CHAPTER 6.4 EVALUATION OF BIOCIDES IN OILFIELD ENVIRONMENTS USING FLUORESCENT *IN-SITU* HYBRIDIZATION

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ABSTRACT

Microbiologically influenced corrosion and souring of oilfield reservoirs are process frequently provoked by the sulphate-reducing bacteria. The most common method applied in the industry for preventing or controlling the deleterious effect caused by the presence of microorganisms is the addition of chemical agents (biocides) aimed at killing the microorganisms or inhibiting the microbial growth. Traditionally, biocide selection and testing are based on NACE standard TM0194 which implies the use of culturing for enumerating the bacteria surviving the treatment.

To overcome culturing limitations, we used Fluorescent in-situ Hybridization to assist in the evaluation of biocides applied in water production treatment plants. Biocides were based on THPS (40% (B1); 75% (B2)) and 40% of a mixture (1/1, v/v) of THPS and benzalkonium chloride (B3) applied at two concentrations: 50 and 400 mg/L. The relation between the number of cells visualized with the fluorescent probes Eub338 and SRB385 (for eubacteria and SRB populations respectively) and the DAPI-stained cells (PR%) was used as an indication of the biocide efficiency. B1 and B3 gave a high PR% indicating that the chemical induced the metabolic cell activity. Only the highest B2 concentration showed effectiveness on eubacteria and SRB populations. Thus, through the application of FISH we were able to distinguish concentration effects of the THPS, discriminating sublethal from net inhibitory effects.

The possibility of including FISH into the protocols for the control of the biocides in water treatment plants could improve the biocide selection and the adjustment of their concentration in order to maintain the water system with a low density of metabolically active cells. This would avoid the misuse of chemicals with their consequent economic and ecological impacts.

Keywords: biocides, biocorrosion, fluorescent *in-situ,* hybridization, sulphate-reducing bacteria

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INTRODUCTION

A wide range of microorganisms, including both aerobic and anaerobic bacteria and fungi could promote the corrosion process, which reduces the lifetime of industrial materials[1]. Microbiologically influenced corrosion (MIC) and souring of oilfield reservoirs are process frequently provoked by the sulphate-reducing bacteria (SRB). The SRB group's metabolism generates substantial amounts of hydrogen sulfide and insoluble ferrous sulfide in the presence of iron. This often leads to deleterious effects manifested in the form of reduced injectivity of formation water, reservoir souring and plugging and pitting of susceptible steel pipes as well as increased sulfur content in the produced hydrocarbon[2].

Secondary oil recovery (SOR) is a procedure whereby freshwater or seawater is injected into petroleum reservoirs to support pressure and push oil toward producing wells. The water treatment process is chosen according to the source of the water supplied[3]. Because the microbial challenges and environmental parameters of these water sources vary, different microbial control strategies and treatments are required for each source. The most common method applied in the Argentinean industry for preventing or controlling the deleterious effect caused by the presence of microorganisms is the addition of chemical agents (biocides) aimed at killing the microorganisms or inhibiting the microbial growth. Tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and benzalkonium salts (BAC) are among the biocides of choice in the oil industry. THPS can damage the cell membrane and inhibit the lactate dehydrogenase enzyme required for SRB metabolism[4], while BAC interacts with guanine nucleotide triphosphate-binding proteins (G proteins), thereby affecting signal transduction in a variety of cell types and processes[5]. Also, they react with membrane phospholipids causing membrane disorganization, leakage of cell contents, and eventually cell lysis.

Traditionally, biocide selection and monitoring are based on NACE standard TM0194[6] which implies the use of culturing for enumerating the bacteria surviving the treatment. Conventional culture-based methods for studying SRBs are time consuming as a result of slow bacterial growth rates and do not necessarily reflect the role of uncultivated bacteria in the oilfield operation[7]. Fluorescent *in-situ* Hybridization (FISH) is a molecular technique applied to environmental samples, which uses fluorescently-labeled oligonucleotide probes that hybridize specifically to its complementary 16S rRNA target sequence within the intact cell. A major advantage of FISH compared to other microbial ecology techniques is that the abundance of the detected microorganisms can be directly determined. Most frequently, the relative abundance of specific community members is estimated by counting (using an epifluorescence microscopy) the cells stained with a general DNA-binding dye and the cells hybridized with a specific probe. FISH has previously been used for direct identification and quantification of bacteria from natural samples without culturing in drinking water and biofilms[8], acid mine drainages[9] and activated sludge[10].

