Bioremediation of soils contaminated by PAHs: Mutagenicity as a tool to validate environmental quality

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HIGHLIGHTS

- Mutagenicity tests of soils submitted to bioremediation qualifies risk evaluations.
- The efficiency of bioremediation depends on the partial biodegradation and byproducts formed.
- Mutagenicity as an alternative among the classical evaluations of soil quality.
- Study with real samples of soils contaminated by the presence of mixtures of PAHs.
- Evaluation of bioremediation must take the mutagenicity of nitrocompounds into account.

ABSTRACT

Bioremediation can be used as one of the decontamination techniques for areas contaminated by polycyclic aromatic hydrocarbons (PAHs). However the effective biodegradation of these compounds must take into account the possible toxic and mutagenic effects that might persist. In this study the mutagenic potential of soil samples from an area contaminated by wood preservatives was evaluated. The area had already been submitted to a simulated bioremediation process in a microcosm, using two different inoculums (1 and 2), and comparing them to the decay of PAHs. Organic extracts were prepared before and after bioremediation, where the 16 PAHs considered a priority by USEPA were analyzed and tested using the Salmonella/microsome assay. The extracts were analyzed in strains TA98, TA97a and TA100 (+S9mix/-S9mix), YG1041 and YG1042. Considering Inoculum 1 only as bioaugmented and Inoculum 2 also stimulated and enriched, the concentrations of PAHs and mutagenic effect were different. The former identified a greater reduction of mutagenesis and a smaller decrease of PAHs while the latter showed greater mutagenic power even associated with the greatest reduction of PAHs. The possible generation of degradation byproducts with high mutagenic power after a partial biodegradation process can be considered. In strains YG1041 and YG1042 the mutagenesis values before bioremediation were 747 and 567 rev/g soil, respectively. Although the efficiency of bioremediation was observed, the associated damage indicates that the analysis of contaminants and their relationship with mutagenic effects are a fundamental stage for the effective evaluation of the risks and efficiency of bioremediation processes.

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1. Introduction

The increase in the number of contaminated areas worldwide has clear environmental implications. Among the main classes of organic pollutant that are a matter of concern around the world are the PAHs — polycyclic aromatic hydrocarbons. These substances are...
present in a large number of contaminated areas and there is an urgent need to intervene in order to remediate them. The risk is due to the toxic, mutagenic and carcinogenic characteristics of some representatives of these substances, such as the 16 PAHs listed as priority pollutants by USEPA (Keith and Telliard, 1979; Simarro et al., 2011). Some existing techniques are able to degrade them in the environment. They include bioremediation (Baquero-Posada and Ortega-Calvo, 2011; Silva et al., 2009; Anderson et al., 2009), which can promote biodegradation until the complete mineralization of these compounds to CO₂ and water (Cerniglia, 1992; Moscoso et al., 2015). However, during this process byproducts can be formed as a result of the initial oxidation of the aromatic rings that exist in the PAHs. Thus commonly monitored compounds are not the only products present during a bioremediation process, and effects of other derivatives that have not been investigated may appear (Anderson et al., 2009; Hu et al., 2012). A few of the metabolites formed in the degradation may be as toxic as PAHs, or even more, such as nitro and oxy-PAHs (Lemieux et al., 2009; Lundstedt et al., 2007). An effective and more practical strategy indicates the need to follow the bioremediation processes with the evaluation of possible biological effects (Moscoso et al., 2012; Loibner et al., 2004) instead of expensive monitoring only via chemical methods. Therefore it is obvious that the evaluation of persistent risk after remediation processes—traditionally performed only through the chemical analysis of the 16 PAHs as target compounds—could be insufficient, since their reduction does not necessarily ensure the reduction of toxicity and/or mutagenicity in the sample considered (Anderson et al., 2009).

