MICROSCOPY APPLIED TO BIOFILMS IN DRINKING WATER CLOSED LOOP

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SUMMARY: During water distribution operation, tubing material is submitted to permanent contact with aquatic microbial species. They colonise the network surface forming biofilms which frequently cause corrosion on metallic substrata. The present study approaches the analyses of laboratory biofilms formed on water pipe materials from several (SEM, ESEM, CLSM) microscopy technique point of view and also from a microbial ecological analysis using DGGE for the study of the communities formed on each substrate. Microbial interactions with the different materials were described using an ample range of magnification. Three commercial metals (Fe, Zn, Cu) and polypropylene tubing used as substrata revealed very different behaviours. Even when it was possible to distinguish different attack morphologies and the biofilms 3-D structures formed on all materials it was not already established a clear correlation with the genetic profile of each community.

Key words: MIC; biofilm morphology; microscopic techniques; topography; DGGE.

1. INTRODUCTION

Microorganisms, included several species of bacteria, are present in natural and artificial aqueous environments. These microorganisms tend to attach and grow on the immersed surfaces developing a biofilm (Dexter, 2003). The biofilm is an interacting, organised 3-D structure constituted by microorganisms and their extracellular polymeric substance (EPS) with waters channels and multiple layers of cells (Costerton et al., 1999; Jenkinson & Lappin-Scott, 2001). Many problems in drinking water networks such as corrosion, increased resistance to biocides and persistence of pathogenic species are due to the presence of biofilms (Berry et al., 2006).
Electron microscopy techniques as scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM) have been important for high resolution visualization of bacterial biofilms outer surface. SEM observation requires the biofilm fixation, staining, drying and conductively coating prior to imaging under high vacuum, which may alter its structure (Beech, 1996; Priester et al., 2007). On the other hand ESEM observation requires moderate vacuum in moist containing atmosphere and no coating, preserving the original morphology (Little et al., 1991; Walker et al., 2001).
Alternatively, confocal laser scanning microscopy (CLSM) in combination with different staining protocols is increasingly considered an important tool allowing not only the outer but also the inner spatial distribution visualization and cell quantification in biofilm structure. Imaging bacterial biofilm with CLSM combined with other techniques such as cryoembedding, Raman microscopy, provide additional information about biofilm components (Stewart el al, 1995; Wagner et al, 2009). The use of appropriated software or mathematical models allows rebuilding the 3-D image of biofilm (Lewandowski, 2000; Pitts & Stewart, 2008).
The development of molecular techniques has allowed studying microbial communities (Muyzer, 1999). Among these techniques denaturing gradient gel electrophoresis (DGGE) is commonly used for genetic fingerprint analysis of microbial community composition, diversity and dynamics (Green et al., 2009).
The main purpose of this work was to shed light on biofilm inner and outer structures effect on the attack susceptibility of tested substrata, by means of diverse microscopic, analytic and molecular biology techniques.

2. EXPERIMENTAL

2.1. Materials

The most frequently used tubing materials for water distribution were selected for this study. Commercial iron (instead of cast iron), Zn (instead of galvanized steel), copper base hidrobronze and polypropylene (PP) were used.
Tests samples were cut to 10 mm x 10 mm after rolling to 0.2 mm the respective row commercial material for minimizing laminar water flux perturbation. Surface finishing was done by manual abrasion up to 1,000 grade emery paper.
La Plata drinking water was used as microbial source and aqueous medium for colonisation during dynamic testing of materials.

2.2. Techniques

2.2.1. Drinking water closed loop.

A laboratory simulated distribution system (Fig.1), with the drinking water flowing at laminar flux alternatively 30/60 minutes work/stop periods to simulate a domestic network operating cycles was used. After 30 days test period a transparent support with 7 replicates of each of the 4 materials was extracted in sterile conditions from the loop.

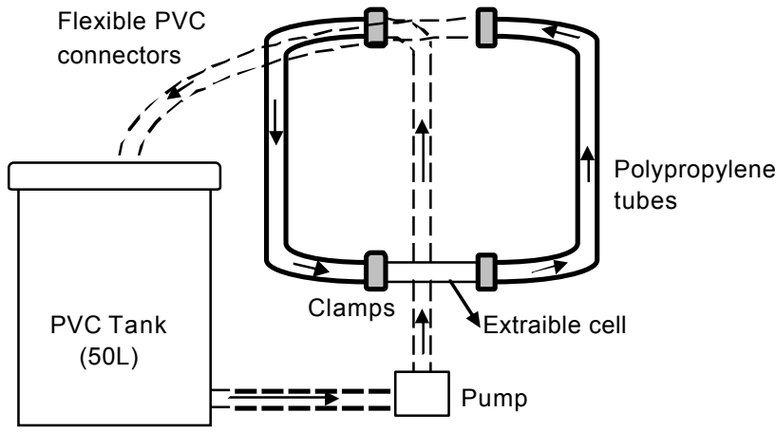

Figure1. Scheme of the drinking water closed loop.

