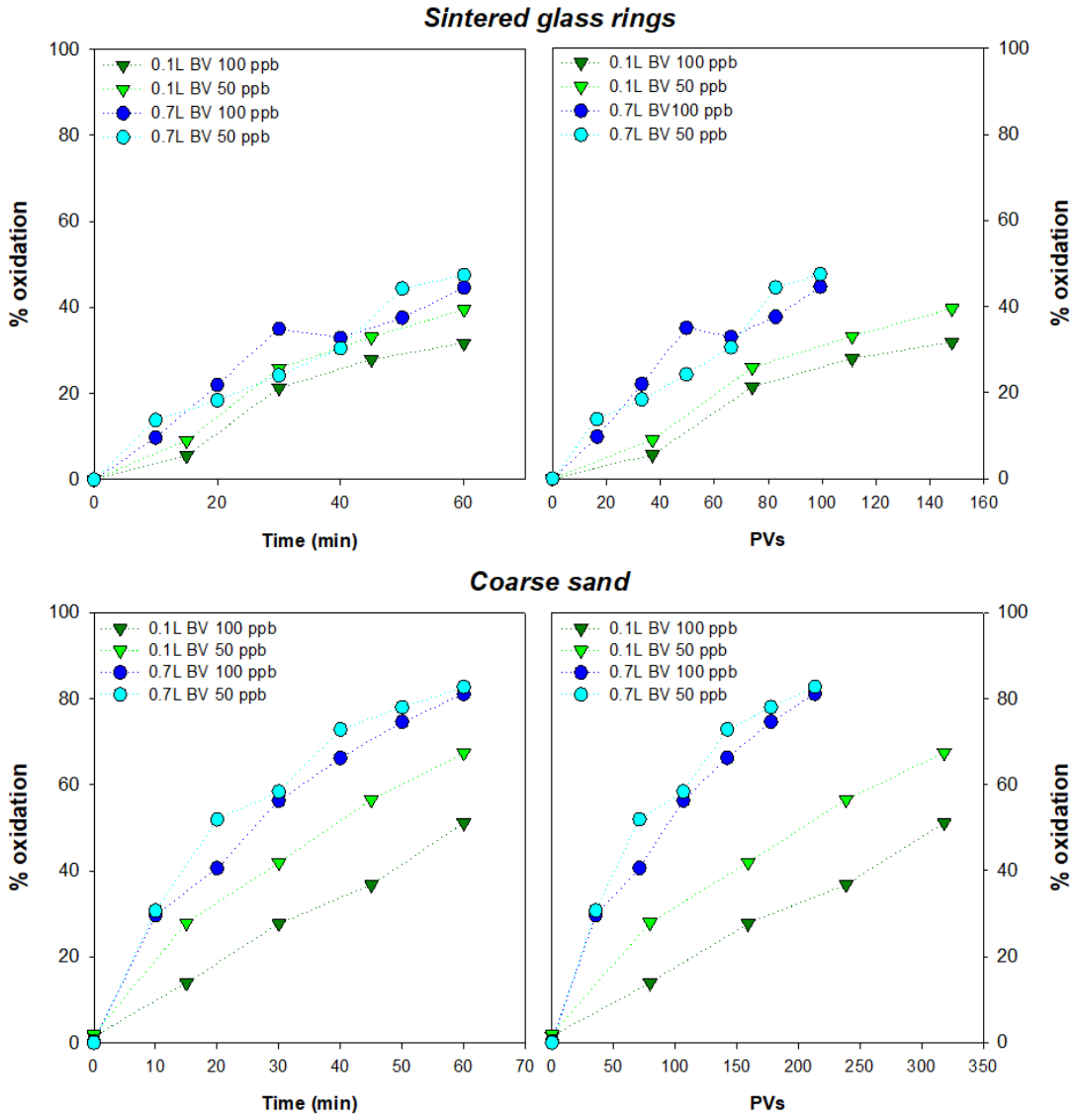

Figure 24. Arsenite oxidation in biofilters with BV 0.1 L and 0.7 L by testing different initial As(III) concentration.

Larger biofilters showed the same ability in oxidizing an initial amount of 50 or 100 µg/L As(III) for both materials. After 1 hour glass biofilters oxidized about 45% and sand filters 80% of As(III). As shown in Figure 24, 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.

5.3.5 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. 25).

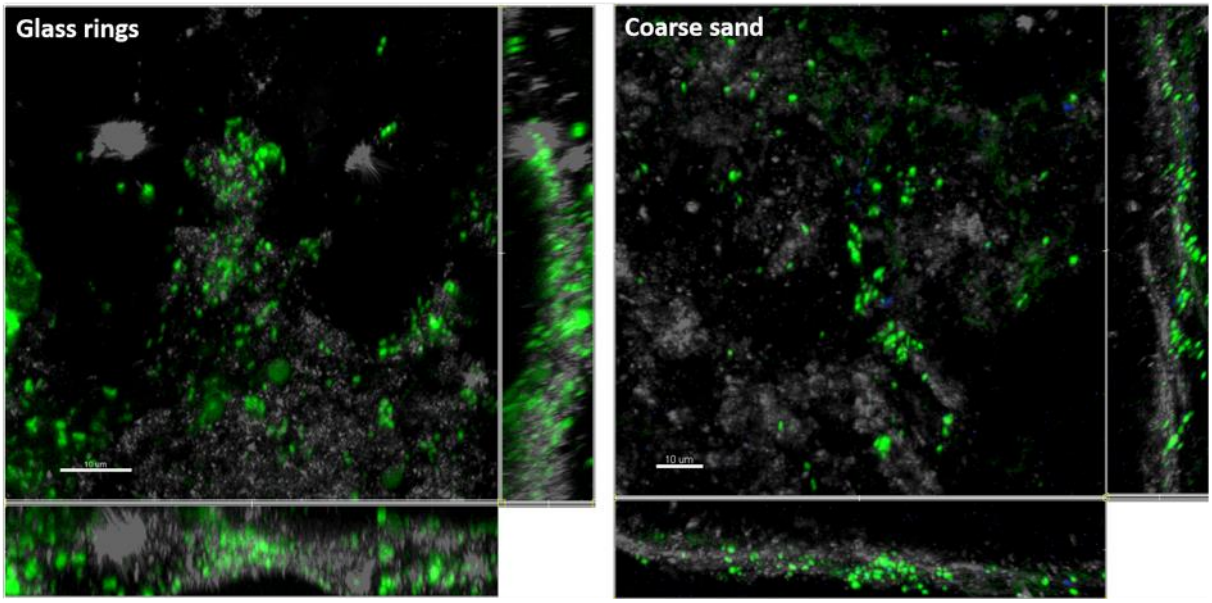

Figure 25. CLSM combined images showing the spatial distribution (X−Y, X−Z, and Y−Z planes) of Bacteria (green) identified by CARD-FISH in biofilms grown on glass rings and coarse sand. The hybridized bacterial cells were excited with the 488 nm line of an Ar laser (excitation) and observed in the green 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).

The prokaryotic abundance was similar (16.4 ± 3.9 x 10^6 cells/g of filling material) in biofilms grown on glass rings in spite of the different acclimation period imposed (Table 8). 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 and 7.4 x 10^6 cells/g, 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%).

Table 8. Prokaryotic abundance (cells/g) 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.

		PAB 10^6 cells/g	**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

5.3.6 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. 26). 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 x10^8 ± 8.9 x 10^7 gene copies/g and 1.7 x 10^{10} ± 3.6 x 10^9 gene copies/g. The genes for the As(III) membrane efflux pump (*arsB*) showed lower values on average around 2.2 x 10^6 ± 3.9 x 10^5 gene copies/g.

The gene *aioA*, involved in As(III) oxidation, was reported in all biofilm samples with values between 5.7 x 10^5 ± 2.3 x 10^5 and 1.8 x 10^6 ± 3.0 x 10^5 gene copies/g in glass biofilms. The abundance of *aioA* differed considerably between STA and LTA sand biofilms, ranging between 1.8 x 10^6 ± 1.2 x10^5 and 6.0 x 10^6 ± 5.5 x 10^4 gene copies/g.