The aim of this work was to evaluate the utility of FISH in the selection of an appropriate biocide to control the microbial population present in the water treatment plant of an oil secondary recovery plant in an Argentinian oilfield

MATERIALS AND METHODS

Test fluid and Inoculum source. Water samples from two water treatment plants of secondary oil recovery placed in Mendoza and Neuquén provinces, Argentina were used as source of the targeted microorganisms and as fluid medium for biocide challenges. Samples from Mendoza treatment plant were taken at two points: near the water entrance to the plant (EW), and before the exit to the field (SF). Neuquén plant was sampled at a point corresponding to the storage water tank located before the pump to the field (STk). Table 1 shows the physicochemical properties of the waters determined following standard methods. Enrichment cultures in of these water samples prepared in Postgate´s B medium[1] (with the addition of 50 g/L NaCl to approximate the salinity of the produced waters) incubated at their corresponding temperature were tested as inoculum in the biocide inhibition assays. Additionally, a SRB consortium (SRB-OT) of our laboratory culture collection was used.

Chemicals. Three available commercial biocides were used. Biocide 1: 40% (w/w) tetrakis (hydroxymethyl) phosphonium sulfate (THPS); Biocide 2: 75% (w/w) THPS and Biocide 3: 40% of a mixture of THPS and benzalkonium chloride (BAC), 1:1 (v/v).

Table 1. Physicochemical propierties of the three water samples originated in oil fields

Properties / [unit]	EW	SF	STk
PH	7.0	6.7	7.20
Conductivity / [mS/cm]	108.1	107.4	163095.21
Carbonate / [mg/L]	< 1	< 1	< 1
Bicarbonate / [mg/L]	366	329	889.28
Chloride / [mg/L]	43249	42540	68577.08
Sulfate / [mg/L]	1258	1261	1857.00
Calcium / [mg/L]	1063	1043	1763.52
Magnesium / [mg/L]	49	146	807.42
$Na^+ + K^+$ / [mg/L]	27476	26843	41882.71
Total Dissolved Solids / [mg/L]	73461	72162	113283.48
Total Suspended Solids/[mg/L]	59.2	2.7	94.63
Temperature / [°C]	62	60	30
Dissolved oxygen / [ppb]	60	< 10	163.33
Total Sulphide / [mg/L]	9.5	27.9	2.07
Dissolved Sulphide / [mg/L]	6.8	26.1	No determined

Experimental design for the biocide inhibition assays. The assays were done in 10-mL bottles filled with filter-sterilized field water. Anaerobic conditions were achieved by adding thyoglycolate-ascorbic acid solution. A 1% inoculum was added to each bottle. A known volume of a distilled water-based biocide stock solution was added in the reaction bottles. Distilled water instead of biocide stock solution was added to control bottles.

Incubation time (4 h) and temperature (30 or 60°C) were chosen to match field conditions. At the end of the incubation time, 1-mL samples were withdrawn from each bottle to determine the cultivable bacterial counts by extinction method, in R2 broth and Postgate´s B (plus 50 g/L NaCl). Tubes were incubated 3 weeks in the dark at the selected temperature before scoring. The remaining volume was filtered and processed for hybridization. Triplicate assays for each biocide at each concentration were done.

Fluorescence in-situ hybridization. A volume of water sample was filtered onto 0.2 µm nitrate cellulose membrane and fixed in 4 % paraformaldehyde for 2-4 h at 4°C. The volume to be filtered was adjusted in order to get an adequate number of cells on the membranes, usually 10 mL of the sample or 10mL of a 1/10 dilution were used. The membranes with the fixed samples were washed three times with 3 mL phosphate buffered saline [PBS; 130 mM sodium chloride, 10 mM sodium phosphate buffer (pH 7.2)] and stored at –20 ºC.

For hybridization, a section of the membrane filter was dehydrated by successive dipping in 50, 80 and 98% ethanol for 3 min and allowed to air dry. The piece of filter was place on a clean microscope slide and cover with 8 µL of hybridization buffer (20 mM Tris-HCl pH 7.4, 0.9 M NaCl, 0.01% sodium dodecyl sulfate (SDS) and 35% formamide) and 1µL of the fluorescent oligonucleotide probe solution (25ng/mL).

The Eub338 probe was used to detect most members of domain Bacteria and Non338, (complementary to Eub338) was used as the negative control for non-specific binding The SRB385 probe was used to detect most species of SRB belonging to δ-proteobacteria[2]. Al the probes were 5P end-labelled with Cy3.