2.2.2. Energy dispersive X-ray analysis (EDX) and topography characterisation of deposits.

Different techniques applied provided complementary vision of biofilm growth process. From very low, at the naked eye, 40x aspect, with a stereoscopic magnifier (Arcano trinocular stereoscopic ANT WT 4130) up to 300,000 x enlargement with a Zeizz Supra™ 40 SEM, having the largest field depth and best three-dimensional appearance, different detailed observation was performed. Intermediate magnification with a FEI Quanta 200 ESEM coupled to EDS analysers was applied for most observation and elemental analyses respectively. A Philips SEM 505 coupled to an EDAX ultra thin window analyser was used for analysis of elemental contaminants of biofilms and corrosion products formed. SEM observations were always preceded by fixation with glutaraldehyde 2.5% in phosphate buffered saline (PBS), dehydration with ethanol 20 to 100%, critical point drying and surface conductive ultra thin coating on all samples.
Biofilms inner structure as cell cluster matrix surrounded by interstitial channels was observed by CLSM. The used microscope was an Olympus FV 300 with acquisition soft FLUOVIEW 3.5 version. Each image was taken at 100 x on 5 µm slices in the complete thickness along biofilms at 488 nm wave length. Cells were dyed with acrydyne orange reagent 0.01 % in PBS. The 3-D images were reconstructed by ImageJ software.

2.2.3. Surface analyses

Once the biofilms, EPS and corrosion product deposits were eliminated with a commercial pickling solution, microbial surface alterations were also investigated. Relationship between the attack and biofilm deposit elemental composition on each material was also performed using a FEI Quanta 200 ESEM coupled to an EDAX.
Very initial microbial colonisation and corrosion attack were observed up to 300,000 x magnification with the Zeizz Supra™ 40 SEM.

2.2.4. Microbial characterization

DNA from planktonic bacteria was obtained by filtering 1L water from the circuit tank through 0.22µm sterile membrane. To characterise the attached bacteria (biofilm) on each material, biofilm along with EPS and corrosion products were removed from 4 samples by scrapping with sterile scalpel and poured into 1ml sterile physiologic solution. One of them was used to analyse culturable sessile bacterial community in nutritive broth, the remaining 3 were combined and centrifuged at 13000g for 15min and the supernatant discarded. Both total and culturable sessile and total planktonic DNA were extracted using E.Z.N.A. Soil DNA kit (Omega bio-tek) following the manufacture's instructions. DNA was amplified by the polymerase chain reaction (PCR) (Mastercycler® ep, Eppendorf) using 341F with a GC clamp and 907R primers (Ishii and Fukui, 1993) with the following program: initial temperature 94 ºC for 4 min, then 10 cycles: 94 ºC for 30 sec, 62 ºC for 45 sec, 72 ºC for 60 sec, then 25 cycles: 94 ºC for 30 sec, 57 ºC for 45 sec, 72 ºC for 60 sec and finally 10min at 72ºC. The presence of PCR product was confirmed by 1.2 % w/v agarose gel electrophoresis and SybrGold staining with a negative control.
DGGE was performed using DGGE 2401 equipment (CBS Scientific Co) in a 6 % w/v polyacrylamide gel with a 30-70 % denaturant gradient (100 % denaturant is 7 M urea and 40 % v/v formamide). Electrophoresis was performed in TAE buffer for 16 hs at 60 ºC, at 100 V. The gels were stained with SybrGold for 40 min and observed and photographed in a UV transillumination Universal Hood II (Bio Rad). For statistical evaluation, DGGE fingerprints/profiles were analysed with the software GelCompareII. The banded based Dice coefficient was used to calculate the similarity matrix with a position tolerance of 1%, for clustering the unweighted pair group method with arithmetic mean (UPGMA) was applied.