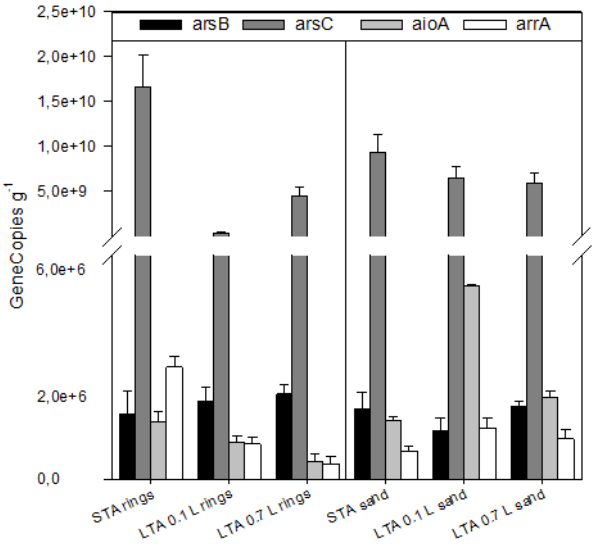

Figure 26. Abundance of arsenic-related functional genes estimated by qPCR in biofilms (gene copies/g). *arsB*, arsenite transporter; *arsC*, arsenate cytoplasmic reductase; *aioA*, arsenite oxidase; *arrA*, respiratory arsenate reductase; STA "material", biofilm established on glass rings or coarse 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.

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 x 10^6 ± 4.1 x 10^4 gene copies/g) than in sand biofilms (on average 1.2 x 10^6 ± 3.6 x 10^5 gene copies/g). The occurrence of As-related functional genes was reported also in groundwater used in the experiments (Fig. 27).

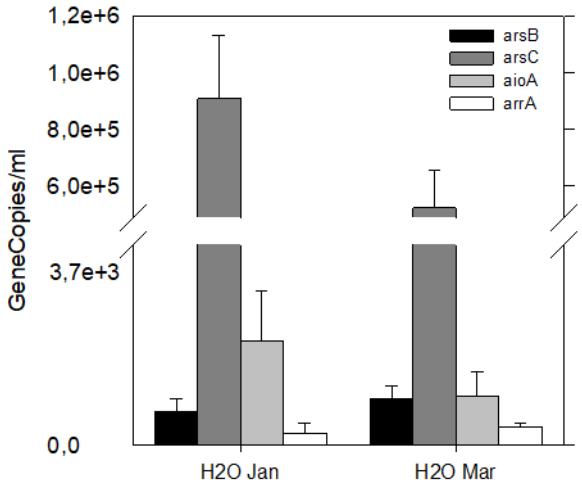

Figure 27. Arsenic-related functional genes abundance (gene copies/ml) estimated by qPCR in groundwater used for the kinetic tests sampled in two different periods (January and March). *arsB*, arsenite transporter; *arsC*, arsenate cytoplasmic reductase; *aioA*, arsenite oxidase; *arrA*, respiratory arsenate reductase.

5.3.7 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 (Appendix 8). A high microbial diversity was observed in all biofilm samples as highlighted by the Simpson index (range between 0.9 and 1). The Shannon index showed values higher in glass biofilms (on average 4.0) than in sand ones (on average 3.4) and in particular these index values were higher in biofilms grown in biofilters with a BV of 0.1 L (range between 3.4 and 4.3) rather than in 0.7 L ones (range between 3.3 and 3.7). *Proteobacteria* was the most abundant phylum showing values up to 40.7% and 45.8% of total OTUs in glass biofilms and up to 33.5% and 39.2% in sand biofilms. This phylum was mainly represented by *Alphaproteobacteria*, affiliated with orders *Caulobacterales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Sphingomonadales*, and *Betaproteobacteria* mostly belonging to orders *Burkholderiales*, *Nitrosomonadales* and *Rhodocyclales* (Fig. 28). Among

these orders the occurrence of several OTUs affiliated with aerobic and heterotrophic genera was reported (e.g. *Phenylobacterium*, *Hirschia*, *Woodsholea*, *Bradyrhizobium*, *Rhodobacter*, *Roseomonas*, *Limnobacter*, *Leptothrix*, *Noviherbaspirillum*) as well as to aerobic and autotrophic genera (i.e. *Hydrogenophaga*, *Variovorax).*

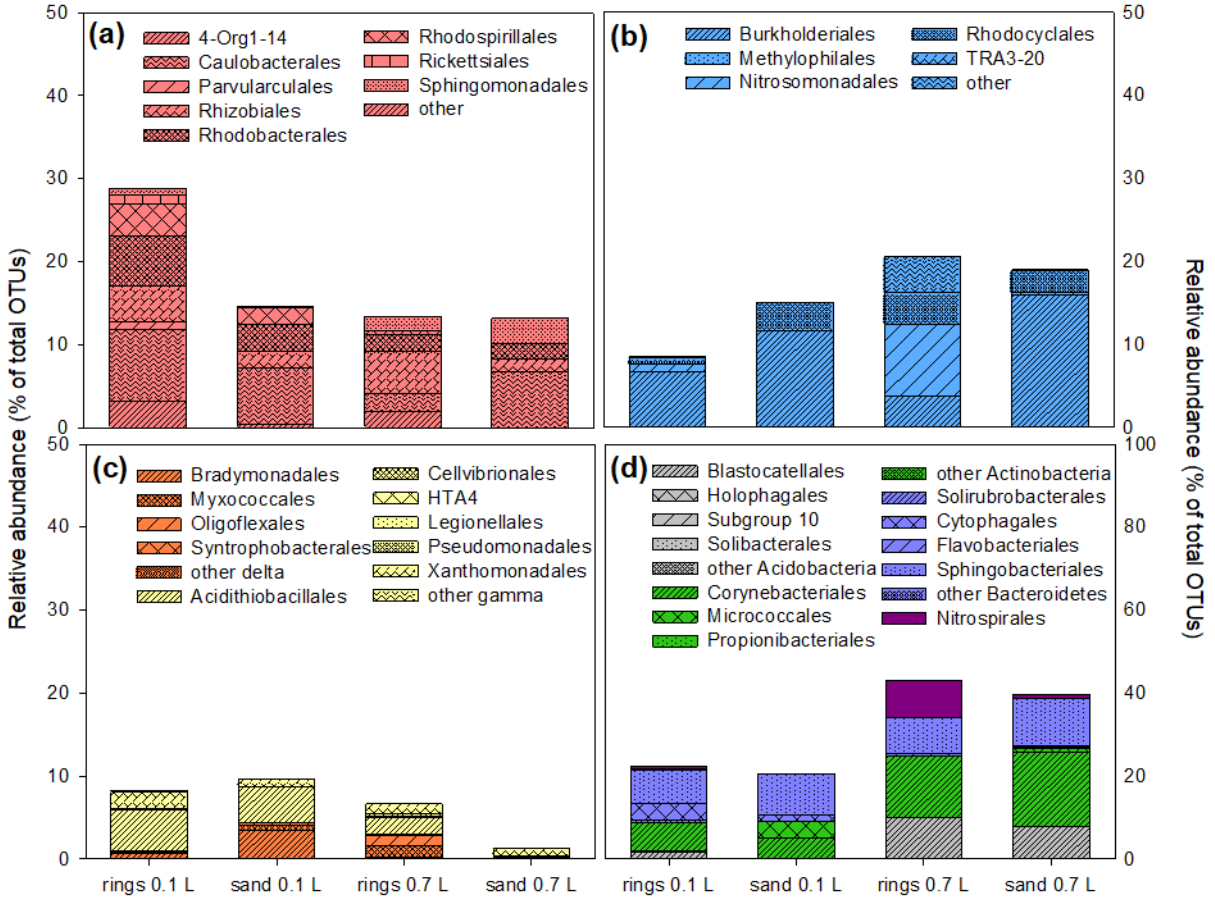

Figure 28. Operational taxonomic units (OTUs) relative abundance (%) estimated by high-throughput sequencing in long-term acclimated (LTA) biofilms with BV 0.1 L and 0.7 L. Clusters at order level in proteobacterial classes *Alphaproteobacteria* (panel a), *Betaproteobacteria* (panel b), *Delta-* and *Gammaproteobacteria* (panel c) are represented. The graphical representation of order composition within phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Nitrospirae* is reported in panel d.