The slide was placed in a hybridization chamber, equilibrated with the hybridization buffer and incubated at 46º C during 2 h. The hybridization mixture was gently removed with several milliliters of the washing buffer (20 mM Tris-HCl pH 7.4, 0.1% SDS and NaCl) prewarmed at 48°C. The stringency of the washing step was adjusted with 18mM NaCl in the washing buffer. The slides were immersed for 20min in the washing buffer at 48°C. Then they were briefly washed with distilled water, air-dried, stored in the dark.

For the determination of the total number of bacteria in the samples, filters on the slides were stained with 5 µL of the general bacterial stain DAPI (4, 6-diamidino-2-phenylindole dihydrochloride) (1 µg/mL). The slides were covered with antifading solution, and fluorescence was detected with a DMLB microscope coupled to a DC100 camera (Leica Microscopy System) and filter sets 01 (for DAPI staining), and 15 (for Cy3 probes). Among 15 to 20 images were analyzed per sample.

RESULTS AND DISCUSSION

According to the NACE Standard TM0194-2004[6], the organisms used to challenge a biocide to be applied in an oil field should be the bacteria normally found in the oil field waters (which would be the test fluid). Alternatively, up to a 1% inoculum of a fully grown culture originating from the oil field may be used. The hybridization of the water samples with the Eub338 probe done immediately after reception in our laboratory (one week after collection) showed a low number of bacteria stained with DAPI (Figure 1, above left) with very low percentage of the cells emitting the probe-conferred fluorescence. (Figure 1, above, right). This behavior could be attributed to the low metabolic activity of

the cells present in the water samples. Taking into account that the objective of our work was to evaluate the biocide efficacy in the bacteria population present in the oil field environment using FISH, such low hybridization efficiency would reduce the degree of certainty of the results. Thus, enrichment cultures were done. Hybridization of these samples yielded a higher intensity of the fluorescence signal and an increase in the hybridization ratio (**Figure 1**, below). The ratio between the number of cells visualized using the fluorescent probes and the DAPI-stained cells defined the Probe Ratio (PR%). This value was near 80% for the Eub338 probe. This detection yield was comparable to those obtained for activated sludge[4] and sea sediment[5]. Thus, it was decided that the enrichment cultures were inoculated (at 1%) in the test fluid for biocide testing.

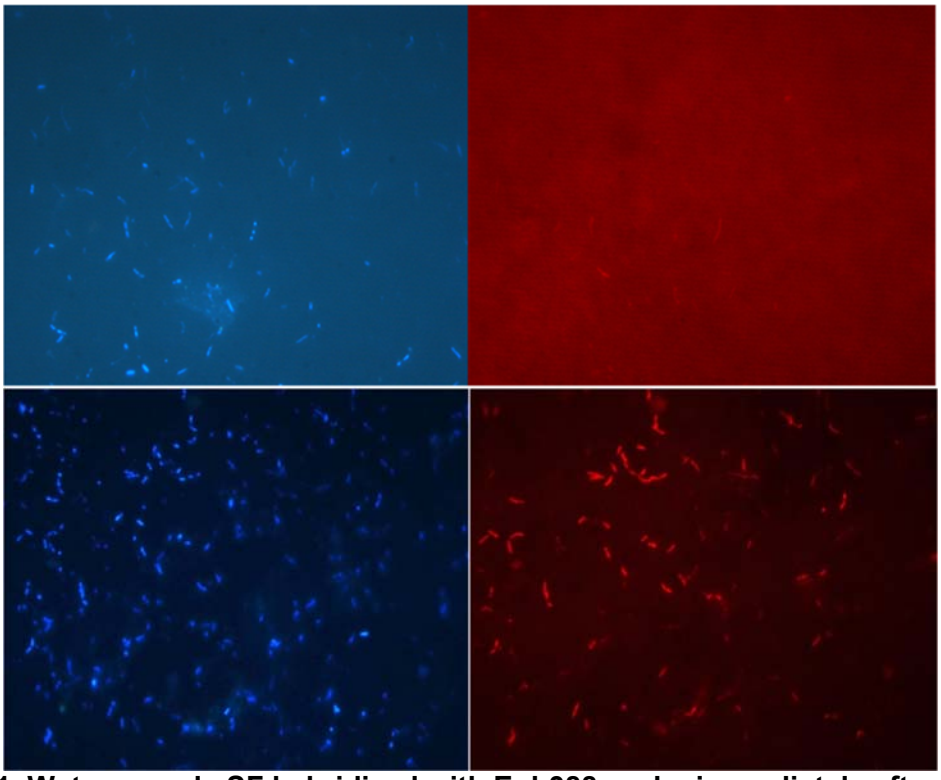

FIGURE 1. Water sample SF hybridized with Eub388 probe immediately after received (above) or after enrichment in Postage´s B medium (below). Left images: DAPI fluorescence; right images: Cy3 fluorescence

