CHAPTER 6.1 INFLUENCE OF AS (V) ON THE DIVERSITY OF BIOFILMS FORMED ON DIFFERENT SUBSTRATA

S.E. Rastelli (1,2) and **M.R. Viera *(1,3)**

(1) Centro de Investigación y Desarrollo en Tecnología de Pinturas (CIDEPINT; CICPBA-CONICET) Calle 52 entre 121 y 122 (1900) La Plata, Argentina.
(2) Facultad de Ciencias Naturales y Museo (UNLP). Av. 60 y 122. (1900) La Plata, Argentina.
(3) Facultad de Ciencias Exactas (UNLP), calle 47 y 115. (1900) La Plata, Argentina

ABSTRACT

Microorganisms, including bacteria, present in natural and artificial aquatic environments, tend to attach to and grow on immersed surfaces developing a biofilm. Many problems in drinking water networks such as corrosion, persistence of pathogenic species and increased resistance to biocides are due to the presence of biofilms. Arsenic is a contaminant widely distributed in the Argentinean underground water. Despite arsenic's toxicity, a number of microorganisms are capable of growing in arsenic environments playing an important role in the process of arsenic mobilization. The aim of this work was to study the influence of As (V) on the bacterial planktonic community and biofilms structures grown on different drinking water distribution materials.
To simulate a water distribution system, two tanks with a closed loop of polypropylene (PP) tubes were built and filled with drinking water. As(V) (5 mg L^{-1}) was added in one of the tanks. Coupons of four materials were place in the loops for biofilm formation: commercial iron (Fe), commercial zinc (Zn), copper (Cu) and PP. Bacterial planktonic and sessile communities were analysed by culture (heterotrophic plate counts) and molecular (DNA extraction, PCR, sequencing, DGGE) techniques.
Bacterial counts on Fe and Zn were higher than those obtained on Cu and PP and, except for Cu, they were higher in the presence of 5mg.L^{-1} of As(V). Culturable As-tolerant bacteria able to grow in the presence of high As(V) concentration (up to 1g.L^{-1}) were obtained from all the biofilms except Cu-biofilms, which grew in the presence of up to 300mg.L^{-1} As(V). It was possible to isolate and identify 60 colonies corresponding: 40% to the Class Bacilli, 40% α-Proteobacteria (both Classes were found in all the biofilms), 10% Actinobacteria (detected in biofilms formed on Fe in the absence of As, Cu and PP in the presence of As), 8% β-Proteobacteria (found on Fe, Zn and PP biofilms in the presence of As) and 2% γ-Proteobacteria (detected only in biofilms formed on Zn in the absence of As). The DGGE profiles of the planktonic bacterial communities were qualitative and quantitative affected by the presence of arsenic. In general, the planktonic community developed in the water without As showed higher richness and diversity indices, indicating that the presence of a toxic element induced a selection of the species in the water with Arsenic. In the case of the sessile communities, the trends were not so clear. The clustering analysis of the sessile communities showed that the nature of the substrata was a more important factor for the establishment of the community than the presence of arsenic in water.

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*Author for correspondence: m.viera@cidepint.gov.ar

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INTRODUCTION

Microorganisms, including bacteria, present in natural and artificial aquatic environments, tend to attach to and grow on immersed surfaces developing a biofilm[1]. Many problems in drinking water networks such as corrosion, persistence of pathogenic species and increased resistance to biocides are due to the presence of biofilms[2]. The presence of bacterial biofilms on the inner surface of water distribution pipes could lead to a deterioration of the water quality and its subsequent impact in public health.

Another problem related with water quality is the presence of chemical contaminants of diverse origin. Amongst them, arsenic (As) is being increasingly detected in distribution water services, which generate serious sanitary and social problems impacting large urban areas throughout the Planet. In Argentina, As is of great concern due to its natural occurrence in high concentrations in groundwater in large areas of the country[3]. Despite As toxicity, a number of microorganisms are capable of growing in arsenic environments. These microorganisms could be involved in arsenic mobilization and may play a role in arsenic removal[4]. The presence of toxic agent as arsenic in the water phase could induce changes in the bacterial planktonic and sessile communities[5,6]. The aim of this work was to study the influence of As on biofilm formation in water distribution systems. The structures of the planktonic and the sessile communities were analyzed. The presence of Arsenic tolerant-microorganisms was assayed.