Furthermore, OTUs affiliated to anaerobic heterotrophic or mixotrophic genera, such as *Azoarcus* and *Acidovorax*, were found. *Delta-* and *Gammaproteobacteria* were mainly represented by orders *Bradymonadales*, *Acidithiobacillales* and *Xanthomonadales*; the latter order was mainly represented by members of facultative anaerobic heterotrophic genus

Pseudoxanthomonas. Additionally, the occurrence of OTUs affiliated with *Actinobacteria* (7.3 - 18.8% of total OTUs), *Bacteroidetes* (9.2 - 12.5% of total OTUs) and *Acidobacteria* (up to 10%) was revealed. *Acidobacteria* and *Bacteroidetes* were mainly represented by the aerobic heterotrophic families *Blastocatellaceae*, *Cytophagaceae* and *Chitinophagaceae*.

Other phyla were overall found at minor extent with some exceptions as for example *Nitrospirae* in glass biofilter with BV 0.7 L (9% of total OTUs), *Ignavibacteriae* in sand biofiter with BV 0.7 L (19.3% of total OTUs), *Planctomycetes* and *Armatimonadetes* in glass biofilter with BV 0.1 L (10.2 and 7.2% of total OTUs), and *Cyanobacteria* in sand biofiter with BV 0.1 L (27.3% of total OTUs). The relative abundance of OTUs in groundwater differed considerably (Appendix 8 and Fig. 29). In this sample *Proteobacteria* and *Nitrospirae* represented up to 97% of total OTUs, mainly affiliated with genera *Nitrosomonas* (on average 65.4% of total OTUs) and *Nitrospira* (on average 28.5% of total OTUs).

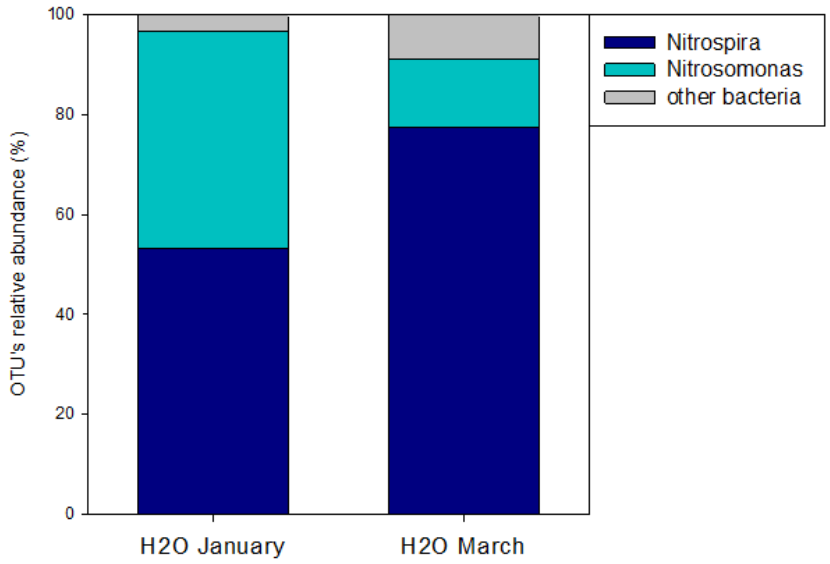

Figure 29. Operational taxonomic units (OTUs) relative abundance (%) estimated by high-throughput sequencing in groundwater used for the experiments.

5.3.8 Discussion

The groundwater mixed microbial communities were able to promote a highly efficient and rapid As(III)-oxidation through the development of biofilms enriched in arsenite oxidizers and specific As-related functional genes.

A similar oxidation efficiency was observed between the two filling materials when the same treated pore volumes are considered. However, the halved porosity of coarse sand compared to glass rings allowed a higher number of recirculations through the biofilters within the same operational time. This may explain the markedly different oxidative efficiencies observed between glass and sand biofilters. It is worth to noting that arsenic was not retained during the operation of the biofilters, diversely from previous studies (Li et al., 2016; Wan et al., 2010).

Various hydraulic and chemical conditions were here tested for the first time to evaluate the potentialities of microbial As(III) oxidation in biofilters by using native microbial population occurring in As-contaminated groundwater.

Interestingly, the highest oxidation efficiencies occurred within few hours (2-3 hours) and this finding sustains the feasibility of coupling this biological process with the As(V) physical-chemical removal treatment.

The use of various flow rates strongly affected the oxidative performance of the biofilters, mainly due to the different treated pore volumes rather than the contact time between water and filling materials. Biotic oxidation was independent from contact time, most probably due to the imposed very short contact times in selected filter columns. In particular, under the tested operation conditions, a higher volume of the glass biofilter, a longer duration of the treatment or a higher number of tank recirculations would be required to achieve the same oxidative efficiencies observed within three hours by using sand biofilters.

Furthermore, the biological As(III) oxidation was found to be mostly affected by the initial As concentration rather than As speciation, at natural groundwater concentrations range. The latter suggests the process feasibility of treating natural groundwater containing both As(III) and As(V) species common in volcanic regions (Crognale et al., 2017b; Fazi et al., 2016b). The initial concentration of As(III) mostly impacted the oxidation rate rather than the efficiency. In particular, the highest oxidation efficiency was observed at the lowest As(III) concentration, in line with previous evidences on pure cultures (Bahar et al., 2016; Debiec et al., 2017).

Overall, the oxidation rates were in line with those obtained by using pure or enrichment cultures from low As-content groundwater (Casiot et al., 2006) but lower compared to As(III)-oxidizing microorganisms from high As-content environments (Battaglia-Brunet et al., 2002; Debiec et al., 2017; Li et al., 2016; Wan et al., 2010). The latter evidence suggests that the overall process performance is likely expected to increase when As-richer groundwaters are treated. Autochthonous mixed microbial communities, already acclimated to high As contamination, might form high-performing As(III) mixed biofilm under the same operating conditions here investigated.

In line with the evidences reported in Gude et al. (2018), this study showed that a long exposure time to As(III) promoted the formation of biofilms with marked capabilities to withstand As and to oxidize As(III). The highest oxidation efficiencies of biofilm grown on sand can be also related to the larger colonization surface available for microorganisms compared to glass rings. The analysis performed on biofilm grown at different acclimation periods clearly showed the increase of As(III) oxidation efficiencies with increase of the cell densities.

The accurate quantification of microbial cells in water biofilters was recently considered essential for substrate utilization kinetics and theoretical system modelling, with flow cytometry proved as a useful tool for biomass characterization and the comprehensive

interpretation of microbiome surveys (Vignola et al., 2018; Props et al., 2017). The relative abundance of cells with different morphology and nucleic acid content was used as a gross parameter to rapidly assess distinct microbial phenotypic traits in water systems (Amalfitano et al., 2018; Props et al., 2018). The occurrence of LNA and HNA groups in different proportions was reported to reflect the activity state of the microbial communities (Lebaron et al., 2002), but also a different phylogenetic community composition (Proctor et al., 2018).

In this study, we found a higher relative abundance of HNA cells in LTA sand compared to the LTA glass biofilters, thus underlying that the observed oxidation performances could rely on either functional or structural traits of microbial communities. Interestingly, the high abundance of HNA cells was already reported in As-rich aquatic environments (Crognale et al., 2017b; Fazi et al., 2016b).

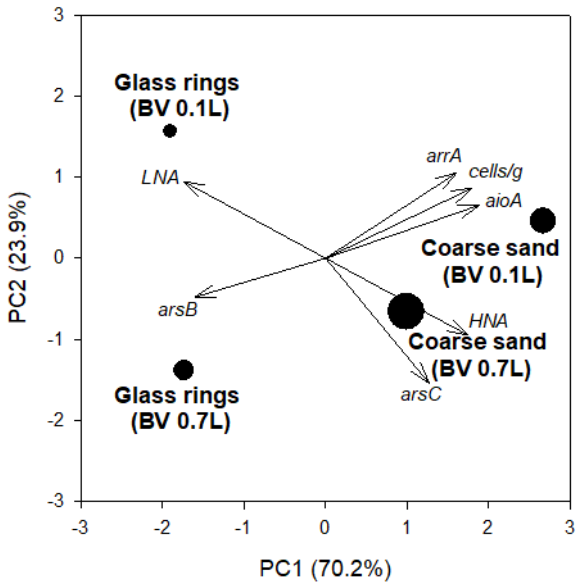

Figure 30. Principal Components Analysis biplot performed using quantification data of As-related functional genes (*aioA*, *arrA*, *arsB*, *arsC*), prokaryotic abundance (cells/g) and the relative abundance of low and high nucleic acid content cells (HNA and LNA). The vector length is proportional to the correlation between corresponding parameter and the PCA axis 1 and 2. The symbol size indicating biofilm samples are proportionally related to the average oxidation rate.