Biocide inhibition assays. Two inhibition experiments were done. In the first one, SF was used as test fluid with SRB-OT as inoculum. In this experiment, the viable bacterial population was tested by hybridization using Eub338 probe. In the second experiment, EW was used as test fluid and the enrichment culture originated in this sample was the inoculum. In this case, the bacterial population and the SRBs were detected by hybridization using the Eub338 and SRB385 probes. The efficiency of the biocides was measured through the evaluation of the PR%. A lower PR was expected in the presence of the biocide in relation with the control systems, as an indication of the deleterious effect of the biocide on the bacteria.

Assay 1- Biocide inhibition assay in SF as fluid test and the Eub338 probe. In this case, the inhibitory effects of the three biocides (B1, B2 and B3) at two concentration levels (50 mg/L and 400 mg/L) were evaluated. Both concentrations are usually used in water treatment in the secondary oil recovery process. The results of the viable counts performed in R2 broth and Postage´s B medium for the enumeration of the total bacteria and the SRB showed that the biocides are effective in reducing the number of viable bacteria (**Table 2**), as their number is reduced from 10^3-10^4 cell/mL in the control systems to only 1-10 cells/mL in the presence of 50mg/L of any biocide. At the highest concentration used all the tubes were negatives.

TABLE 2. Viable counts determined by the dilution to extinction method after biocide treatment using SF water sample. The biocides were applied at 50 and 400 mg/L

	Biocide (mg/l)	THPS (mg/l)	BAC (mg/l)	Extinction tubes (R2broth/PGB) 1(1-10)	2(10-10^2)	3 (10^2-10^3)	4 (10^3-10^4)
Control		-	-	+/+	+/+	+/+	+/+
B1	50	20	-	+/+	-/-	-/-	-/-
	400	160	-	-/-	-/-	-/-	-/-
B2	50	37.5	-	+/-	-/-	-/-	-/-
	400	300	-	-/-	-/-	-/-	-/-
B3	50	10	10	+/-	-/-	-/-	-/-
	400	80	80	-/-	-/-	-/-	-/-

Figure 2 shows the PR% values obtained after 4 h of incubation. The first two columns represent the values obtained in the control systems after 15 min and 4h of incubation. A decrease in PR was observed after 4h, attributed to the loss of metabolic activity of the cells during the incubation at 60 °C in the water. Because of this, it was not possible to estimate the percentage of surviving bacteria after the biocide treatments compared to the control. However comparisons amongst biocides were still possible and, interestingly, very different behaviors were detected. As it can be seen, B1 at the lowest concentration gave a high ratio indicating an induction of the metabolic cell activity. At the highest concentration, this biocide reduced the number of metabolically active cells, but not at a level as low as the control. On the other hand, B2 provoked significant inhibitory effects at both concentrations. A different behavior was observed with B3, which did not produce a significant effect at 50 mg/L and induced the metabolic cell activity at 400 mg/L.

The metabolic activation of the bacterial cells observed in the presence of B1 and B3 could be associate to an induced response of the bacteria to an environmental stress (in this case, it could be the presence of a sublethal concentration of biocide). In this situation, bacteria could express numerous stress mechanisms to face with the biocide. Adopting a stress resistant phenotype frequently involves growth arrest and the adoption of a metabolically downregulated state[11]. Maintaining functional growth machinery, such as ribosomes, represents the highest energetic expenditure for stressed cells, which therefore divert their resources towards survival rather than growth when conditions deteriorate[12].

