Evaluation of EPS Application in Microbial Consortia Growing in Aqueous Co-Contaminated Systems: The Trend to Natural Attenuation

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Abstract: The needing of a bacterial extracellular polymeric substance (EPS) application in bioremediation of an aqueous system containing glucose and/or gasoline and/or heavy metals (Cd^{2+}, Zn^{2+} and Cu^{2+}) by two different microbial consortia was studied. At the low concentrations (1.00 ppm of each metal), it was observed an inhibitory effect on the metabolism of the "consortium 1", as well as the application of EPS has improved the cellular growing in media containing glucose and/or gasoline as carbon sources, indicating that using this substance decreases the negative effect caused by the presence of heavy metals. In the other hand, the application of small concentrations of EPS was evaluated, and results show that a little increase in this substance concentration leads to an improvement of 39 % in cellular growing of the "consortium 2", indicating the potential use of the EPS in a system with gasoline and metals. Once many microorganisms can produce this kind of substance during cultivation, our results show that a system with low concentrations of hydrocarbons and metals could be susceptible to natural attenuation, without human intervention in the environment, especially if the process is conducted in a larger period of time.

Key Words: EPS, heavy metals, co-contaminated systems, natural attenuation.

INTRODUCTION

In co-contaminated systems, the presence of heavy metals can inhibit the natural microbiota involved in the degradation of organic compounds and affect biodegradation rates. The level of inhibition will depend on the concentration and availability of the heavy metals and is dependent on the action of complex processes controlled by multiple factors including the nature of the metals, media and microbial species. Heavy metals inhibit microorganisms by blocking essential functional groups or interfering with essential metal ions incorporation of biological molecules. In some cases microorganisms are resistant to some heavy metals through different possible mechanisms [1].

Effective strategies to enhance organic biodegradation in the presence of toxic metals include reducing the bioavailable concentration of the toxic metal and reducing interactions of the toxic metal with the cell. In their work, Sandrin *et al.* (2000) [2] carried out an experimental system to determine the effect of a rhamnolipid, which is an EPS produced by *Pseudomonas aeruginosa* 9027, on the capability of a metal-sensitive microorganism to degrade organic contaminant. Their results suggested that rhamnolipid reduces cadmium induced inhibition of naphthalene degradation through a combination of cadmium complexation and release of lipopolysaccharide (LPS) from the cell.

Extracellular polymeric substances (EPS) play an important role in aggregation of bacterial cells in flocks, stabilization of the biofilm structure, retention of water, formation of a protective barrier that buffers harmful effects, but also play a very important role in biosorption of heavy metals, and are produced by most bacteria. EPS comprise a mixture of polysaccharides, mucopolysaccharides and proteins, which depends on the strain and its culture conditions. They contain ionizable functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups, which enable EPS to sequester heavy metals. Ion exchange, complexation with functional groups of negatively charged, adsorption and precipitation are the mechanisms involved in metal biosorption onto EPS [3].

The clean-up of petroleum-contaminated groundwater (i.e. water saturated subsurface zones) continues to be a challenge. According to Van Stempvoort *et al.* (2007) [4], one approach that has been recently recognized for groundwater applications is natural attenuation. Natural attenuation as a management strategy for contaminated soils and sediments hinges on the notion that some transformation processes involving the in situ microbial metabolism of the target compounds are self-sustaining, appropriate with regard to type and sufficient in magnitude to control the risk associated with resident pollutants [5]. Biotechnological approaches to the abatement of toxic metal and organic pollution consist of selectively using and enhancing the natural processes to treat particular wastes [6].

The aim of this work was to investigate the effect caused by the presence of a bacterial EPS in aqueous systems containing low concentrations of heavy metals (Cd^{2+}, Zn^{2+} and Cu^{2+}) and gasoline, in some cases glucose, in the metabolism of two different microbial consortia, reflected in their growing curves.