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 30, 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 to 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 widespread in the genome of many 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).

Even though autotrophy was most likely the main mechanism under the operation conditions imposed to the biofilters, the simultaneous occurrence of heterotrophic oxidation cannot be excluded. Heterotrophic arsenite oxidising bacteria might grow on the groundwater endogenous organic carbon or on the carbon released by naturally occurring cell decay.

The presence of *arrA* genes mainly in STA glass biofilters might 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). Although highly oxygenated water (DO = ~8 mgL^{-1}) was recirculated in the biofilters, the formation of small anoxic niches in the inner biofilm layers promoting As(V)-reductive transformations cannot be excluded.

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. 31).

The composition of microbial communities in bioreactors differed considerably from groundwater used for the experiments, suggesting an adaptation to high As(III) concentrations (Fig. 28 and Appendix 8). 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 in bioreactors and household filter sand used for the treatment of As-contaminated groundwater (Gude et al., 2018; Li et al., 2014, 2016). The large occurrence of OTUs affiliated with Burkholderiales, at higher extent in sand biofilms, is most likely related to the observed As(III) oxidation since these microorganisms are well known for their capability to resist and oxidize As(III) (Li et al., 2014). Furthermore, the occurrence of OTUs affiliated with Bradyrhizobium, Rhodobacter, and Roseomonas, confirmed the active role of microbial communities in As(III) oxidation (Bagade et al., 2016; Battaglia-Brunet et al., 2006; Li et al., 2016; Sultana et al., 2012).

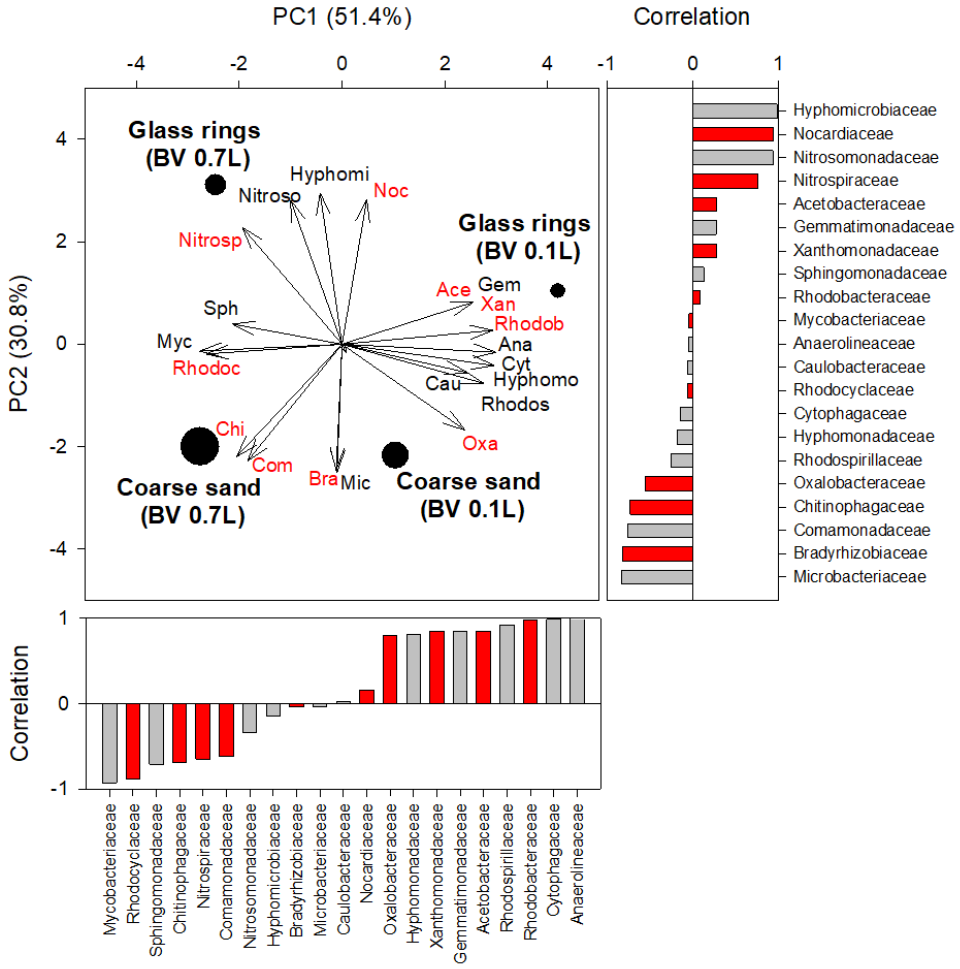

Figure 31. Principal Components Analysis biplot representing the typifying As-related microbial composition at family level. Length of arrows represents the correlation between corresponding parameters and PCA axis 1 and 2. The symbol size indicating biofilm samples are proportionally related to the average oxidation rate. Histogram plots show the contribution of each variable (vector projection values) expressed as the correlation with the x- and y-axis. Families known for their involvement in As-resistance and As(III) oxidation processes are reported in black and red, respectively. Families not involved in As-transformations and with an occurrence <1% are not considered in this analysis. Ace, *Acetobacteraceae*; Ana, *Anaerolineaceae*; Bra, *Bradyrhizobiaceae*; Cau, *Caulobacteraceae*; Chi, *Chitinophagaceae*; Com, *Comamonadaceae*; Cyt, *Cytophagaceae*; Gem, *Gemmatimonadaceae*; Hyphomi, *Hyphomicrobiaceae*; Hyphomo, *Hyphomonadaceae*; Mic, *Microbacteriaceae*; Myc, *Mycobacteriaceae*; Nitroso, *Nitrosomonadaceae*; Nitrosp, *Nitrospiraceae*; Noc, *Nocardiaceae*; Oxa, *Oxalobacteraceae*; Rhodob, *Rhodobacteraceae*; Rhodoc, *Rhodocyclaceae*; Rhodos, *Rhodospirillaceae*; Sph, *Sphingomonadaceae*; Xan, *Xanthomonadaceae*.

Thesis conclusions

Overall, the PhD research activity has allowed to describe the native microbial communities in As-rich aquatic environments and to highlight the high potentialities of the biological As(III)-oxidation processes for water treatment.

This study provided the structure and composition of the water microbiomes exposed to a natural range of geogenic As contamination. The main metabolic traits and potentialities of microorganisms involved in As transformation were also elucidated. Since the occurrence and distribution of arsenic-related genes were not previously exhaustively explored in waters of geothermal origin, this work provided field insights on the metabolic activities and potentialities of microbial communities related to arsenic detoxification, reduction and oxidation. The As detoxification were found to prevail over As metabolic processes since the microbial communities were able to withstand high As levels, without using it for energetic metabolism. Concomitantly, the intriguing occurrence of novel thermophiles able to tolerate high As concentrations was found in the screened geothermal waters. This study also elucidated the microbial community dynamics in water and biofilm occurring in geothermal environment along a natural temperature, oxygen and pH gradient in the presence of a constant high total As content. Notably, complex and stratified microbial communities with a high level of biodiversity inhabited the biofilm in volcanic hot spring and were actively involved in the oxygenic and anoxygenic phototrophy.

The laboratory-scale experiments 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 systems. 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 biofilter start-up can be easily performed by recirculating As-contaminated groundwater within a reasonable period of time without the addition of specialized As(III) oxidizing bacteria. The native groundwater bacteria were indeed able to withstand high As concentration and easily formed highly As(III) oxidizing biofilms.

In conclusion, this study allowed to better understanding the capabilities of autochthonous microorganisms in arsenic treatment processes in naturally As contaminated waters by exploiting the diversity and wide distribution of functional genes controlling arsenic transformation in such environments. This study has contributed to expand the current knowledge on microbial As-transformations capabilities by providing information of pivotal interest in the future application of biological processes in the removal of As from drinking water.