3. RESULTS

3.1. EDX and topography characterisation of deposits.

Since the beginning of water circulation test, heterogeneously distributed microbial colonisation could be observed on all exposed materials. After some days, mixed up with the biofilms and biological EPS deposits, increasing amounts of corrosion products and spurious solids coming with the drinking water appeared especially on Fe and Zn samples. At the end of each test series the aspect of deposits presented very scattered distribution amongst all the materials replicates (Fig.2).

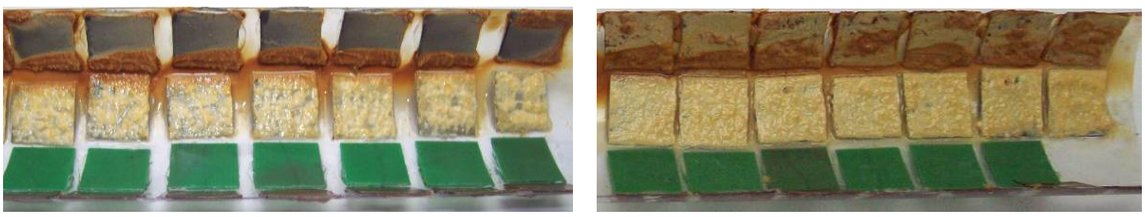

Figure 2. Heterogeneous aspect of deposits on extracted samples from two test series

Starting with a stereoscopic magnifier at 40 x the aspect of various steps were appreciated during biofilm formation on Fe and Zn, characterised by two or more superposed phases, separated by a cleavage plane. The outer phase is much thicker presenting a blistered structure and a space between the inner phase and the metal substrata can be appreciated. Details were further observed with SEM-EDAX up to 1,000 x on all materials showing a single phase biofilm on hidrobronze and PP. At the latter magnification also microorganisms colonizing bare surface areas could be clearly seen in Fig. 3.
The EDX composition of spheroid structures observed on the hidrobronze biofilms revealed Cu_2O formation and water contaminants.

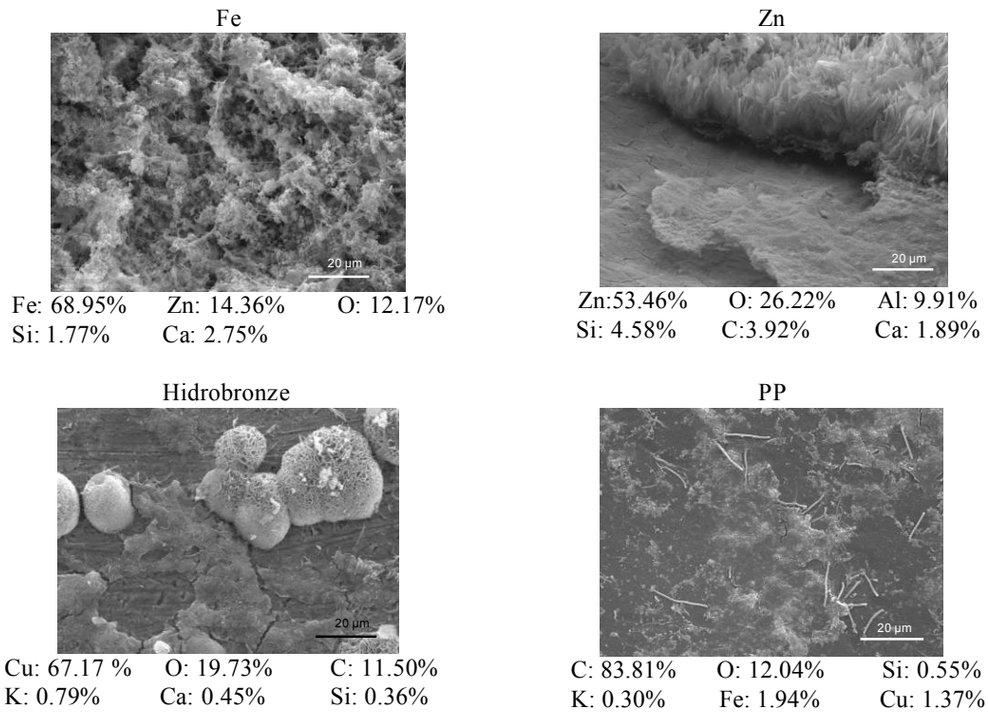

Figure 3. Colonization of materials samples exposed to drinking water circuit and EDS.