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MATERIALS AND METHODS

Production, Extraction and Partial Purification of EPS

In order to produce the EPS, it was used the Gram-positive spore-forming bacterium *Paenibacillus polymyxa*, isolated from fermented sausages, as described by Piuri *et al*. (1998) [7]. The microorganism was grown in a solid medium of following composition (adapted from Sharma *et al*. (2001) [8]): sucrose 5.00 g/L; yeast extract 0.15 g/L; KH2PO4 0.50 g/L; (NH4)2SO4 1.0 g/L; MgSO4.7H2O 0.41 g/L; agar 30.00 g/L, pH 7.0, over 2 days at 30ºC. After this time, the culture was kept under refrigeration at 10 ºC. For activating and obtaining the pre-inoculum, subsequent culturing took place in 400 mL of a medium of same composition described above, in absence of agar, and incubated for at 30ºC on a rotatory shaker at 150 rpm, for 24 hours. The initial cell concentration was 0.01 g/L.

The growing cells were heated until 100 ºC for 15 minutes for releasing the cell surface adsorbed EPS. After that, the medium was concentrated with a rotary vaporizer and further centrifuged at 3000 rpm for 30 minutes, for cell removing. The supernatant volume was measured and three volumes of cold ethanol were added for EPS precipitation, and the mixture was kept under refrigeration for 24 hours. After new centrifugation, at same conditions described above, precipitated EPS was dialyzed under refrigeration with distilled water overnight. Partial purified EPS was dried in a stove at 50 ºC until constant weight.

Propagation of Microbial Consortia

Microbial consortia used in these experiments were obtained through two different methods. In the first one, according to Thompson *et al*. (2005) [9], 5.0 g of a soil sample were mixed to 100 mL of a mineral medium, with the following composition: NaCl 5.00 g/L; KNO_3 3.00 g/L; K_2HPO_4 1.00 g/L; $(NH_4)_2SO_4$ 1.00 g/L; $NH_4H_2PO_4$ 1.00 g/L; $MgSO_4.7H_2O$ 0.20 g/L and pH 7.0. Five grams per liter of glucose was added to stimulate the total microbial population. The authors report that this procedure enhances the chances of obtaining isolates that could metabolize gasoline later. The mixture was kept under agitation of 150 rpm at 30 ºC for 48 hours, in a New Brunswick Scientific Shaker. After this period of time, a sample of 10 mL was taken from the medium and transferred to 100 mL of mineral medium, also containing 5.0 g of a soil sample and 5.0 g/L of glucose. This new system was kept under agitation at 150 rpm and 30 ºC for 24 hours. Following this procedure, a sample of 10 mL was take from the last flask and transferred to a new one, containing 100 mL of mineral medium, 1.0 g/L of glucose and 1.0 % (v/v) of gasoline, obtained from Refinaria Duque de Caxias (REDUC, RJ), in order to select a population capable to consume gasoline. This system was also kept under agitation of 150 rpm and 30 ºC, for 4 days. This last step was repeated once, and the maintenance of the consortium was made in liquid mineral medium, with 0.5 g/L of glucose and 0.1 % (v/v) of gasoline, under refrigeration. The microbial consortium obtained with this technique was called "consortium 1".

The second technique used a methodology similar to the first one, but it was not added glucose in any phase of propagation, and 1.0 % (v/v) of gasoline was used in all steps, in order to simulate hydrocarbons contaminated environment. Each mixture was kept under agitation for 4 days, at 150 rpm and 30 ºC. This new consortium was called "consortium 2".

Cellular Growing in Co-Contaminated System

Experiments involving "consortium 1" were carried out in duplicate, in 200 mL of mineral medium, amended with glucose 0.5 g/L and/or gasoline 0.10 % (v/v) as carbon source (s). Some flasks contained 1.00 ppm of each heavy metal (Cd (II), Zn (II), and Cu (II)), and other flasks contained EPS at final concentration of 0.05 g/L. Table **1** shows the conditions of the experiments. Samples were taken from each medium also in duplicate for protein quantification, in order to build cellular growing curves to evaluate the influence of heavy metals and EPS in the curves profile. The values of protein concentration presented in all Figs. (**1-5**) are the average of the four data, and the bars in graphic are referred to the standard errors.