The efficiency of bioremediation processes must take into account the total effects of the contaminants, such as their interactions, synergy, or antagonism (Loibner et al., 2004). Among the bioassays that allow this integral evaluation is the Salmonella/ microsome assay — Ames Test. The Salmonella/microsome test is widely used for environmental diagnosis (Coronas et al., 2013; Da Costa et al., 2012; Da Silva Jr. and Vargas, 2009; Meyer et al., 2015; Vargas et al., 1993, 2008; White and Claxton, 2004). However, the bioremediation processes do not yet fully incorporate the need to evaluate the soils by genotoxicity and mutagenicity tests among the work strategies. Besides the possibility of using them to diagnose contaminated areas, these assays can be used to follow the efficiency and biodegradation of pollutants through bioremediation. It should be underscored that when the mutagenicity of contaminated soils submitted to bioremediation processes is evaluated, the risk evaluations that are commonly based on chemical tests are expanded and the processes to evaluate the efficiency of bioremediation are qualified (Anderson et al., 2009; Lundstedt et al., 2007; Kuppusamy et al., 2017).

Nevertheless, not all studies show a reduction in the toxic and/or mutagenic effects. Alexander et al. (2002) found increased genotoxicity after evaluating soil submitted to biological treatment, although there were significant changes in the concentration of PAHs present. In studies evaluating contaminated soil after bioremediation experiments on a laboratory scale, rates of reduction of up to 74% for PAHs were found, and the different bioremediation strategies were equivalent. However there was an increase of mutagenic power in some strains tested (Brooks et al., 1998; Hughes et al., 1998). In a study by Hu et al. (2012) when cell strains were tested to evaluate the variation of toxicity and genotoxicity after bioremediation, increased damage was observed, although the concentration of the 14 PAHs analyzed was reduced. It is highlighted that the results found for mutagenicity will reflect the biological effects when the contaminants are completely degraded, i.e., mineralized, or only partially transformed (Cerniglia, 1992; Lundstedt et al., 2003), enabling the evaluation of the generation of byproducts formed from the incomplete metabolism of the PAHs and their associated risks. Moreover, the majority of studies on toxicity and biodegradation of PAHs are conducted with a single compound, or a few representatives of the class aiming to minimize the variables. However, PAHs are present in the contaminated areas as complex mixtures (Cerniglia, 1992; Hwang and Cutright, 2002). It is therefore necessary to perform studies with real samples from contaminated soils where the mixture of these contaminants is present, in order to verify the effective potential of biodegradation and associated risks.

Therefore, the objective of this study was to look at the potential for mutagenic risks and PAHs concentration in soils from a contaminated area after they were submitted to bioremediation experiments previously performed in microcosms.

2. Material and methods

2.1. Contaminated soil

The soil samples used were collected at a site contaminated by wood preservatives at Triunfo, in the State of Rio Grande do Sul, Brazil and obtained after finalizing bioremediation simulation experiments after a 60-day period as described in Pohren et al. (2016). In this process, quantities of soils were submitted to biodegradation assays in respirometers and monitored for microbial respiration by the production of CO₂. In these experiments, two inoculums prepared from bacteria and fungi from that area were tested. These soils were originally from an old deactivated wood preservatives plant that used in this several chemicals such as pentachlorophenol, creosote and copper chrome arsenate, and the contaminated soil was submitted to the experiments and research in this study prioritizing contamination by PAHs (Pohren et al., 2012; Fepam, 2010).

The contaminated soil used to perform this study was previously submitted to bioremediation experiments described by Pohren et al. (2016). In the present stage of research it was evaluated for mutagenicity and the chemical analysis of total PAHs, and initially it presented the following characteristics: 48.3% sand, 17.6% silt, 12.85% clay, pH 6.3, organic matter content 3.4%, PCP 1.1 mg/kg concentration of the contaminant pentachlorophenol (PCP), 26 mg/kg of As, 40 mg/kg of Cu, 43 mg/kg of Cr and concentration of Cr₆⁺ less than 1 mg/kg. Despite the presence of metals in the soil samples, the evaluations regarding the influence of the PAHs as chemical markers were prioritized, given the potential risks. Thus, the tests to evaluate bioremediation were performed only with organic extracts.

After the end of the biodegradation period, the soils submitted to the experiments were pooled, homogenized and stored at 4 °C protected from light until the organic extractions, performed in order to look at the efficacy of bioremediation. The soil samples evaluated for mutagenicity and risk of PAHs were identified according to the bioremediation experiments performed previously (Pohren et al., 2016) where: 1SC contaminated soil - Control; 2SC (soil + Inoculum 1); 3SC (soil + PAHs pool); 4SC (soil + Inoculum 1 + PAHs pool); 6SC (soil + Liquid Fertilizer); 8SC (soil + Inoculum 1 + Liquid Fertilizer); 9SC (soil + Inoculum 2) and 10SC (soil + Inoculum 2 + PAHs pool).