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List of publications

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2. **Crognale** S, Venturi S, Tassi F, Rossetti S, Rashed H, Cabassi J, Capecchiacci F, Nisi B, Vaselli O, Morrison HG, Sogin ML, Fazi S (2018) Microbiome profiling in extremely acidic soils affected by hydrothermal fluids: the case of Solfatara Crater (Campi Flegrei, southern Italy). FEMS Microbiol Ecol 94(12). doi.org/10.1093/femsec/fiy190
3. Plewniak F, **Crognale** S, Rossetti S, Bertin P (2018) A genomic outlook on bioremediation: the case of arsenic removal. Front Microbiol 9:820. doi:10.3389/fmicb.2018.00820
4. **Crognale** S, Amalfitano S, Casentini B, Fazi S, Petruccioli M, Rossetti S (2017) Arsenic-related microorganisms in groundwater: a review on distribution, metabolic activities and potential use in arsenic removal processes. Rev Environ Sci Biotechnol 16:647–665. doi: 10.1007/s11157-017-9448-8
5. **Crognale** S, Zecchin S, Amalfitano S, Fazi S, Casentini B, Corsini A, Cavalca L, Rossetti S (2017) Phylogenetic structure and metabolic properties of microbial communities in arsenic-rich waters of geothermal origin. Front Microbiol 8:2468. doi: 10.3389/fmicb.2017.02468
6. Fazi S, **Crognale** S, Casentini B, Amalfitano S, Lotti F, Rossetti S (2016) The arsenite oxidation potential of native microbial communities from arsenic rich freshwaters. Microb Ecol 72:25-35 doi: 10.3389/fmicb.2017.02468
7. Fazi S, Amalfitano S, Casentini B, Davolos D, Pietrangeli B, **Crognale** S, Lotti F, Rossetti S (2016) Arsenic removal from naturally contaminated waters: a review of methods combining chemical and biological treatments. Rend Fis Acc Lincei 27:51-58 doi: 10.1007/s12210-015-0461-y

In preparation:

- Fazi S, **Crognale** S, Amalfitano A, Casentini B, Rossetti S. Biofilm characterization in As-rich hydrothermal environments (in prep.)
- Casentini B, Falcione FT, Amalfitano S, **Crognale** S, Fazi S, Rossetti S. The influence of hydraulic parameters on ZVI corrosion and arsenic removal efficiency: chemical and microbiological aspects (in prep.)
- Casentini B, Falcione FT, Amalfitano S, Di Pietro E, Lazzazara M, **Crognale** S, Fazi S, Rossetti S. Removal of arsenic in drinking water by iron and lantanum oxy-hydroxides: from lab to pilot study (in prep.)

Appendix

Appendix 1. Main functional genes involved in arsenic resistance and metabolism (adapted from Andres and Bertin, 2016)

Mechanism	Gene	Description	Reference
As(III) OXIDATION	***aioA***	As(III) oxidase (large and catalytic subunit)	Lett et al. 2012; van Lis et al. 2013
	aioB	As(III) oxidase (small subunit)	Lett et al. 2012; van Lis et al. 2013
	aioC	type_c cytochrome	Santini et al. 2007; Branco et al. 2009
	aioD	molybdenum cofactor	Branco et al. 2009; Slyemi and Bonnefoy 2012
	aioX	periplasmic As(III)-binding protein	Cai et al. 2009; Liu et al. 2011
	aioS	two-component signal transduction histidine kinase	Kashyap et al. 2006; Sardiwal et al. 2010
	aioR	response regulator	Kashyap et al. 2006; Sardiwal et al. 2010
ANAEROBIC As(III) OXIDATION	***arxA***	anaerobic As(III) oxidase molybdopterin subunit	Zargar et al. 2010, 2012
	arxB	anaerobic As(III) oxidase iron-sulfur subunit	Zargar et al. 2010, 2012
	arxB'	iron-sulfur protein of unknown function	Zargar et al. 2010, 2012
	arxC	membrane subunit involved in anchoring and electron transferase	Zargar et al. 2010; van Lis et al. 2013
	arxD	chaperone	Zargar et al. 2010; van Lis et al. 2013
	arxX	putative periplasmic As(III)-binding protein	Zargar et al. 2012
	arxS	putatitive two-component signal transduction histidine kinase	Zargar et al. 2012
	arxR	putative response regulator	Zargar et al. 2012
As(V) RESPIRATORY REDUCTION	***arrA***	respiratory As(V) reductase (large subunit)	Saltikov and Newman 2003; Kruger et al. 2013
	arrB	respiratory As(V)reductase (small subunit)	Saltikov and Newman 2003; Kruger et al. 2013
	arrC	membrane subunit involved in anchoring and electron transferase	Duval et al. 2008; van Lis et al. 2013
	arrD	chaperone	van Lis et al. 2013
ARSENIC UPTAKE	***Pit***	unspecific phosphate transporter	Willsky and Malamy 1980a, b
	Pst	specific phosphate transporter	Rosen and Liu 2009
	GlpF	aquaglyceroporin (glycerol facilitator)	Sanders et al. 1997; Meng et al. 2004
As(V) CYTOPLASMIC REDUCTION	***arsR***	transcriptional repressor	Wu and Rosen 1991; Slyemi and Bonnefoy 2012
	arsB	As(III) transmembrane carrier pump	Tisa and Rosen 1990; Rosen 2002

	arsC	As(V) reductase	Mukhopadhyay et al. 2002
	arsD	transcriptional repressor and As(III) chaperone	Wu and Rosen 1993; Lin et al., 2006
	arsA	anion stimulated ATPase	Tisa and Rosen 1990; Rosen 1999
	arsH	organoarsenical oxidase	Chen et al., 2015a
	arsN	putative acetyltransferase	Chauhan et al. 2009
	arsP	organoarsenical permease	Chen et al. 2015b
	arsTX	thioredoxin system	Achour et al. 2010
	arsO	putative flavin-binding monooxygenase	Wang et al. 2006
	acr3	As(III) transmembrane carrier pump	Tisa and Rosen 1990; Rosen 2002
METHYLATION	***arsM***	As(III) methyltransferase	Qin et al. 2006; Ajees and Rosen 2015
DEMETHYLATION	***arsI***	C·As lyase	Yoshinaga and Rosen 2014

Appendix 2. Primers sets used for PCR amplification of arsenic-related genes

MECHANISM	GENE	PRIMER NAME	PRIMER SEQUENCE (5'-3')	Reference
AsIII OXIDATION	***aioA***	aoxBM1-2F	CCACTTCTGCATCGTGGGNTGYGGNTA	Quéméneur et al., 2008
		aoxBM3-2R	TGTCGTTGCCCCAGATGADNCCYTTYTC	
		aoxBM3-1R	TGCCCCAGATGATGCCYTTYTCRWA	
		aoxBM2-1R	GGAGTTGTAGGCGGGCCKRTTRTGDAT	
		AOX-F-A2	TGCATCGTCGGCTGYGGNTAY	Zargar et al., 2012
		AOX-R-E2	TTCGGAGTTATAGGCCGGNCKRTTRTG	
		aroA #1F	GTSGGBTGYGGMTAYCABGYCTA	Inskeep et al., 2007
		aroA #1R	TTGTASGCBGGNCGRTTRTGRAT	
		aroA #2F	GTCGGYYGYGGMTAYCAYGYYTA	
		aroA #2R	YTCDGARTTGTAGGCYGGBCG	
		aroA95f	TGYCABTWCTGCAIYGYIGG	Hamamura et al., 2008
		aroA599r	TCDGARTTGTASGCIGGICKRTT	
AsV CYTOPLASMIC REDUCTION	***arsC***	P52F	AGCCAAATGGCAGAAGC	Bachate et al., 2009
		P323R	GCTGGRTCRTCAAATCCCCA	
		amlt-42-F	TCGCGTAATACGCTGGAGAT	Sun et al., 2004
		amlt-376-R	ACTTTCTCGCCGTCTTCCTT	
		smrc-42-F	TCACGCAATACCCTTGAAATGATC	
		smrc-376-R	ACCTTTTCACCGTCCTCTTTCGT	
		arsCF	AACAGTTGCCGCAGCATTCT	Anderson and Cook, 2004
		arsCR	ATGCGCTCCAGCTCACGCTT	
		Q-arsC-f1	GATTTACCATAATCCGGCCTGT	Saltikov et al., 2005
		Q-arsC-r1	GGCGTCTCAAGGTAGAGGATAA	
		arsCGP-Fw	TGCTGATTTAGTTGTTACGC	Villegas-Torres et al., 2011
		arsCGP-Rv	TTCCTTCAACCTATTCCCTA	
		arsC 4f	TCHTGYCGHAGYCAAATGGCHGAAG	Escudero et al., 2013