Table 1. Experimental Conditions Used for "Consortium 1"

Experiment	Mineral Medium Containing			
	Glucose 0.5 g/L	**Gasoline 0.1 % (v/v)**	**Metals 1 ppm**	**EPS 0.05 g/L**
G	yes	no	no	no
Gas	no	yes	no	no
GGas	yes	yes	no	no
GasEPS	no	yes	no	yes
GGasEPS	yes	yes	no	yes
GM	yes	no	yes	no
GMEPS	yes	no	yes	yes
GasM	no	yes	yes	no
GasMEPS	no	yes	yes	yes
GMGas	yes	yes	yes	no
GMGasEPS	yes	yes	yes	yes

In order to verify the ideal concentration of EPS that can improve cellular growing in an aqueous system co-contaminated with heavy metals and gasoline, an experimental design was carried out, using the conditions presented in Table **2**. "Consortium 2" was used in these experiments, because it was obtained under conditions that are similar to what is found in contaminated systems resulted by gasoline leaking, and glucose probably is not present.

The experimental design was made with quintuplicate of central point (Experiment 9).

All experiments were carried out in duplicate, in 50 mL flasks, containing 20 mL of mineral medium. Samples were taken from each medium for protein quantification, in order to build cellular growing curves to evaluate when the protein concentration is higher, and so using this time as the re-

sponse. Results were evaluated with the software DESIGN EXPERT 6.0.4.

Table 2. Experimental Conditions Used for "Consortium 2"

Experiment	Gasoline Concentration (% (v/v))	Each Metal Concentration (ppm)	EPS Concentration (g/L)
1	0.10	1.0	0.01
2	0.10	1.0	0.05
3	0.10	5.0	0.01
4	0.10	5.0	0.05
5	1.00	1.0	0.01
6	1.00	1.0	0.05
7	1.00	5.0	0.01
8	1.00	5.0	0.05
9	0.55	3.0	0.03

Biomass Quantification

Once it is not possible to get homogenous samples when the experiments involve gasoline or other hydrocarbons as carbon source, microbial growing was indirectly determined by intracellular protein quantification, according to Sandrin *et al.* (2000) [2], with the use of Lowry's method, after biomass hydrolysis with 10 % (v/v) of NaOH 1 M at 100 ºC for 10 minutes. After reaction, absorbance was determined with an espectrophotometer CamSpec, model M302, at 660 nm. Protein concentration was obtained by the average of absorbance values of the samples obtained in the experiments. In order to relate samples absorbance to protein concentration, a BSA (bovine serum albumine) standard curve was built.

RESULTS

Experiments with "Consortium 1"

The standard errors observed in protein and glucose concentration values (Figs. **1-4**) are small and not considered as relevant. It is possible to observe, in Fig. (**1**), the inhibitory effect caused by the presence of heavy metals on the microbial metabolism, evidenced in the growing rate decrease in the experiment involving glucose and metals (GM curve), when compared to experiment involving only glucose (G curve).

After a relatively large period of time, corresponding to lag phase in experiment involving glucose and metals (GM), it is possible to note a little cellular growing, represented by the protein concentration, which indicates that consortium microorganisms are adapted to the presence of metals in the medium. The presence of EPS in medium containing glucose and metals resulted in a growing curve identified as GMEPS. It is possible to note that the cellular growing was not as sharp as G curve, related to experiment involving just glucose. In the other hand, it was not as small as in GM curve, related to experiment involving glucose and metals. This intermediate growing indicates that somehow EPS was able to attenuate the inhibitory effect caused by metals in the microbial metabolism, probably due to metals adsorption or complexation, decreasing their bioavailability to biomass.