In these previously performed bioremediation experiments it is highlighted that liquid fertilizer from the agricultural degradation of grapevine residues was added to treatments 6SC and 8SC. In addition, to treatments 3SC, 4SC and 10SC, was added a pool of PAHs, phenanthrene, fluoranthene, pyrene, 1-nitropyrene and 2-nitrofluorene (all of them with a purity of >98%, Sigma–Aldrich) and a final concentration in the soil of 4 mg/kg of each PAH. The Inoculums added to the soil indicating the use of the microorganisms inherent to the bioremediation experiments are characterized...
as representing: i) Inoculum 1: bioaugmentation effect; ii) Inoculum 2: bioaugmentation effect + biostimulation and enrichment. Bioaugmentation was performed with autochthonous microorganisms, biostimulation by adding inorganic nutrients and enrichment with the PAHs pool as described in Pohren et al. (2016).

2.2. Organic extraction of soils

Organic extracts of the soils collected at the industrial site and of the soils submitted to bioremediation in microcosms were prepared after the biodegradation experiments. These extracts were obtained according to the USEPA method 3550C (2007); the soil for testing was homogenized with an inox spatula; the solvent dichloromethane – DCM was added in 15 g of this sample and it was taken for extraction in ultrasound. The liquid was filtered in a chromatographic column concentrated at 40 °C and stored in a freezer until the biological assays were performed to evaluate the mutagenic potential and quantifications of PAHs.

2.3. Chemical analyses

The PAHs were quantified in the samples of microcosm soils after the period of biodegradation and compared to the results of the contaminated soils from the area before they were submitted to the biodegradation experiments. The organic extracts were analyzed for this using the GC/MS (Perkin Elmer model Clarus 600 Quadruple system, SIR mode), and the 16 priority PAHs were quantified by USEPA (Iarc, 2011), according to the USEPA method SW846/8270-D (1996). The concentration of the pentachlorophenol (PCP) contaminant was also evaluated before and after some of the biodegradation experiments. This contaminant was analyzed by GC/MS, after organic extraction, according to USEPA SW846-8270D. 3550C. Also in the initial characterization the sample of contaminated soil was analyzed for the content of metals of interest – As, Cu and Cr – through ICP-OES (PERKIN ELMER/ OPTIMA 7300 DV).

2.4. Evaluation of mutagenicity using a Salmonella/microsome assay

Mutagenicity was evaluated in the organic extracts of the contaminated soil – SC, before being submitted to respirometric assays, and in the samples after the microcosms for biodegradation were finished, identified as: SC, 1SC, 2SC, 3SC, 4SC, 6SC, 8SC, 9SC and 10SC.

In order to evaluate mutagenicity, the Salmonella/microsome test was used with the microsuspension method, Kado test (Kado et al., 1983; Umbuzeiro and Vargas, 2003), which is a modification of the Ames test (Maron and Ames, 1983). The Salmonella typhimurium, TA98 and TA97a strains were used; the TA98 strain is sensitive to PAHs and to most of the environmental mutagens (Zieger et al., 1985); TA97a being described as the most sensitive to heavy metals (Pagano and Zeiger, 1992) and PAHs (Maron and Ames, 1983), and TA100. Strains were also tested with a high production of nitroreductase and O-acetyltransferase enzymes (Hagiwara et al., 1993), which are specific for the diagnosis of nitrocompounds: YG1041 and YG1042.

The mutagenicity of the extracts was evaluated at six concentrations: 10, 20, 40, 80, 120 and 160 mg soil equivalent, as defined in previous studies (Pohren et al., 2012). All the tests were performed in the presence and absence of a P450 metabolism system in vitro, S9mix (MOLTOX SA) induced by AROCLOR 1234, except for the YGs strains performed without a metabolism system. The assays were performed in duplicate and with the inclusion of negative controls (nutrient medium – 100 µl/plate, and the solvent used in the assay: 5 µl DMSO/plate) and positive according to the strain and treatment used (detailed in Pohren et al., 2012). Before the mutagenicity test the cell survival assay was performed, where samples that presented a percentage survival of less than 60% when compared to the negative control were considered cytotoxic (Vargas et al., 1993; Umbuzeiro and Vargas, 2003). Only the concentrations considered non-toxic to the samples were looked at in the analysis of the results.