		arsC 4r	GCNGGATCVTCRAAWCCCCARTG	
		arsC 5f	GGHAAYTCHTGYCGNAGYCAAATGGC	
		arsC 5r	GCNGGATCVTCRAAWCCCCARNWC	
		arsC 6f	CACVTGCMGRAADGCRARRVVDTGGCTCG	
		arsC 6r1	YKYCRYCBRYVADRATCGG	
		arsC 6r2	TTRWASCCNACGWTAACAKKHYYKYC	
		arsC 6r3	YYVHWYTSKTSTTCRYKRAASCC	
	arsB	arsB-f	ATGGCAACCGAAAGGTTTAG	Anderson and Cook, 2004
		arsB-r	GTTGGCATGTTGTTCATAAT	
		arsB#1F	GGTGTGGAACATCGTCTGGAAYGCNAC	Achour et al., 2007
		arsB#1R	CAGGCCGTACACCACCAGRTACATNCC	
	acr3	dacr1F	GCCATCGGCCTGATCGTNATGATGTAYCC	Achour et al., 2007
		dacr1R	CGGCGATGGCCAGCTCYAAYTTYTT	
		dacr5F	TGATCTGGGTCATGATCTTCCCVATGMTGVT	
		dacr4R	CGGCCACGGCCAGYTCRAARAARTT	
AsV RESPIRATORY REDUCTION	***arrA***	arrAf	AAGGTGTATGGAATAAAGCGTTTGTBGGHGAYTT	Malasarn et al., 2004
		arrAr	CCTGTGATTTCAGGTGCCCAYTYVGGNGT	
		HAArrA-D1f	CCGCTACTACACCGAGGGCWWYTGGGRNTA	Kulp et al., 2006
		HAArrA-G2R	CGTGCGGTCCTTGAGCTCNWDRTTCCACC	
		AS1F	CGAAGTTCGTCCCGATHACNTGG	Song et al., 2009
		AS1R	GGGGTGCGGTCYTTNARYTC	
ARSENIC METHYLATION	***arsM***	arsM-F1	TSGAYATGACSSVBGMSAT	Cordi et al., 2015
		arsM-F2	HDNGYSATCGGNSTSGAY	
		arsM-R1	AGRTTGATSACRCARTTSS	
		arsM-R2	GWSAGRTTGATSACRCARTT	
		arsMF1	TCYCTCGGCTGCGGCAAYCCVAC	Jia et al., 2013
		arsMR2	CGWCCGCCWGGCTTWAGYACCCG	

Appendix 3. Details of the bacteria strains, sampling locations, GenBank accession number, arsenic transformation abilities (AsIII oxidation and AsV reduction) and arsenic-resistance genes (arrA, arsB, arsC, aioA) described in the test.

STRAIN[a]	SITE	ARSENIC TRANSFORMATION		ARSENIC-RELATED GENES				REFERENCE
		AsIII oxidation	AsV reduction	*arrA*	*arsB*	*arsC*	*aioA*	
				Proteobacteria				
				gammaproteobacteria				
Enterobacter sp.	Taiwan	-	+	-	+	+	-	Liao et al., 2011
Psychrobacter sp.	Taiwan	-	+	-	-	+	-	Liao et al., 2011
Psychrobacter sp.	Taiwan	-	+	-	+	-	-	Liao et al., 2011
Vibrio sp.	Taiwan	-	+	-	-	+	-	Liao et al., 2011
Citrobacter sp.	Taiwan	-	+	-	+	+	-	Liao et al., 2011
Pseudomonas sp.	Taiwan	-	+	-	+	+	-	Liao et al., 2011
Pseudomonas sp. (2)	Taiwan	-	+	-	-	-	-	Liao et al., 2011
Pseudomonas sp. (5)	Italy	n.d.	n.d.	n.d.	+	n.d.	n.d.	Davolos & Pietrangeli 2013
Enterobacter AF3	Italy	n.d.	n.d.	n.d.	+	n.d.	n.d.	Davolos & Pietrangeli 2013
Escherichia sp. AF4	Italy	n.d.	n.d.	n.d.	-	n.d.	n.d.	Davolos & Pietrangeli 2013
Pseudoxanthomonas sp.	India	+	+	n.d.	+	+	n.d.	Sarkar et al., 2013
Pseudomonas sp. (3)	Italy	-	+	n.d.	-	+	-	Corsini et al., 2014
Pseudomonas sp. (4)	Italy	-	+	n.d.	+	-	-	Corsini et al., 2014
Acinetobacter	India	+	+	-	+	+	+	Paul et al., 2015a
Pseudomonas sp.	India	-	+	+	+	+	-	Paul et al., 2015a
Pseudomonas sp.	India	-	+	-	+	+	-	Paul et al., 2015a
Acinetobacter calcoaceticus	Italy	+	n.d.	n.d.	n.d.	n.d.	n.d.	Fazi et al., 2016
				betaproteobacteria				
Comamonas sp. (2)	Italy	n.d.	n.d.	n.d.	+	n.d.	n.d.	Davolos & Pietrangeli 2011
Comamonas sp. BI44	Italy	n.d.	n.d.	n.d.	-	n.d.	n.d.	Davolos & Pietrangeli 2011
Delftia sp. BA2	Italy	n.d.	n.d.	n.d.	+	n.d.	n.d.	Davolos & Pietrangeli 2011
Acidovorax sp. BA3	Italy	n.d.	n.d.	n.d.	+	n.d.	n.d.	Davolos & Pietrangeli 2011
Acidovorax sp. (2)	Italy	n.d.	n.d.	n.d.	-	n.d.	n.d.	Davolos & Pietrangeli 2011

Variovorax sp- BI2	Italy	n.d.	n.d.	n.d.	-	n.d.	n.d.	Davolos & Pietrangeli 2011
Achromobacter sp. (2)	India	+	-	n.d.	-	-	n.d.	Sarkar et al., 2013
Hydrogenophaga sp. (2)	India	+	-	n.d.	-	-	n.d.	Sarkar et al., 2013
Alcaligenes faecalis	India	+	-	n.d.	+	-	n.d.	Sarkar et al., 2013
Achromobacter spanius	Italy	-	+	n.d.	-	+	-	Corsini et al., 2014
Achromobacter sp.	Italy	+	-	n.d.	-	-	+	Corsini et al., 2014
				alphaproteobacteria				
Bosea sp.	Taiwan	+	-	-	-	-	+	Liao et al., 2011
Ochrobactrum sp. (3)	India	+	+	n.d.	+	+	n.d.	Sarkar et al., 2013
Brevundimonas sp. As5-16	India	-	+		+	+	n.d.	Sarkar et al., 2013
Rhizobium	India	+	+	n.d.	+	+	n.d.	Sarkar et al., 2013
Aliihoeflea sp.	Italy	+	-	n.d.	-	-	+	Corsini et al., 2014
Brevundimonas	India	-	+	+	+	+	-	Paul et al., 2015a
Rhizobium	India	-	+	-	+	+	-	Paul et al., 2015a
Phyllobacterium	India	-	+	-	+	+	-	Paul et al., 2015a
Azospirillum	Australia	+	-	n.d.	n.d.	n.d.	+	Bahar et al., 2016
				Actinobacteria				
Arthrobacter sp. (3)	India	+	-	n.d.	-	-	n.d.	Sarkar et al., 2013
Rhodococcus ruber	Italy	+	-	n.d.	-	-	-	Corsini et al., 2014
Rhodococcus sp. (2)	Italy	-	+	n.d.	-	-	-	Corsini et al., 2014
Rhodococcus sp.	India	-	+	+	+	+	+	Paul et al., 2015a
Arthrobacter	India	+	+	-	+	+	-	Paul et al., 2015a
				Firmicutes				
Bacillus sp. (2)	Taiwan	-	+	-	-	+	-	Liao et al., 2011
Staphylococcus	India	-	+	-	+	+	-	Paul et al., 2015a

[a] The number of isolates is reported in brackets; -, not detected; +, detected; n.d., not determined.

Appendix 4. Physical-chemical parameters analyzed in arsenic-rich waters of geothermal origin.