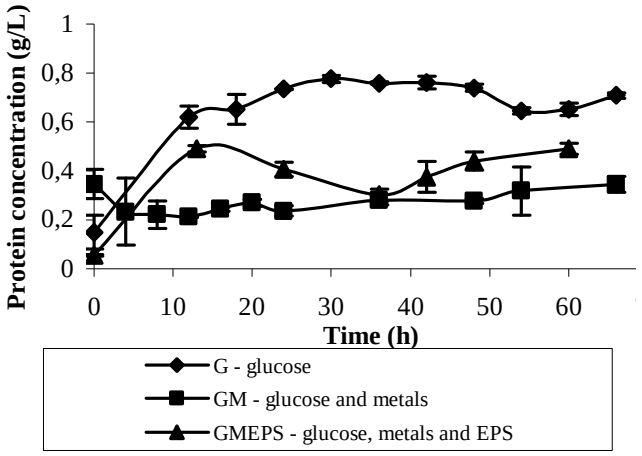


Fig. (1). Growing curves for "consortium 1" in media containing only glucose as carbon source, with or without metals and EPS.

Glucose consumption curves are presented in Fig. (**2**). It is possible to note that glucose consumption was slower in medium containing metals (GM curve), characterizing the metabolism inhibition. Similarly, glucose consumption in medium containing metals and EPS (GMEPS curve) was faster, indicating the attenuation of inhibitory effect caused by the EPS presence in medium with a little difference when compared to experiment containing only glucose (G curve).

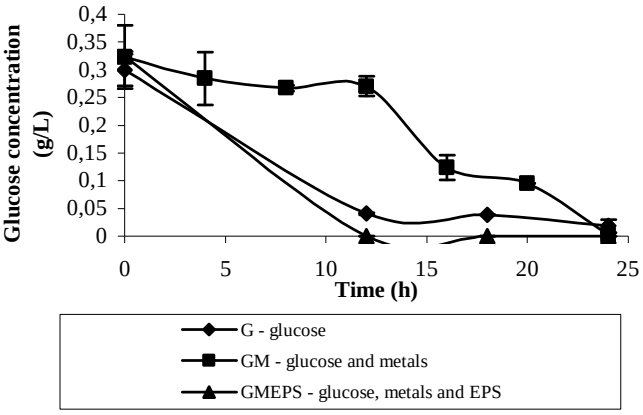


Fig. (2). Glucose consumption by "consortium 1" in media containing glucose, with or without metals and EPS.

Fig. (**3**) presents the consortium growing profile in media containing glucose and gasoline as carbon sources, and heavy metals and/or EPS. Experiment involving glucose, gasoline and EPS (GGasEPS curve) was carried out to compare the cellular growing to experiment involving only glucose and gasoline (GGas curve), in order to verify the possible consumption of EPS as a carbon source. It is possible to note that both curves are very similar, what lead us to believe that EPS was not consumed during growing.

Similarly to experiments carried out only with glucose, inhibitory effect caused by metals presence in solution is clear (GMGas curve), as well the presence of EPS has at-

tenuated this effect, confirmed by a larger cellular growing (GMGasEPS curve). In the other hand, gasoline presence associated to glucose lead to a smaller effect of inhibition