The results were analyzed looking at the mutagenic activity expressed by the number of revertants per dry grass soil equivalent (rev/g soil equivalent) calculated through the linear portion of the dose-response curve by the SALANAL statistical program (Salmonella Assay Analysis, version 1.0 - RTP, North Carolina, USA) selecting the linear model or Bernstein (Umbuzeiro and Vargas, 2003; Bernstein et al., 1982). The sample was considered mutagenic when statistical significance was observed in the regression analysis (p ≤ 0.05) and in ANOVA (p ≤ 0.05). In order to verify the relationship between the mutagenic responses and the PAHs concentration, Pearson correlations were performed in the statistical program “Statistical Package For The Social Sciences” (SPSS/PASWSTAT), version 18. Principal Components Analysis (PCA) was also performed using PC-Ord software to look at how the strains tested are correlated to the PAHs.

3. Results and discussion

3.1. PAHs: alterations in PAHs concentrations before and after biodegradation

When we consider the concentration of PAHs in the soil samples after the period of the biodegradation experiment (Fig. 1-a), it is observed that these diminished in all treatments. The values obtained after the bioremediation experiments for 8SC and 9SC were highlighted, and the control soil showed an excellent reduction compared to the carcinogenic PAHs even without having received inoculums – once again evidencing the possibility of natural attenuation, which is valid according to some studies (Marin-Morales et al., 2016).

In 8SC the addition of liquid fertilizer promoted the degradation of contaminants together with the action of Inoculum 1. The presence of nutrients from the agricultural degradation of grapevine residue may have contributed to the presence of humic acids – detected at a concentration of 6.4% - making it easier for the Inoculum to act on the PAHs present. Therefore, it is noted that nutrient addition may help the performance of a bioaugmented inoculum - Inoculum 1. As presented by Hwang and Cutright (2002), in a study with bioaugmentation and biostimulation in contaminated soil, better results were found in the reduction of phenanthrene and pyrene with the bioaugmentation technique, and when these were evaluated together instead of isolated in solution. Another effect to be considered is the production of biosurfactants by bacteria used in the bioaugmented consortium. One of the bacteria present in the inoculums used (Pohren et al., 2016), Pseudomonas aeruginosa is recognized as a producer of a lipopeptide structure that acts as a biosurfactant (Bezza and Chirwa, 2016), favoring the bioremediation process.

Among the 16 priority PAHs listed by USEPA, only eight are considered carcinogenic (Fig. 1-b). They are: benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene and indeno(1,2,3-cd)pyrene, which are classified as 2B. Due to the risk profile of these PAHs, the biological responses found must be associated with the concentrations of carcinogenic PAHs that persisted after the bioremediation experiments.
However in a study by White (2002) about the genotoxicity of complex mixtures of PAHs, it is underscored that some substances could modulate the effects of known mutagens, as in the case of the action of anthracene and naphthalene that might interfere in the effect of compounds such as benzo(a)pyrene. Thus it should be emphasized that the mutagenicity responses will depend on the entire load of pollutants present in the samples of contaminated soils, and the potential antagonistic, synergistic and/or additive effects resulting from the complex mixture.

When we consider the action of the inoculums, Inoculum 2 proved more effective to reduce the contaminants, including the carcinogens. However, greater mutagenicity was found in the same treatment, maintaining the mutagenic responses in this soil even after bioremediation — including a considerable increase in one of the strains. According to Cerniglia (1992),acenaphthene, pyrene, benzo(a)anthracene and benzo(a)pyrene are genotoxic in the Ames Test, and the latter are also carcinogenic. In another study, mutagenic potencies were found in TA98 + S9 caused by phenanthrene, fluoranthene, pyrene and benzo(g,h,i)perylene (Maertens et al., 2008). Thus, the mutagenic responses found may be related to these other compounds (Fig. 2 - a).