Very diverse aspects in cross section, revealed by a 45º SEM stage tilt, showed structures corresponding to various compounds on the different material surfaces, especially evident on Zn (Fig. 4).

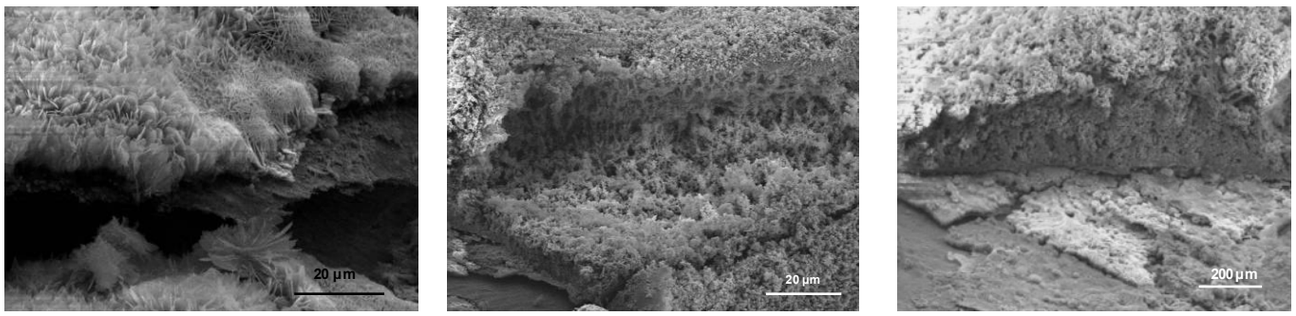

Figure 4. Deposits on Zn samples.

All tubing material samples showed bacterial presence in contact with each material substratum (Fig. 5). That allowed a close correlation with soft metallic attack. This contact showed evidence of very diverse structure on the respective failure induced, being possible to appreciate the low aggressiveness detected on several metals.

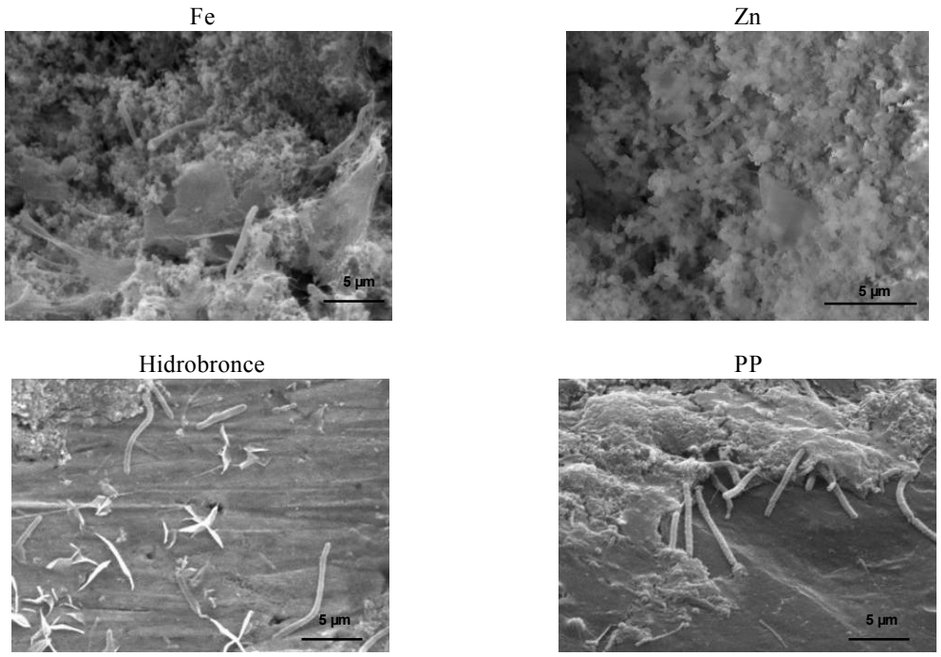

Figure 5. Bacteria in biofilm in contact with material substrata.

Respect to PP the thinnest single phase biofilm was observed, similar to the external phase formed on metallic substrata. Its low thickness allowed detecting isolated microbes at the biofilm bottom in contact with the substratum material as can be seen in Figs. 6 b)-c). Comparing with non exposed material in (Fig. 6 a) the surface failures detected near to microbial cells could not be attributed to microbial effects, but to spurious deposits and corrosion products from the other materials.