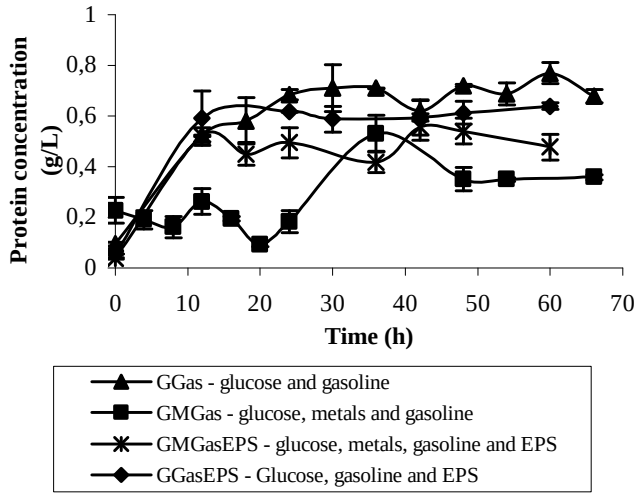


Fig. (3). Growing curves for "consortium 1" in media containing glucose and gasoline as carbon sources, with or without metals and EPS.

caused by metals (GMGas curve), when compared to experiment without gasoline (GM curve), presented in Fig. (**1**). Experiments carried out with metals and/or EPS (GMGas and GMGasEPS curves) presented results similar to maximum growing obtained in medium containing glucose and gasoline (GGas curve) an containing only glucose (G curve) (Fig. **1**).

Similarly to observed in Fig. (**2**), presence of metals slows glucose consumption, and EPS presence attenuates this effect (Fig. **4**).

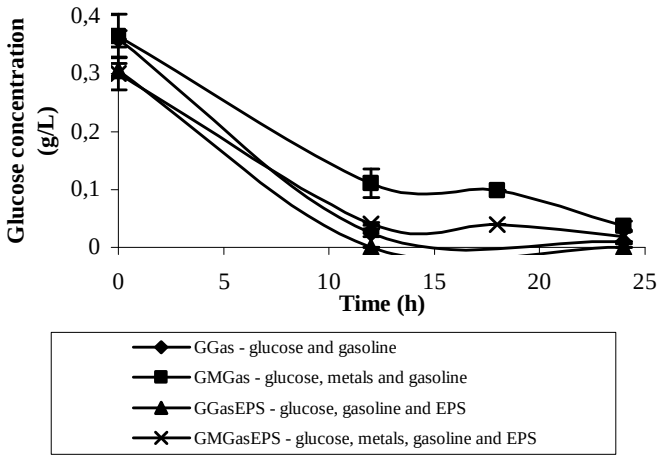


Fig. (4). Glucose consumption by "consortium 1" in media containing glucose and gasoline, with or without metals and EPS.

Results related to experiments involving gasoline as only carbon source, in media containing or not metals and/or EPS are presented in Fig. (**5**). The standard errors observed in protein concentration in these experiments are probably due to the small cellular growing of the consortium in media without the presence of glucose. In these experiments, cellular growing was smaller than in experiments containing glucose (Figs. **1** and **3**). It is possible to note that lag phase in all cases was longer, indicating the needing of adaptation of microorganisms to cultivation conditions.

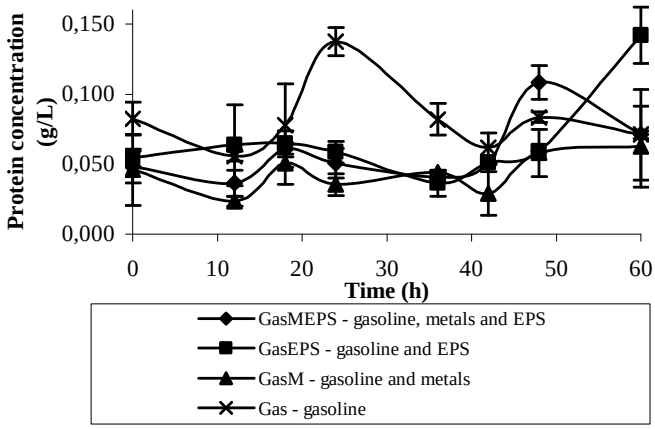


Fig. (5). Growing curves for "consortium 1" in media containing only gasoline as carbon source, with or without metals and EPS.

Again, EPS presence minimized inhibitory effect caused by metals to microbial metabolism, once the substance addition has improved cellular growing (GasMEPS curve), when compared to experiments involving only gasoline and metals (GasM curve).