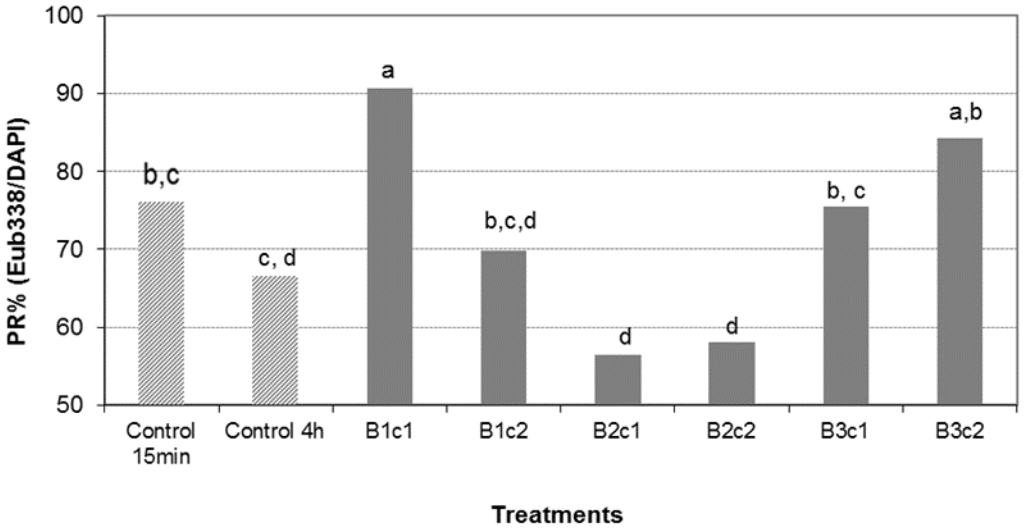

FIGURE 2. PR% values for the Eub338 probe after 4h of incubation with the biocides B1, B2 and B3 at two concentrations: 50 mg/L (c1) and 400 mg/L (c2). (Equal letters indicate no significant difference)

The highest THPS concentration tested (300 mg/L present in 400 mg/L B2) was effective against heterotrophic and SRB population reducing significantly the cultivable cell number to below the detection limit. Although, no significant differences with the control were observed by hybridization, a tendency to provoke cell death could be inferred for 300 mg/L THPS. It has been reported that THPS had little inhibitory effect on E. coli, up to 243.6 mg/L whereas at 487.2 mg/L it lowered CFU/mL by 3 log units after one hour of contact[13]. According to our results, the TPHS effective concentration was lower; however, the FISH results indicated that some cells remained metabolically active in the fluid test.

When THPS was applied along with BAC (B3), a sublethal effect, similar to that obtained when THPS was applied at the lowest concentration (20 mg/L) was observed. The result suggested that there was no synergic effect among both chemicals on the water bacteria community. Mixtures of biocides with a different mode of action are likely to be synergistic. However, in agreement with our results, other authors found that mixtures of THPS with other agents did not exhibit any synergistic effect[14].

Assay 2- Biocide inhibition assay in EW as fluid test with Eub338 and SRB385 probes. In this case, the three biocides were applied at one concentration (200 mg/L) and their inhibitory effects were determined on the total bacterial population and on the SRB populations by counting the cells hybridized with the Eub338 and SRB385 probes. Figure 3 shows the PR% values obtained for 15 min and 4h of incubation. In this experiment, it was not observed a decrease in PR in the control system, after the incubation. The presence of 200 mg/mL of the B1 and B3 produced an increase in PR, associated with a higher metabolic cell activity in the survival cells after the treatment. No significant effect on the total bacterial population was observed with the same concentration of B2.

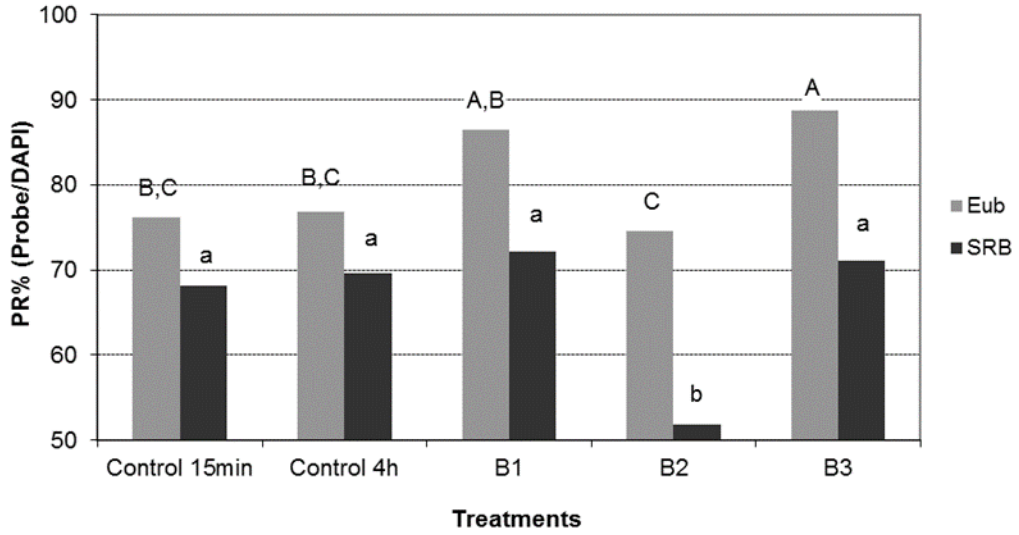

FIGURE 3. PR% values of both probes (Eub338 and SRB385) in EW water after 4h of incubation with the biocides B1, B2 and B3 at 200 mg/L. (Equal letters indicate no significant difference)