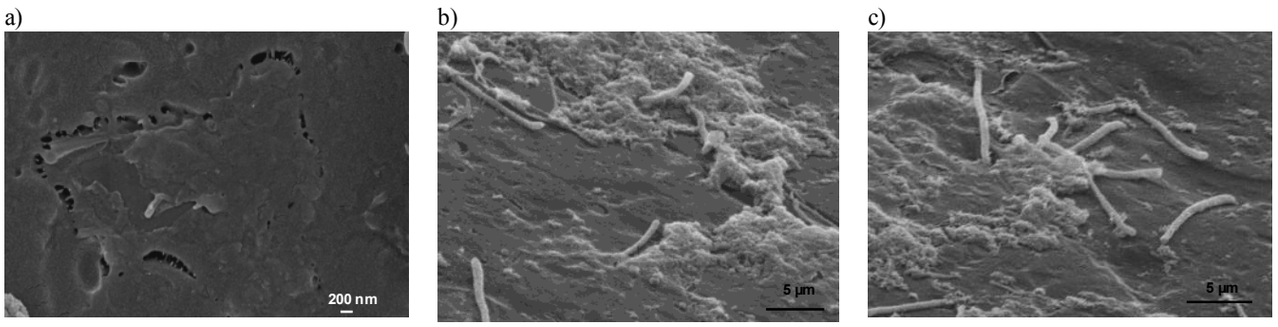

Figure 6. **a)** PP surface original defects. **b)-c)** Single biofilm on PP. Microbes in contact with the substratum.

The uneven aspect at the naked eye of samples retired after circulation test (Fig. 2) suggested the need of surface morphological biofilms analysis at diverse magnification and also through cuts in deepness. Using CLSM inner structure deconvolution was performed on randomly selected sites at a fixed depth as in sequences shown in Figs. 7 and 8.

Biofilms formed on all tubing materials showed to be very heterogeneous amongst replicates. Morphology along biofilms, as particle form, size and relative density revealed different distribution on random areas of each material as shown in Fig. 7. Single images at various deepness taken along biofilm profile showed evidence of different cells spatial distribution (Fig. 8).

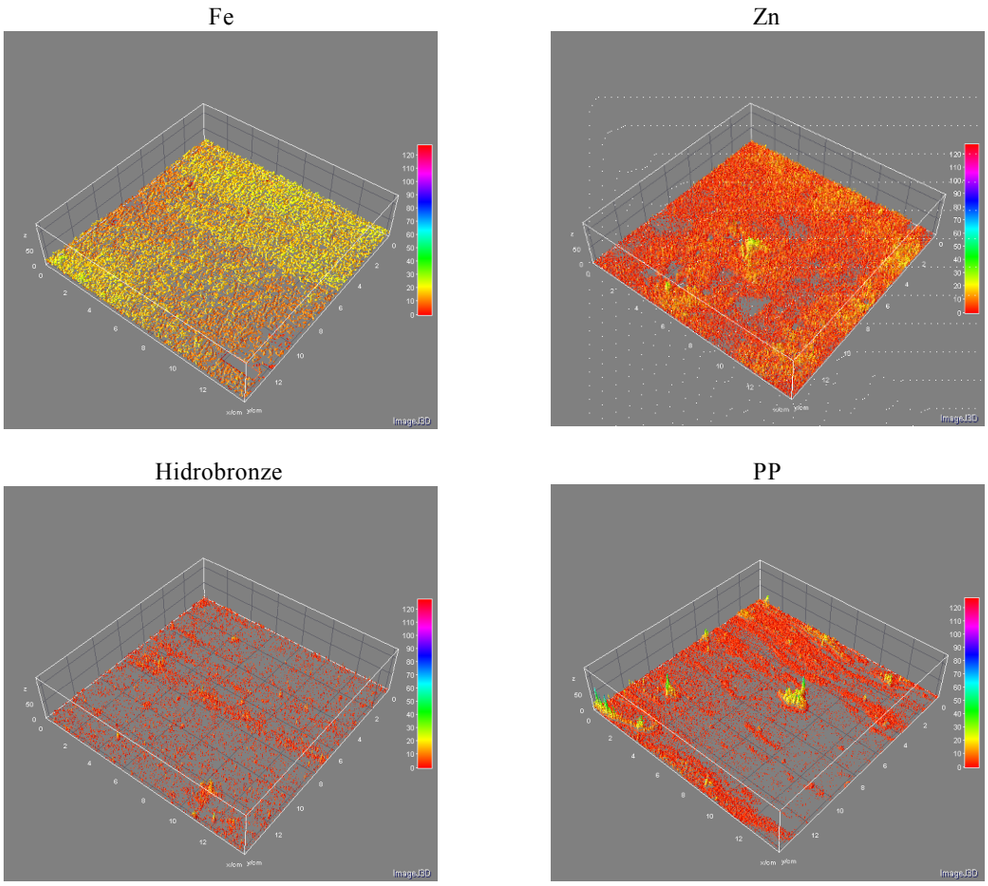

Figure 7. 3-D structure of biofilm formed on all materials after exposure to circuit.