The fluctuations that generated protein concentration peaks in all experiments could be due to microorganisms adaptation to utilization of several gasoline hydrocarbons as substrates (benzene, toluene, xylenes, polyaromatic hydrocarbons, etc.), or due to experimental errors, once the values of protein concentration were very low.

Experiments with "Consortium 2"

"Consortium 2" was used in experiments involving only gasoline, once it was propagated from a medium containing only gasoline as carbon source, as cited above.

According to these results, a period of time of 14 hours was chosen as response, and protein concentration values were used for model generation.

Fig. (**6**) presents the Outlier T graphic for cellular growing at 14 hours. It is possible to verify that every data are inserted in confidence interval.

Table **4** presents the analysis of variance (ANOVA) for the most adequate model for cellular growing at 14 hours, related to gasoline concentration (C_{Gas}), metals concentration (C_M) and EPS concentration (C_{EPS}).

Values of Prob > F smaller than 0.0500 indicate that model terms are significant, and values higher than 0.1000 indicate that terms are not significant. In this case, the term EPS concentration (A) and its combinations (EPS concentration and metals concentration – AB; and EPS concentration and gasoline concentration – AC) are significant. Also, it is possible to note that the effect of metals concentration combined to gasoline concentration is relatively significant, once Prob > F value is between 0.0500 and 0.1000. Value of Prob > F for lack of fit test indicates that it is not significant re-

Table 3. Cellular Growing of "Consortium 2" in Experiments Used for Experimental Design

Time (h)	Protein Concentration (g/L)								
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9*
0	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
6	0.018	0.019	0.011	0.017	0.014	0.000	0.018	0.018	0.020
14	0.041	0.020	0.023	0.019	0.037	0.041	0.046	0.037	0.034
24	0.030	0.029	0.020	0.016	0.027	0.015	0.027	0.035	0.032
30	0.022	0.035	0.010	0.035	0.009	0.000	0.053	0.055	0.024

* average of 5 experiments.

Table 4. Analysis of Variance – Cubic Reduced Model for Cellular Growing of "Consortium 2" in 14 Hours

Factor	Statistics					
	Degrees of Freedom	Mean Square	F Value	Prob > F	Estimated Coefficient	Standard Error
Model	7	2.552E-004	12.99	0.0130		
Intercept	1				0.051	1.79E-003
C_{EPS} (*A*)	1	9.461E-004	48.17	0.0023	0.011	1.567E-003
C_M (*B*)	1	5.513E-005	2.81	0.1692	-2.625E-003	1.567E-003
C_{Gas} (*C*)	1	4.050E-005	2.06	0.2243	-2.250E-003	1.567E-003
AB	1	1.620E-004	8.25	0.0454	4.500E-003	1.567E-003
AC	1	4.061E-004	20.68	0.0104	7.125E-003	1.567E-003
BC		1.361E-004	6.93	0.0580	4.125E-003	1.567E-003
ABC		4.050E-005	2.06	0.2243	-2.250E-003	1.567E-003
Residue	4	1.964E-005				
Lack of fit	1	4.537E-005	4.10	0.1360		
Pure error	3	1.106E-005				
					$R^2 = 0.9579$	

lated to the pure error, what is a favorable indication, because it is interesting the fitting of the model.