											As speciation			ANIONS					
	T (°C)	pH	EC (µS/cm)	DO (mg/L)	DO %	DOC (mg/L)	HCO_3^- (mg/L)	FeTOT (mg/L)	FeII (mg/L)	S^{-2} (mg/L)	AsTOT (µg/L)	AsIII (µg/L)	AsIII/ AsTOT %	F^- (mg/L)	Cl^- (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^- (mg/L)	SO_4^{-2} (mg/L)
PAL	58	6.44	5680	0.2	n.d.	0.9	1160	0.38	0.17	1.5	362.1	107.4	29.6%	3.0	12.9	n.d.	1.3	n.d.	1554.8
SSC	57.8	6.41	5660	0.2	n.d.	1.3	1121	0.27	0.3	2.1	351.9	12.0	3.4%	2.8	12.6	n.d.	n.d.	n.d.	1670.4
CAR	55.5	6.49	4610	0.2	n.d.	0.9	1026	0.67	0.46	0.1	329.2	172.5	52.4%	3.2	13.5	n.d.	n.d.	n.d.	1263.9
OLI	24.9	6.65	724	1.33	16.2	1.7	708	0.54	0.04	0	22.9	0.0	0.0%	3.0	15.3	n.d.	6.4	n.d.	20.0
BEL	21.7	7.04	356	3.16	37.8	0.6	232	0.84	0.11	0	152.1	5.4	3.6%	4.0	19.6	n.d.	5.9	n.d.	33.6
ANG	18.7	6.28	250	7.21	82.3	0.3	195	0.78	0.18	0	182.4	8.9	4.9%	1.5	9.1	n.d.	3.2	n.d.	15.8
FON	18.2	6.69	269	7.95	87.2	0.3	122	0.15	0.07	0	51.9	1.0	2.0%	1.8	19.2	n.d.	20.0	n.d.	21.3
VICO	14	8.28	352	9.06	92.88	2.5	244	0.61	0.19	0	20.9	7.4	35.5%	1.0	16.8	n.d.	1.1	n.d.	81.8

	CATIONS																					
	Be (µg/L)	B (µg/L)	Al (µg/L)	V (µg/L)	Cr (µg/L)	Mn (µg/L)	Ni (µg/L)	Cu (µg/L)	Zn (µg/L)	Se (µg/L)	Sr (µg/L)	Cd (µg/L)	Sb (µg/L)	Cs (µg/L)	Ba (µg/L)	Hg (µg/L)	Pb (µg/L)	U (µg/L)	Li (µg/L)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)
PAL	2.6	1206	6.5	0.5	0.7	23.2	0.2	0.4	15.1	0.2	14270	<0.1	0.2	57.2	45.0	< 0.1	0.2	0.3	167	36.2	165	34.7	761
SSC	2.6	1183	34.0	0.5	0.7	16.7	0.3	0.3	2.1	0.1	14260	<0.1	0.1	55.1	37.0	< 0.1	0.1	0.1	162	35.7	162	34.3	702
CAR	2.5	1178	2.1	0.3	0.7	27.0	0.2	0.2	0.2	0.1	12650	0.1	0.1	59.0	44.1	< 0.1	0.2	0.2	176	35.6	127	36.0	556
OLI	0.3	73	14.0	3.4	0.8	2.5	0.6	4.4	23.0	3.0	3614	<0.1	0.9	85.0	268.0	< 0.1	0.3	8.1	55.8	21.4	12.2	10.6	106
BEL	0.6	582	10.1	6.1	0.7	7.1	0.4	0.2	5.4	0.1	295	< 0.1	0.4	11.0	19.0	< 0.1	0.2	1.2	128	27.3	4.6	26.9	26.7
ANG	1.1	1127	70.9	13.2	0.8	3.3	0.3	0.2	594.0	0.2	288	<0.1	0.9	4.1	1.8	< 0.1	0.2	2.1	82.9	22.5	9.2	26.8	17.6
FON	0.5	215	3.8	16.0	0.7	0.3	0.2	1.3	8.5	0.1	192	< 0.1	1.4	5.6	7.6	< 0.1	0.2	5.5	24.2	17.8	5.9	19.4	16.7
VICO	0.2	1176	336.0	1.4	0.7	1.8	0.2	1.7	20.0	0.2	373	<0.1	0.2	2.4	11.0	< 0.1	0.2	3.7	25.2	24.1	14.7	25.5	25.9

Appendix 5. Simper test results.

Taxon	Av. dissim	Contrib. %	Cumulative %	Mean abund. 1	Mean abund. 2
unassigned	3317	43.35	43.35	4.21	54.3
Betaproteobacteria	2514	32.85	76.19	45.2	24.6
Bacteroidia	861.3	11.25	87.45	14.7	0
Nitrospira	682.5	8.917	96.37	14.4	0.0998
Gammaproteobacteria	176.6	2.308	98.67	3.78	13.3
Alphaproteobacteria	44.27	0.5785	99.25	6.35	0.203
Deltaproteobacteria	41.95	0.5481	99.8	3.34	4.21
Acidobacteria	4.786	0.06253	99.86	1.75	0
Epsilonproteobacteria	4.42	0.05776	99.92	0.0321	1.69
Clostridia	3.11	0.04063	99.96	1.67	0.451
Cyanobacteria	0.7869	0.01028	99.97	0.773	0
Bacilli	0.4398	0.005747	99.98	0.0547	0.399
other bacteria	0.3862	0.005046	99.98	0.424	0.0588
Thermotogae	0.2076	0.002713	99.98	0.231	0.0147
Sphingobacteriia	0.147	0.001921	99.99	0.315	0
CF2	0.1341	0.001752	99.99	0.183	0
other Proteobacteria	0.09903	0.001294	99.99	0.201	0
Phycisphaerae	0.09288	0.001214	99.99	0.263	0
Chlorobia	0.09234	0.001206	99.99	0	0.175
Ignavibacteria	0.08594	0.001123	99.99	0.147	0
Holophagae	0.0721	0.0009421	99.99	0.162	0
OM190	0.0656	0.0008571	100	0.183	0
Gemmatimonadetes	0.06154	0.0008041	100	0.177	0
Planctomycetacia	0.06122	0.0007999	100	0.209	0
Pla4_lineage	0.04878	0.0006374	100	0.126	0
Fibrobacteria	0.04223	0.0005518	100	0.103	0
Elusimicrobia	0.03534	0.0004617	100	0.147	0
OPB35_soil_group	0.02925	0.0003822	100	0.0209	0.103
BD7-11	0.02788	0.0003643	100	0.0835	0
TA18	0.01996	0.0002608	100	0.0706	0
vadinHA49	0.01499	0.0001959	100	0.0847	0
Thermoleophilia	0.01017	0.0001329	100	0.0569	0
Actinobacteria	0.009973	0.0001303	100	0.0499	0
RB25	0.004792	6.26E-05	100	0.0467	0
BSV13	0.001756	2.29E-05	100	0.0209	0
Verrucomicrobiae	0.001517	1.98E-05	100	0.0221	0
other_ARMATIMONADETES	0.001486	1.94E-05	100	0.0193	0
Opitutae	0.001485	1.94E-05	100	0.0193	0
Spartobacteria	0.0006598	8.62E-06	100	0.0128	0
Deferribacteres	0.0006492	8.48E-06	100	0	0.0147
other_Bacteroidetes	0.0002911	3.80E-06	100	0.00853	0
Coriobacteriia	0.0001946	2.54E-06	100	0.00698	0
ARKICE-90	0.000165	2.16E-06	100	0.00642	0
WCHB1-32	0.000165	2.16E-06	100	0.00642	0
Armatimonadia	3.23E-05	4.23E-07	100	0.00284	0
Fusobacteria	0	0	100	0	0
Cytophagia	0	0	100	0	0
Candidatus_Methylacidiphilum	0	0	100	0	0

Caldilineae	0	0	100	0	0
BD2-2	0	0	100	0	0
Chloroplast	0	0	100	0	0
4C0d-2	0	0	100	0	0
Acidimicrobiia	0	0	100	0	0
Pla3_lineage	0	0	100	0	0
SB-1	0	0	100	0	0
Lentisphaeria	0	0	100	0	0
Flavobacteria	0	0	100	0	0

Appendix 6. Physical-chemical parameters of water samples from “Carletti thermal pools”.