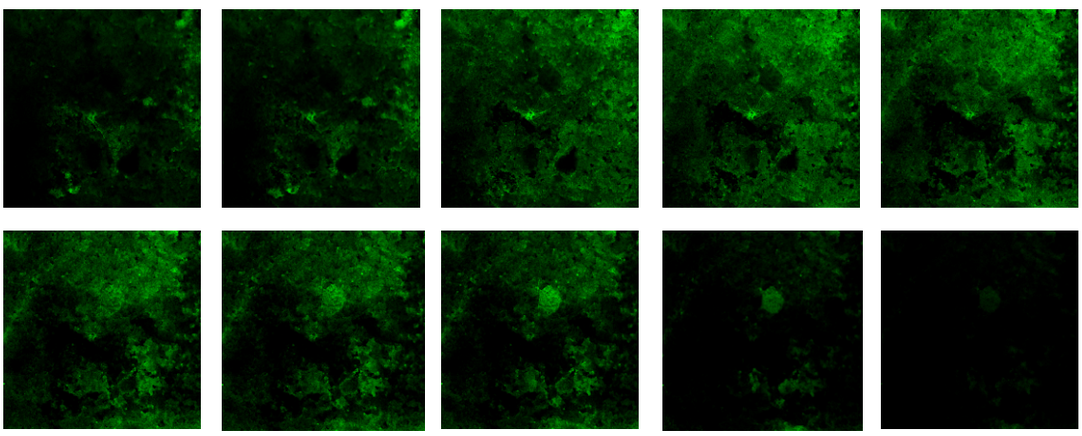

Figura 8. Space-time on a randomly chosen area of a biofilm on Zn by CLSM sequence selected amongst 34 slices.

3.2. Surface analysis

After accumulated deposits removal from different metallic samples SEM/ESEM analyses showed the attack morphology for high magnifications on the different substrata, shown in Fig. 9. The attack morphology seems to depend not only from the prevalent microbe in the biofilm and respective counting but especially from the substratum susceptibility and metallurgical structure. Fe and Zn in Figs. a and b) produced hemispherical localised attack with corrosion products accumulated on the bottom, while the localised attack of the hidrobronze attack shown in Fig. 9 c) reveals the alloy crystallography.