Estimated coefficients of metals concentration (B), gasoline concentration (C), and the combination of these effects with EPS concentration (ABC) are smaller than their errors, indicating that these factors are not statistically significant. So, the simplified codified mathematic model for cellular growing at 14 hours (C_c) – expressed in terms of protein concentration – is given by the by the Eq. 1:

$$C_c = 0.051 + 0.011A + 4.500.10^{-3}AB + 7.125.10^{-3}AC + 4.125.10^{-3}BC$$

The final equation (Eq.2) in terms of actual factors is:

$$C_c = 0.078 - 0.435C_{EPS} + 0.181C_{EPS}C_M + 1.167C_{EPS}C_{Gas} + 8.333.10^{-3}C_MC_{Gas}$$

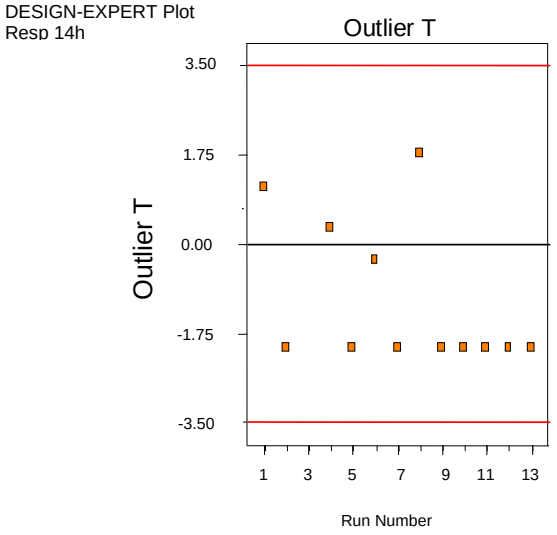
With the tools provided by the software, it is possible to determine the cellular growing variation (inside the interval of the experimental design) when the concentrations of metals and gasoline are maximum, with variations of EPS concentration between the maximum and the minimum used in the experimental design. These data are show in Table **5**.

It is possible to observe that at conditions of high metals and gasoline concentrations, an increase in the EPS concentration from 0.03 to 0.05 g/L improved the cellular growing in 39 %. It means that an increase of the substance concentration favors the cellular growing, probably due to a decrease in the possible toxic effect caused by heavy metals presence in the system.

DISCUSSION

The inhibitory effect caused by heavy metals has been reported by other authors. In their work, Giller *et al.* (1998) [10] cited that laboratory ecotoxicological studies suggest

that the addition of metals some days after substrate addition often results in a decrease of respiratory response for a wide range of substrates, such as glucose, cellulose, straw and plant residues. The amount of carbon mineralized over a short period such as 24 hours after the addition of glucose, for example, has been found to be extremely sensitive to addition of even small amounts of metal salts in the laboratory, which could be due to an increase in the lag time. This kind of inhibition has been observed in cultivations with other carbon sources. Amor *et al.* (2001) [1] evaluated the effect of different concentrations of cadmium, zinc and nickel on the biodegradation of toluene, ethylbenzene and o-xylene by a *Bacillus* strain. According to Maslin and Maier (2000) [11], the presence of cadmium in a medium containing phenantrene increased the lag period of the growing curve of microorganisms isolated from two different soil samples.



Fg. (6). Outlier T graphic for cellular growing of "consortium 2" after 14 hours of cultivation.

Table 5. Maximum Values of Cellular Growing of "Consortium 2" (C_c), in Terms of Protein Concentration, Obtained with Gasoline and Metals Maximum Concentration, with Variation of EPS Concentration

	C_{EPS} (g/L)	C_M (ppm)	C_{Gas} (%v/v)	C_c (g/L)
Lower EPS concentration provided by optimization	0.03	5.0	1.0	0.0508
Higher EPS concentration provided by optimization	0.05	5.0	1.0	0.0704

Microbial growing after a period of time of exposure to heavy metals was also investigated by Díaz-Raviña e Bååth (1996) [12]. The authors added zinc to agricultural soils in their experiments, and they observed an increase in metal tolerance after two days of exposure, and the variations of zinc concentrations did not affect this tolerance. This immediate effect was assigned to quick death of sensitive microbial species, and at long term, this effect is assigned to differences in the competitive and adaptation capacities of survival bacteria.

Results obtained by Sandrin *et al*. (2000) [2] were similar to those observed for "consortium 1" in (Figs. **1** and **2**). They noted an increase in *Burkholderia* sp. growing in relation to media containing only the hydrocarbon and the metal, when a rhamnolipid was added to the medium.