									As speciation			ANIONS					
	Distanza	**T**	**pH**	**DO**	**DO**	**N-NH_3**	**HCO_3^-**	**TOC**	**As Tot**	**As(III)**	**As(III)**	**F^-**	**Cl^-**	**Br^-**	**NO_2^-**	**NO_3^-**	**SO_4^{2-}**
	m	***°C***		***mg/l***	***%***	***mg/L***	***mg/L***	***mg/L***	***µg/L***	***µg/L***	***%***	***mg/L***					
C1	0	52.4	7.25	1.84	34.6	1.10	1180	0.23	175.9	120.7	69%	2.6	18	0.02	0.003	0.07	1153
C2	14	42.6	7.76	5.84	97.5	0.90	1243	0.34	214.8	155.7	72%	2.4	22	0.08	0.002	0.10	1175
C3	28	39.1	8.16	6.39	100.6	0.67	987	0.35	209.3	161.4	77%	2.0	22	0.03	0.006		1182
C4	42	30.8	8.4	8.02	110.1	0.55	965	0.70	218.5	149.6	68%	2.7	23	0.03	0.009		1180
C5	56	25.7	8.63	8.22	103.5	0.62	1007	0.44	201.9	117.9	58%	2.0	22	0.07	0.001	0.07	1196
C6	70	23.5	8.62	8.42	100	0.49	982	0.22	192.5	113.9	59%	2.0	22		0.008	0.06	1187
C7	84	20	8.69	8.41	94.9	0.51	885	0.47	161.7	79.3	49%	1.7	23		0.008	0.49	1199
C8	100	16.4	8.61	9.08	94.6	0.49	854	0.53	186.9	81.6	44%	1.9	22	0.10		0.34	1186

	CATIONS													
	Na	**NH_4**	**K**	**Mg**	**Ca**	**Li**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**Ba**	**Sb**
	mg/L					***µg/L***								
C1	39	1.07	41	136	627	0.025	35	40	0.31	3.4	5.4	6.1	46	7.5
C2	36	0.39	39	131	593	0.021	33	21	<0.14	3.1	5.4	6.2	44	6.4
C3	38	0.78	39	133	561	0.021	27	19	0.16	3.2	4.9	3.3	35	6.7
C4	38	1.45	40	130	541	0.015	23	7.1	0.17	2.8	5.0	4.7	26	2.8
C5	39	1.29	41	135	569	0.022	23	6.8	<0.14	2.7	5.0	5.4	28	2.7
C6	40	0.74	39	135	551	0.015	28	11	<0.14	3.4	6.6	14	26	3.4
C7	37	0.44	41	132	533	0.020	20	6.9	<0.14	1.7	5.1	3.1	25	<2.1
C8	35	0.73	36	128	525	0.018	22	33	<0.14	2.6	5.1	5.7	44	5.7

Appendix 7. Arsenite oxidation in biofilters with BV 0.1 L at different flow rates (70 ml/min, 140 ml/min, 250 ml/min). 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.

Sintered glass rings

	V= 70 ml/min					V=140 ml/min					V= 250 ml/min				
	Time (min)	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**	**Time (min)**	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**	**Time (min)**	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**
T0	0	0	0	0	**0.0**	0	0	0	0	**0.0**	0	0	0	0	**0.0**
T1	15	0.7	11	19	**5.7**	15	1.3	21	37	**5.5**	15	2.3	38	66	**0.3**
T2	30	1.4	21	37	**10.9**	30	2.6	42	74	**21.3**	30	4.6	75	132	**5.1**
T3	45	2.1	32	56	**13.2**	45	3.9	63	111	**27.9**	45	6.9	113	198	**7.4**
T4	60	2.8	42	74	**17.3**	60	5.2	84	148	**31.7**	60	9.2	150	265	**5.9**
T5	75	3.5	53	93	**21.7**	75	6.5	105	185	**34.3**	75	11.5	188	331	**9.4**
T6	90	4.2	63	111	**24.9**	90	7.8	126	222	**40.9**	90	13.8	225	397	**12.5**
T7	105	4.9	74	130	**27.3**	105	9.1	147	259	**44.3**	105	16.1	263	463	**12.5**
T8	120	5.6	84	148	**30.4**	120	10.4	168	296	**48.3**	120	18.4	300	529	**12.4**
T9	135	6.3	95	167	**33.6**	135	11.7	189	333	**59.0**	135	20.7	338	595	**14.4**
T10	150	7	105	185	**35.2**	150	13	210	370	**56.7**	150	23	375	661	**15.2**
T11	165	7.7	116	204	**37.9**	165	14.3	231	407	**63.5**	165	25.3	413	728	**15.9**
T12	180	8.4	126	222	**42.2**	180	15.6	252	444	**69.6**	180	27.6	450	794	**14.1**

Coarse sand

	V=70 ml/min					V=140 ml/min					V= 250 ml/min				
	Time (min)	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**	**Time (min)**	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**	**Time (min)**	**n. tank ricirculations**	**BVs**	**PVs**	**% ox**
T0	0	0	0	0	**0.0**	0	0	0	0	**1.5**	0	0	0	0	**0.0**
T1	15	0.7	11	40	**6.2**	15	1.3	21	80	**13.9**	15	2.3	38	142	**17.5**
T2	30	1.4	21	80	**19.7**	30	2.6	42	159	**27.7**	30	4.6	75	284	**29.9**
T3	45	2.1	32	119	**24.2**	45	3.9	63	239	**36.7**	45	6.9	113	426	**42.4**
T4	60	2.8	42	159	**30.3**	60	5.2	84	318	**51.1**	60	9.2	150	568	**52.9**
T5	75	3.5	53	199	**34.1**	75	6.5	105	398	**57.5**	75	11.5	188	710	**63.0**
T6	90	4.2	63	239	**39.5**	90	7.8	126	477	**63.3**	90	13.8	225	852	**69.8**
T7	105	4.9	74	278	**44.3**	105	9.1	147	557	**70.0**	105	16.1	263	994	**75.5**
T8	120	5.6	84	318	**48.3**	120	10.4	168	636	**71.5**	120	18.4	300	1136	**80.2**
T9	135	6.3	95	358	**53.2**	135	11.7	189	716	**79.4**	135	20.7	338	1278	**83.4**
T10	150	7	105	398	**56.8**	150	13	210	795	**84.0**	150	23	375	1420	**85.9**
T11	165	7.7	116	438	**60.9**	165	14.3	231	875	**86.0**	165	25.3	413	1563	**87.3**
T12	180	8.4	126	477	**67.9**	180	15.6	252	955	**89.0**	180	27.6	450	1705	**89.3**

Appendix 8. Heatmap of phylum in long-term acclimated (LTA) biofilms with BV 0.1 L and 0.7 L and in groundwater used for kinetic experiments. The color intensity and numbers in each cell shows the relative abundance (%) of a phylum in each sample.

	0 .1 L		0 .7 L		Groundwater	
	Glass	Sand	Glass	Sand	January	March
Acidobacteria	2.0	0.0	10.0	7.9	0.0	0.0
Actinobacteria	7.3	9.0	14.9	18.8	0.0	0.0
Armatimonadetes	7.2	0.9	0.7	0.2	0.0	0.1
Bacteroidetes	12.5	11.5	9.2	11.9	0.0	0.1
Chlorobi	0.1	4.4	0.0	1.4	0.0	0.0
Chloroflexi	3.6	1.9	0.9	0.0	0.0	0.1
Cyanobacteria	0.6	27.3	3.9	1.2	0.0	0.3
Elusimicrobia	0.1	0.1	2.7	0.0	0.0	0.0
Firmicutes	0.2	0.0	1.0	3.2	0.0	0.0
Gemmatimonadetes	2.2	0.0	0.0	0.0	0.0	0.0
Hydrogenedentes	0.0	1.2	0.0	0.0	0.0	0.0
Ignavibacteriae	3.1	1.7	3.4	19.3	0.0	0.0
Nitrospirae	0.4	0.0	9.0	1.1	53.2	77.8
Omnitrophica	4.2	0.0	0.0	0.0	0.0	0.3
Parcubacteria	0.3	0.0	0.0	0.0	0.0	0.4
Planctomycetes	10.2	0.9	0.0	0.0	0.0	0.1
Proteobacteria	45.8	39.2	40.7	33.5	45.4	20.1
Saccharibacteria	0.0	0.0	0.0	0.0	0.3	0.1
Spirochaetae	0.0	1.6	0.0	0.3	0.0	0.3
TM6	0.0	0.0	0.4	0.0	1.2	0.1
Verrucomicrobia	0.1	0.1	3.0	0.2	0.0	0.0
Bacteria unknown	0.2	0.1	0.1	1.0	0.0	0.4