The effect of B1 and B3 on the SRB population was not different from that observed in the control system. Instead, a significant inhibitory effect was produced by B2 on this population. In this experiment, the cultivable viable count after 4h of incubation (Table 3) showed that there was no growth in any of the tubes used for enumeration.

TABLE 3. Viable counts determined by the dilution to extinction method after biocide treatment using EW water sample. The biocides were applied at 200 mg/L

	Biocide (mg/l)	THPS (mg/l)	BAC (mg/l)	Extinction tubes (R2broth/PGB) 1(1-10)	2(10-10^2)	3 (10^2-10^3)	4 (10^3-10^4)
Control		-	-	+/+	+/+	+/+	+/+
B1	200	80	-	-/-	-/-	-/-	-/-
B2	200	150	-	-/-	-/-	-/-	-/-
B3	200	40	40	-/-	-/-	-/-	-/-

The THPS effect in this test fluid was similar to that observed in SF, producing metabolic activation of the eubacteria population at the minor concentration (80 mg/L THPS present in 200 mg/L B1) and mixed with BAC (40 mg/L THPS and 40 mg/L BAC present in 200 mg/L B3). Also, no changes in the eubacteria PR% was observed applying 150mg/L THPS (present in 200 mg/L B2) in comparison with the control. However, this concentration of THPS was the only one that significantly reduced the metabolic activity of the SRB population.

The study of the global gene expression resulting from THPS stress on *Desulfovibrio vulgaris* v.H showed that the genes for enzymes related to the energy metabolism were strongly affected[13]. Also, these genes were affected during the treatment with BAC. Although these results were obtained using a pure culture of a typical SRB strain, it

could possible to correlate them with our findings in an enrichment culture of sulphate reducing bacterial community in an actual water production sample. In this sense, we could attribute the increase in the 16SrDNA probe hybridization ratio to an increase in the ribosomal content induced by the application of a sublethal THPS concentration. Likewise, the significant reduction in the hybridization ratio at higher THPS concentration could be attributed to the death of the SRB. In this regard, it is important to point out that even though no culturable cells were detected in the presence of the biocides, at least fifty percent of the cells could be detected by hybridisation with the SRB385 probe. This would mean that survival cells could remain in the water although they were no detected by the conventional cultivable methods.

The use of higher concentrations of biocide might be an alternative to exert its killing action. However, downstream of the treated area there is likely to be a continuum of biocide concentration ranging from the treatment concentration to nil. Thus, sooner or later they can be found in natural environments at low (sub-lethal) concentrations, leading to continuous exposure of water and soil microbial community[15]. As could be shown in our experiment, the usual concentration recommended by the manufacture resulted to be a sublethal dose. This has an unwanted economic and ecological impact. In this sense, the misuse of expensive chemical would condition a habitat where selected tolerant cells may enhance some characteristics potentially useful for colonization of specific environments (biofilm), decreasing the efficacy of biocide treatments and exacerbating the corrosion problems.

Eventually, this scenery could lead to the emergence in the environment of multi-drug resistant strains. Several authors have found a relation between the resistance to biocides and the tolerance to antibiotics[16]. Those results highlight the relevance of the application of sublethal biocide concentrations on the microbial communities from very different habitats.

CONCLUSION

The hybridization assay using ribosomal targeted probes allowed us to detect concentration effects of THPS, discriminating sublethal from net inhibitory effects. Also, it was possible to evidence that the mixture of active compounds such THPS and BAC could not exert a synergistic effect on the heterotrophic and the SRB populations. The possibility of including the FISH technique in the protocols applied in water treatment plants could improve the biocide selection process, contributing to the biocide design targeted to SRB included concentration adjustment in order to maintain the system with an actual low density of metabolically active cells. This would help to avoid the misuse of chemicals with their consequently economic and ecological impacts. This means a cooperative work between the industry and the researchers to improve the protocols for the selection of specific biocides with the ultimate goal of reducing the ecological impact.

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