The behavior of "consortium 1" in medium containing glucose, metals and gasoline (GMGas curve, Fig. **3**), when compared to GM curve in Fig. (**1**), could be explained by the hypothesis presented by Sandrin *et al*. (2000) [2]. According to the authors, the presence of metals can give protection to bacteria, slowing the loss of cell viability in presence of hydrocarbons.

Results of experiments involving only gasoline as carbon source, presented in Fig. (**5**), show that cellular growing under those conditions was small, when compared with experiments containing glucose in medium. Test with gasoline and EPS (GasEPS curve) was carried out in order to verify if EPS presence somehow would facilitate gasoline consumption, when compared to test with gasoline only (Gas curve). Mulligan (2005) [13] relates that some EPS with surfactants properties are able to increase hydrocarbons biodegradation, by enhancing their availability to microorganisms. After 50 hours of cultivation, there is a clear tendency to cellular growing, that can be explained by a probable consumption of gasoline, favored by EPS presence, once this substance could have improved hydrocarbons solubility, facilitating microorganisms attack.

In general, the utilization of "consortium 1" in experiments explicited the importance of using EPS to decrease toxic effects caused by heavy metals in the system. The polymeric substance probably reduces availability of these species to microorganisms, due to possible phenomena of adsorption or complexation [2].

After analysis of the results obtained by the experimental design and indicated in experiments conducted with "consortium 2", it is possible to verify that an optimization of EPS concentration values, according to the contaminated environment, can lead to an intensification of the bioremediation process. This hypothesis is based on the amplification of cellular growing, indirectly indicating a possible improvement of contaminant fractions consumption.

Our results indicate that the application of an extracellular polymeric substance in a co-contaminated system may improve its remediation, probably because the EPS decreases the amount of heavy metal available for microorganisms, leading to an increase in substrate consumption and consequently in cellular growing, which was possibly inhibited when there are available metals in solution, as show in Figs. (**1**-**5**).

There are limited number of researches in literature describing effect of the presence of heavy metals in hydrocarbons contaminated environments, and such effect has been evaluated according to microbial cellular growing, determined by absorbance measures, hydrocarbons quantification, or protein concentration, as we have done in this work [1, 2]. With the use of this simple technique, it is not possible to

make any consideration about the microbial metabolic activity. In order to confirm the related facts and the results obtained in the researches related to this subject it is necessary to use a biochemical approach to evaluate the real metabolic activity of the biomass in this system. Nevertheless, such approach should be conducted in artificial systems in order to simplify the models and the interferences present in a real system for a better understanding of the phenomena, and application of the knowledge obtained in the in situ experiments.

As observed in Figs. (**1**, **3** and **5**), some growing curves related to experiments involving the presence of heavy metals and/or EPS show a tendency to a growing increasing, what lead us to believe that it is possible for the system be naturally bioremediated, if the process is conducted for a larger period of time, without the needing of intervention because of the low tested level of contamination. Under such conditions, the successful application of smalls concentrations of EPS (0.01 to 0.05 g/L) is an interesting result, once the endogenous microorganisms present in a real contaminated environment could be able to produce these substances in situ, as reported by Seo and Bishop (2008) [14], and Van der Aa and Dufrêne (2002) [15]**.** It indicates that a system with low concentrations of these organic and inorganic pollutants could possibly be susceptible to natural attenuation.

CONCLUSIONS

This work aimed to evaluate the possible damage caused by the microbiota presented in a co-contaminated environment. However, it was not observed where the presence of EPS in a co-contaminated system minimizes the inhibitory effect caused by heavy metals on microbial cellular growing of the two studied consortia. Our results suggest that systems under low concentrations of gasoline and heavy metals may be susceptible to natural attenuation, possibly without a great environmental impact, when the process is conducted in a larger period of time.

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