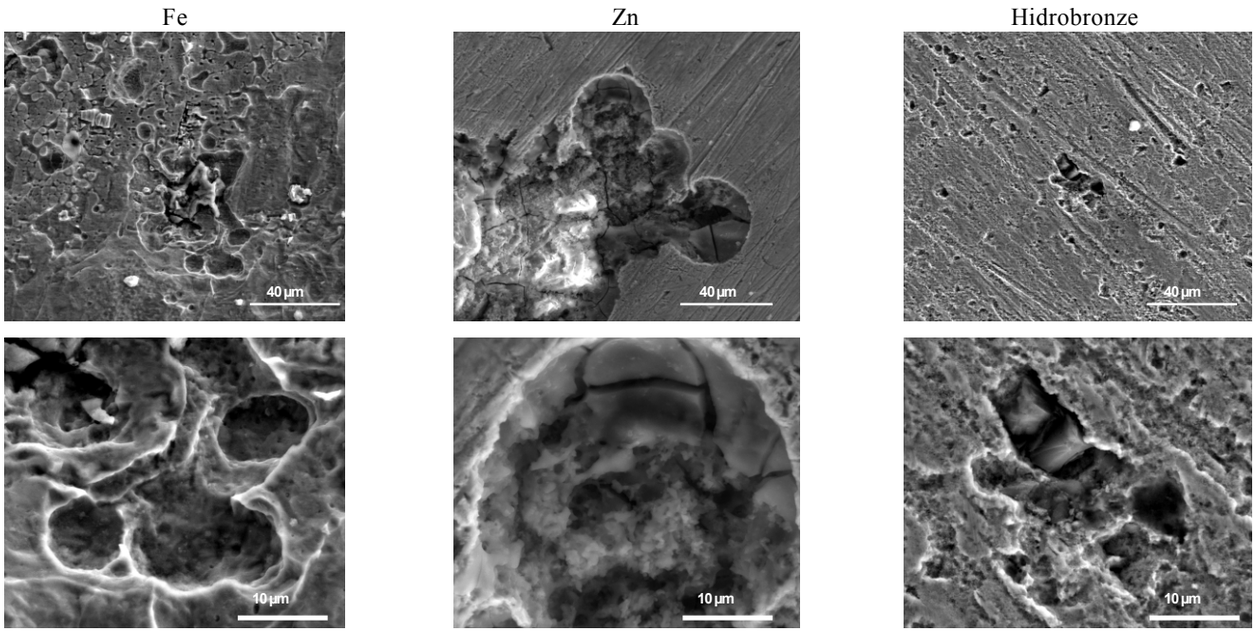

Figure 9. Underlying alterations after biofilm and corrosion product elimination.

3.3 Microbial Characterization

Due to the low number of bacteria in the biofilms, the DNA extraction resulted in a low yield (39-300 ng. ml^{-1}). As a consequence the amplification of DNA from PP and hidrobronce were unsuccesfull.
The DGGE fingerprinting of the PCR products of planktonic and biofilm microorganisms is shown in Fig. 10 and its cluster analysis in Fig. 11. The band positions in the DGGE profile corresponded to a population of individual microbial identities. At least 72 bands can be distinguished in the gel.

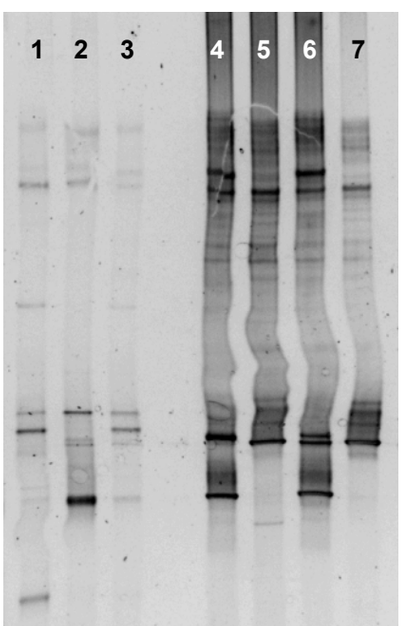

Figure 10. DGGE fingerprinting gel.
REF: 1= total planktonic; 2= total sessile on Fe; 3= total sessile on Zn; 4= culturable sessile on Fe; 5= culturable sessile on PP; 6= culturable sessile on Zn; 7= culturable sessile on Hidrobronze.

The dendrograme of DGGE profiles (Fig.11), clearly show two groups, one for culturables and one for total communities. Considering that each band represents a microbial population, it can be noticed that only those populations identified with red arrows were found on all the samples.

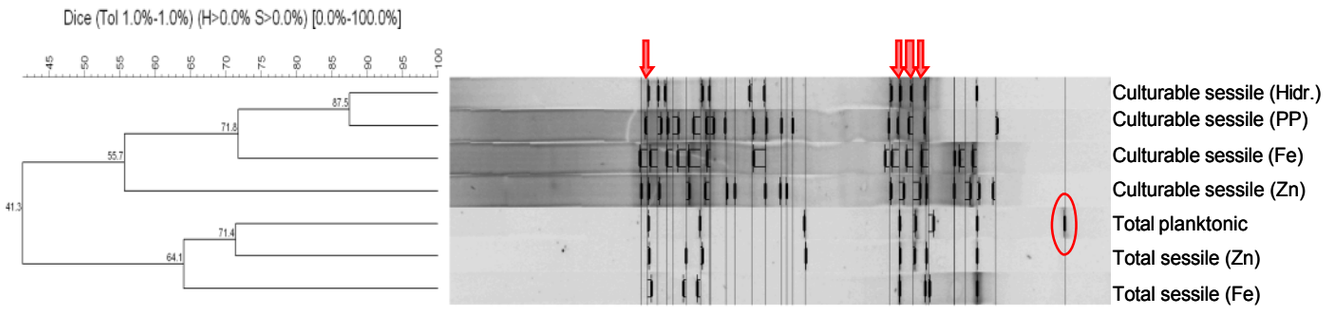

Figure 11. Cluster analysis of the DGGE band profiles. The differences between the DGGE patterns are indicated by similarity percentage.