MATERIALS AND METHODS

Experimental setup. Two laboratory simulated water distribution circuits consisting each in a 50 L polyethylene storage tank and a closed loop of polypropylene tubes (inner diameter: 2.32 cm; length: 200 cm) with a removable 20 cm acrylic cell were used. La Plata City drinking water was pumped from the tank through the loop at a laminar flux with 30/60 minutes work/stop periods along the day and no flow at night to simulate domestic network operating cycles. To study the settlement of bacteria on different water distribution network materials, coupons of 1 cm x 1 cm x 0.02 cm of commercial low carbon steel (Fe), zinc (Zn), copper alloy (Cu) and polypropylene (PP) were placed in the acrylic cell. To study the influence of arsenic, 5 mg L^{-1} As(V) were added in one of the circuits.

Bacterial community characterization. After circulation time (45 days), 4 coupons of each material were withdrawn from each circuit, and replaced by new coupons. Biofilms were scrapped and poured in 1mL physiological solution for enumeration. Arsenic-resistant sessile bacteria was evaluated by culturing in nutritive broth with 50 to 1,000 $mg.L^{-1}$ As(V) by dilution to extinction technique.

To analyze the microbial communities, total DNA of the planktonic and sessile microorganisms was collected. Planktonic DNA was obtained by filtering 1 L water from each tank through a 0.22 µm sterile membrane. For total sessile DNA extraction, the

material scraped from the coupons was centrifuged at 13,000 g for 15 min and the supernatant discarded. Culturable sessile DNA was obtained from 1mL nutritive broth culture of 1/10 dilution. In all the cases, DNA was extracted using a commercial kit (E.Z.N.A. Soil DNA kit) following the manufacturer's instructions. The 16S rRNA gene sequence was amplified by PCR using the universal primers for eubacteria: 341F (with a GC clamp) and 907R[7]. Negative controls (without DNA) were run in all the amplifications and the presence of PCR product was confirmed by 1.2% w/v agarose gel electrophoresis and Sybr® Gold staining. DGGE was performed in a 6% (w/v) polyacrylamide gel with a 30–70% denaturant gradient (100% denaturant is 7 M urea and 40% v/v formamide) loaded with the PCR products (10–15 µL). Electrophoresis was performed in TAE buffer for 16 h at 100 V. The gels were stained, observed and photographed in a UV transillumination. For statistical evaluation, the gels were analyzed using the Gel Compare II software. The band-based Dice coefficient was used to calculate the similarity matrix with a position tolerance of 1%[8]. The unweighted pair group method with arithmetic mean (UPGMA) was applied for clustering. Richness and diversity statistics were calculated from the DGGE profiles of the planktonic and sessile communities by using the number and intensity of the bands in each profile. Phylotype richness (***S***) was calculated as the total number of distinct bands in a DGGE profile. The Shannon-Weiner diversity index (***H***) was calculated considering the proportion of an individual band intensity relative to the sum of all band intensities. Simpson´s index of diversity was calculated from the equation $\boldsymbol{D} = 1 - \Sigma (p_i)^2$ (where pi is the relative intensity of band *i*)[9].

Identification of bacteria. DNA from isolated colonies formed on nutrient agar plates was extracted by suspending, with the aid of an inoculating loop, a colony in 1 mL of sterile distilled water and boiling for 10 min., centrifuged at 13,000 g for 5 min and the supernatant was transferred to a new tube. PCR amplification of almost the whole 16S rRNA gene sequence was carried out using the primers 27F and 1541R. The PCR product was purified and sequenced by MACROGEN (Korea). Sequence data were compared for initial identification, with the closest relatives represented by the retrieved sequences obtained from homology searches using the Blast algorithm at the NCBI (http://www.ncbi.nlm.nih.gov/blast/).

RESULTS AND DISCUSSION

Sessile total and arsenic-resistant bacterial counts. Bacterial biofilms developed on all the tested materials in the presence and in the absence of As (**Figure 1**). However, two features can be pointed out. On one side, the influence of the substrate nature: biofilms developed on Fe and Zn exhibited the highest counts, while biofilms on Cu and PP were one or two orders of magnitude lower. These results are in agreement with the observations made by other authors who found that the number of bacteria attached to iron or steel was higher than that on plastic or copper pipes[9]. On the other side, the influence of the As: higher bacterial counts were found in biofilms developed in the As-containing water except in the case of Cu. Biofilm formation allows microorganisms to survive in the presence of contaminants. In the present case, this hypothesis seems to be confirmed by the higher bacterial counts in those coupons exposed to arsenic-

containing water, except in the case of Cu. Furthermore, the low counts obtained on Cu in both systems could be related to the release of toxic copper ions.

Culturable As-tolerant bacteria were obtained from all the biofilms. These bacteria were able to grow in the presence of up to $1gL^{-1}$ of As(V) except bacteria from Cu-biofilms which could grow up to 300 mgL^{-1} . Arsenic-resistant bacteria have been isolated from both, arsenic rich environments and arsenic-free ones[10]. In our case, we found arsenic resistant bacteria in both systems, with a higher number in the biofilms formed in the arsenic-containing water, indicating a degree of adaptation.

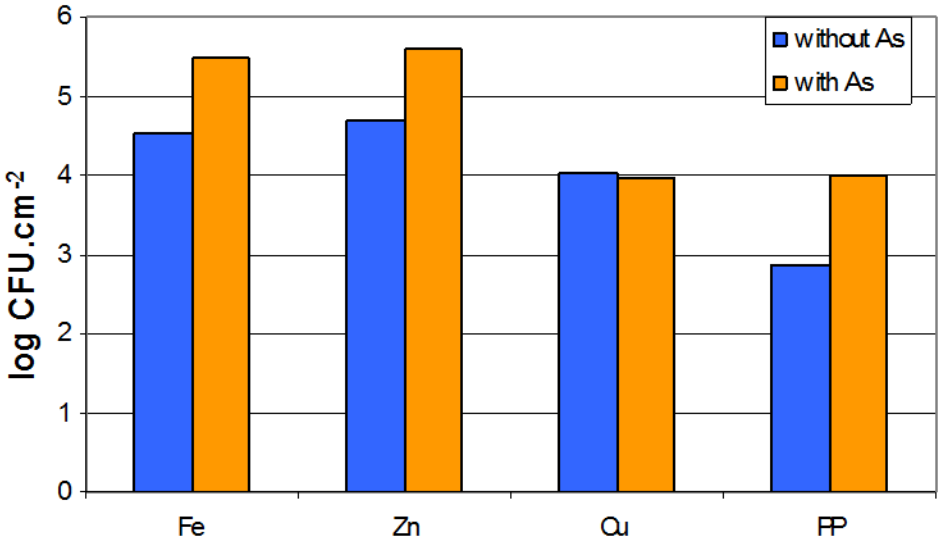

FIGURE 1. Heterotrophic sessile bacteria counts (Log CFU cm^{-2}) of the biofilms formed on the four substrata (Fe, Zn, Cu and PP) in water, with or without 5 mg L^{-1} As(V).

Bacterial community characterization. Direct PCR-DGGE based on the 16S rRNA gene of the planktonic and sessile DNA allowed a description of the structure of microbial communities developed in the water and in the biofilms, respectively. **Figure 2** illustrates the DGGE band patterns corresponding to the planktonic communities presented in both circuits at the end of 7 independent experiments. It can be seen that each sample produced a distinctive DGGE profile, with different number of bands with diverse position and intensity. At the beginning of each experiment, both circuits were filled with the same water source, then As(V) was supplemented in one of them, thus the same populations were originally present in both circuits. The different profiles at the end of each experiment indicated that the presence of arsenic induced qualitative (band position) and quantitative (band intensity) changes in the microbial community.