4. DISCUSSION

Morphological analyses with SEM/ESEM-EDX of this simulated hydrodynamic test on Fe, Zn, Cu base hidrobronze and PP were compared with previous results from the Buenos Aires grey cast iron network (Rosales, 2006). The very similar topography found might suggest that most material deposited on tubing of the distribution networks does not mainly come from the row material forming the system, but from the circulating water spurious solids and biological contaminants not only during short duration (30 days) laboratory simulated loop but also when compared to an urban in service distribution system followed up in different sites for many years.
Main differences found in the latter, considering distant assessed areas in the city, were due to differences in water sources composition, organic assimilable carbon, microbial species, chlorination level and spurious material. On the contrary, the analogies observed between deposits found on all materials in the laboratory loop, triggering different phenotypes proliferation, must be attributed to the different substrata tested in the same cell and water source used.

Biofilms formed on all tubing materials showed very heterogeneous topography as those investigated through CLSM in the literature (Lewandowski & Beyenal, 2003). Cell clusters matrix investigated during CLSM observation, surrounded by interstitial water filled channels, showed very uneven spatial density and revealed a good correlation with the very different barrier effect of deposits towards further corrosion, disinfectant effectiveness and probability of reinfestation events in urban or laboratory simulated distribution system.
The random selection of the area for analysis provided very uneven biofilm compacteness, according to the spatial model proposed Lewandowski.
The application of other dyes would allow focusing different characteristics (like type, amount and distribution of EPS organic substances) or improving image acquisition and resolution.
In a previous paper (Rastelli et al, 2009) comparative anodic polarisation of each metallic material after water exposure revealed changes in the barrier effect of each metallic tubing material due to the different microbial and metallic susceptibility, confirming the highest for Zn, after Fe and the less reactive was found Cu based hidrobronze.

The dendrograme of DGGE profiles clearly shows two groups with a relatively low similarity (41.3 %). One of them, containing the sessile culturable communities, shows a closer association between the communities grown on hidrobronze and PP (87.5 %). The other group includes the total communities (sessile and planktonic). The number of DGGE bands in the gel can be used as an indication of community richness. We found more bands in lanes corresponding to culturable populations of sessile bacteria than those obtained for the total sessile populations on all surfaces. In this case the higher number of bands could be associated with the enhancement of the number of copy of bacterial DNA due to culturing.
It is worth noting that one intense band (black circle, Fig.11) is present only in the total planktonic community and can not be distinguished in any other lane. This could be related with a population present in the water, which is unable to grow attached to any of the assayed materials.
As it was already mentioned, the low amount of DNA recovered did not allow obtaining the community profiles of the biofilms formed on all the substrates. Because of this, the future experiments will be done with coupons with a higher surface area to increase the amount of attached bacteria.

5. CONCLUSIONS

The characterisation techniques applied to the biofilms growing on all tested materials seemed to mainly depend on the respective oligodynamic effect on the colonising bacteria.

The relative total microbial growth on all substrata was in the same magnitude order and dispersion as those found in previous works.

The corrosion attack susceptibility decreased in the order Zn > Fe> hidrobronze> PP.

The attack morphology on all substrata cannot still be correlated with the results of the molecular biology analyses performed.

6. ACKNOWLEDGMENTS

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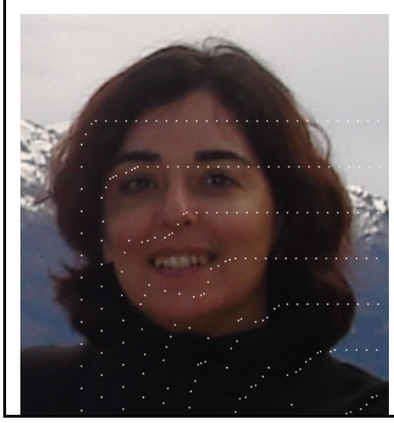
	Rastelli, Silvia E. is Biologist. She has a fellowship from Argentinean research Council (CONICET) to study the relation between arsenic, biofilms and corrosion.
	Viera, Marisa. R. works at CINDEFI, and is a research member of CONICET and Professor of Chemistry at the University of La Plata. Her main research topics are related with bioleaching, bioremediation of heavy metals and biocorrosion.