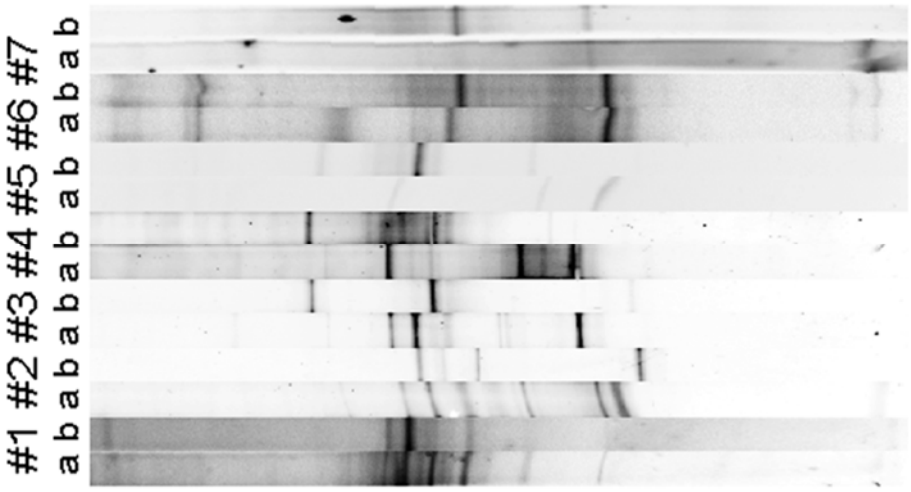

FIGURE 2. DGGE profiles of amplified bacterial 16S rDNA fragments from the planktonic communities in: (a) the circuit without As(V); and (b) with 5 mg/L As(V) in 7 independent experiments.

The DGGE profiles of the biofilms formed on the four materials assayed in the presence and in the absence of As in several independent experiments are shown in Figure 3. All the samples generated a different profile, but several bands were detected in most of the biofilms **(Figure 3**, green arrows), while others were detected in one simple only (blue arrows).

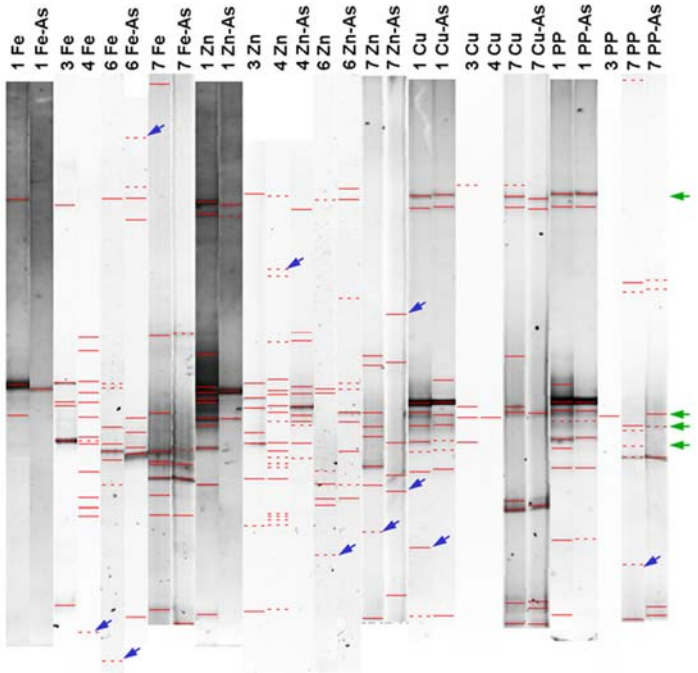

FIGURE 3. DGGE profiles of biofilms growth in presence and absence of As(V) on Fe, Zn, Cu and PP. in seven independent experiments. Green arrows: bands present in all the communities; blue arrows: bands present in one community.

As can be noticed in **Figure 3**, it was not possible to obtain DNA from all the biofilms in all the experiments. Only in experiments 1 and 7 all the profiles could be obtained. The clustering analysis of the 8 profiles in these two experiments was performed (Figure 4). The result shows a clear trend for biofilms formed on a particular material to cluster together with a high similarity regardless the presence of arsenic in the liquid phase. This finding indicated that the nature of the substrata was a more important factor for the establishment of the sessile community than the presence of arsenic. It has been reported that surface properties affected biofilm community composition[11]. Certain materials can release different compounds that can influence biofilm development[12]. In our case, the community profile developed on different materials differed as well as the magnitude of the attack produced on the substrata[13]. A high similitude amongst the established community on the materials less susceptible to bacterial attack (PP and Cu) was observed. These results suggested that a relationship could exist amongst the established community and its deterioration effect on the substratum.

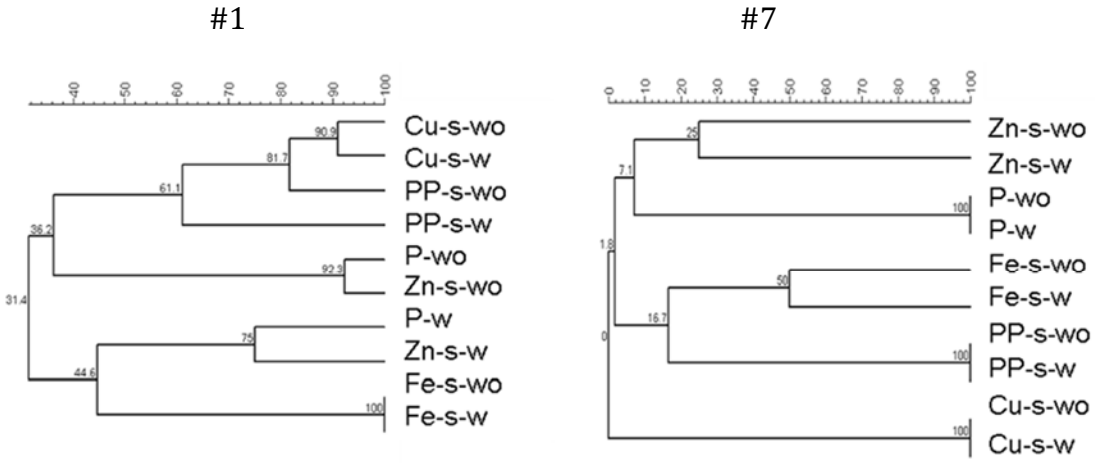

Figure 4. Clustering analysis of the Planktonic (P) and Sessile (s) communities formed on the four materials assayed (Fe, Zn, Cu and PP) in both circuits: with As (w) or without As (wo) in experiments 1 and 7.

DGGE provides an indication but not an absolute measure of the degree of biodiversity in a bacterial community. However, based on the number and intensity of the bands and using of appropriate software, it was possible to obtain richness (S) and diversity (H, D) indices of all the samples (**Table 1**). In general, the planktonic community developed in the water without As showed higher richness and diversity indices, indicating that the presence of a toxic element induced a selection of the species in the water with Arsenic. In the case of the sessile communities, the trends were not so clear. Most of the biofilms gave H and D indices between 2 and 3, values normally found for biofilms formed in water[9], however biofilms with high (H>3 and D close to Dmax), and very low diversity (S=1 and D= 0, only one band) were found.

TABLE 1. Richness (S) and Diversity (H, D) indices for planktonic and sessile communities in different experiments

Planktonic Samples				
Experiment	Richness (S)	Shannon-Weiner Diversity (H)	Simpson diversity (D)	D_{max}
1	10	2.9	0.8	0.9
1-As	5	1.9	0.6	0.8
2	11	3.0	0.8	0.9
2-As	6	2.0	0.7	0.8
3	10	2.5	0.7	0.9
3-As	4	1.4	0.5	0.7
4-As	17	3.6	0.9	0.9
5	18	3.7	0.9	0.9
5-As	5	1.0	0.3	0.8
6	13	3.3	0.8	0.9
6-As	11	3.1	0.8	0.9
7	9	2.8	0.8	0.8
7-As	4	1.5	0.6	0.7
Biofilm Samples				
Experiment	Richness (S)	Shannon-Weiner Diversity (H)	Simpson diversity (D)	D_{max}
1-Fe	3	0.8	0.2	0.6
1-Zn	12	3.5	0.9	0.9
1-Cu	10	2.9	0.8	0.9
1-PP	11	2.9	0.8	0.9
1-Fe-As	1	0	0	0
1-Zn-As	4	1.0	0.3	0.7
1-Cu-As	10	2.5	0.7	0.9
1-PP-As	8	2.2	0.6	0.8
2-Fe	6	2.0	0.6	0.8
2-Zn	9	2.7	0.8	0.8
2-Cu	4	1.5	0.6	0.7
2-PP	1	0	0	0
3-Fe	14	3.5	0.9	0.9
3-Zn	22	3.6	0.8	0.9
3-Cu	1	0	0	0
3-Zn-As	11	2.9	0.8	0.9
5-Fe	9	2.7	0.8	0.8
5-Zn	8	2.8	0.8	0.8
5-Fe-As	9	2.5	0.7	0.8
5-Zn-As	10	2.7	0.7	0.9
7-Fe	9	2.9	0.8	0.8
7-Zn	9	2.7	0.8	0.8
7-Cu	10	3.1	0.8	0.9
7-PP	9	2.6	0.7	0.8
7-Fe-As	6	2.3	0.7	0.8
7-Zn-As	6	2.4	0.8	0.8
7-Cu-As	7	2.7	0.8	0.8
7-PP-As	7	2.4	0.7	0.8

Regarding the nature of the surface, biofilms formed on Zn had the highest diversity indices. Thus this substrate gave the biofilms with the highest number of bacteria and the highest number of bacterial species. However, there is not direct correlation between bacterial counts and diversity. For instance, biofilms formed on Cu and PP gave low bacterial counts and their diversity indices were similar to that found in the others surfaces.

Identification of bacteria. Among all the colonies obtained in nutrient agar plates seeded with the scrapped biofilm, 60 isolates could be identified through the amplification on the 16SrRNA gene (34 of them were already included in the GeneBank under the accession numbers KM349185- KM349219). The bacteria identified belong to genus normally found in water distribution systems[14]. Bacteria belonging to the Class Bacilli (genus *Bacillus, Paenibacillus* y *Staphylococcus)* and α-Proteobacteria (*Brevundimonas*, *Sphingomonas*) were found in all the biofilms, Actinobacteria (*Kokuria, Micrococcus, Janibacter*) were found in biofilms formed on Cu and PP without As, β-Proteobacteria (*Delftia*, *Acidovorax)* were found in biofilms in the As containing water and γ-Proteobacteria (*Acinetobacter*) detected only in biofilms formed on Zn in the absence of As (**Figure 5**).

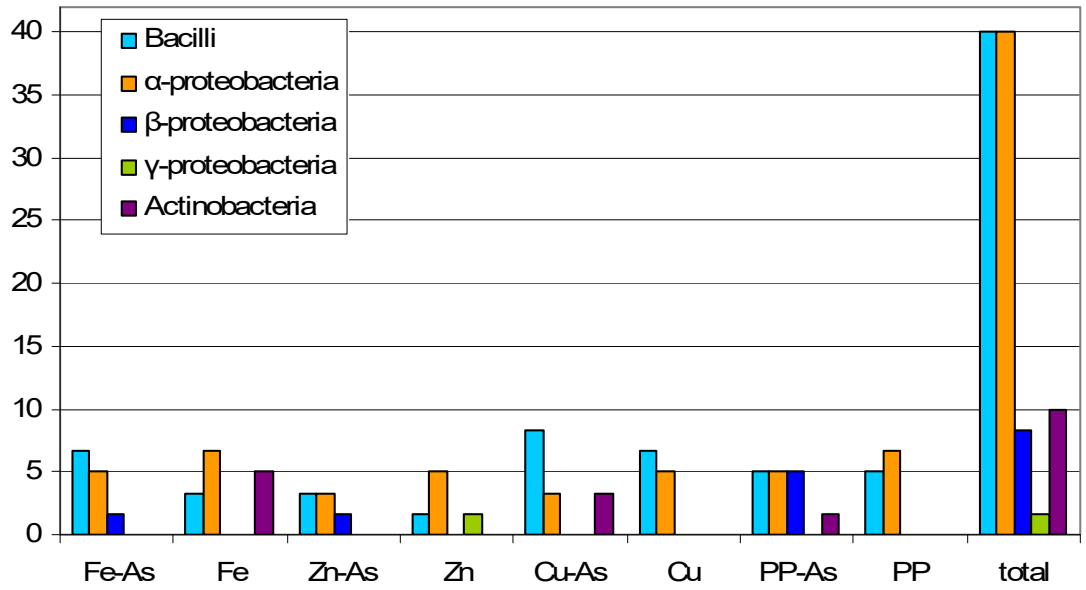

FIGURE 5. Abundance (in percentage) of the different bacterial Classes found on all the materials assayed (Fe, Zn, Cu and PP) in absence or presence of 5 mg.L^{-1}As(V) in the water

CONCLUSION

Bacteria could form biofilms on all the materials tested. The number of bacteria attached to Fe and Zn were higher than those found on Cu and PP. Slightly higher values were found for biofilms developed in the As-containing water, except in the case of biofilms formed on Cu. Culturable As-tolerant bacteria were obtained from most of the biofilms originated in both circuits.

The presence of As(V) in the water induced qualitative and quantitative changes in the planktonic bacterial community. In general, the planktonic community developed in the water without As showed higher richness and diversity indices, indicating that the presence of a toxic element induced a selection of the species. However, for the establishment of the sessile community, the nature of the substrata resulted in a more important factor than the presence of arsenic.

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