For the individual compounds, a relationship was observed between total mutagenicity and the following compounds: benzo(a)anthracene \( r = 0.677 \), benzo(k)fluoranthene \( r = 0.708 \), benzo(a)pyrene \( r = 0.758 \), naphthalene \( r = 0.707 \), chrysene \( r = 0.703 \), dibenzo(a,h)anthracene \( r = 0.716 \), all of them with \( p < 0.01 \) (Fig. 2 - b). It is thus clear that the mutagenicity observed would be correlated with the concentration of carcinogenic compounds.

According to the results obtained in PCA, the percentage of variation explained in the two first axes was 95.9%, with the significance of the axes as \( p = 0.025 \). This analysis enabled visualizing how the strains tested are correlated with some of the PAHs (Fig. 2 - b). The strains TA97a-S9, TA100 + S9, YG1041 and YG1042 showed an inverse relationship to the PAHs responses, thus indicating that the effects observed in these strains could be caused by other

![Fig. 1. Concentration of: a) PAHs detected before and after performing biodegradation experiments; b) carcinogenic PAHs before and after the biodegradation experiments.](image-url)
different PAHs compounds. However, the compounds benzo(a)anthracene, benzo(k)fluoranthene, indeno(1,2,3,cd)pyrene, acenaphthylene, phenanthrene, and fluoranthene are associated with axis 2, to strains TA100 + S9 e YG1042.

CONAMA Resolution 420 (2009) (Table 1), referring to the compounds, benzo(a)anthracene, dibenzo(a,h)anthracene, benzo(a)pyrene, dibenzo(a,h)anthracene and indeno(1,2,3,cd)pyrene, the final concentrations after the bioremediation experiments, which initially presented values greater than the limit of prevention (VP) – even though the concentrations have diminished a lot, continue to surpass the guidance values for warning. This condition signals that, as a result of the persistent concentrations of these compounds in the soil, the latter would not be able to sustain its main functions. This limitation should be understood within the concept of soil multifunctionality, where according to the Dutch Government’s Law of Soil Protection the functions of soil should be considered from the ecological standpoint, of water supply, food, agriculture and others.

On the other hand the compounds benzo(k)fluoranthene, benzo(g,h,i)perylene and naphthalene that had a higher concentration than the prevention value, were reduced to levels lower than VP in an industrial area (VI), and after the degradation experiments there was a considerable reduction, although all the treatments still show values higher than the prevention limit. The most worrisome compound, because it has proven carcinogenic action (Group 1), benzo(a)pyrene, which had a concentration close to the VI range, presented a reduction in all microcosms but remained at higher levels than those of the prevention value for all treatments, except 2SC, with Inoculum 1, reducing the concentration below VP. The biotransformation of this compound is described by bacteria when co-metabolism occurs: the bacteria growing in the presence of benzo(a)pyrene may degrade the benzo(a)pyrene by co-metabolism (Kanaly and

![Image 101x592 to 324x705]

**Table 1** Concentrations of the 16 priority PAHs in biodegradation treatments and regulations applicable to soils.

<table>
<thead>
<tr>
<th></th>
<th>SL Before bioremediation</th>
<th>1SC</th>
<th>2SC</th>
<th>3SC</th>
<th>4SC</th>
<th>6SC</th>
<th>8SC</th>
<th>9SC</th>
<th>10SC</th>
<th>Prevention Value</th>
<th>Industrial Investigation Value</th>
<th>Screening Levels Industrial</th>
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<td>NA</td>
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<td>0.05</td>
<td>0.08</td>
<td>0.05</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
<td>0.07</td>
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<td>NA</td>
<td>NA</td>
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<td>0.00</td>
<td>0.02</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
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<td>0.039</td>
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<td>0.54</td>
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<td>0.37</td>
<td>0.11</td>
<td>0.11</td>
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<td>0.03</td>
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<td>0.20</td>
<td>0.14</td>
<td>0.22</td>
<td>0.10</td>
<td>0.22</td>
<td>0.12</td>
<td>0.052</td>
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<td>0.79</td>
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<td>0.96</td>
<td>0.11</td>
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Concentrations in mg/Kg.


Regional Screening Levels for Chemical Contaminants at Superfund Sites – USEPA.
soil before being submitted to biodegradation. Thus it is evidenced practically the same pattern of strains with a greater effect on the other strains the damage observed diminished. However, there is experiment (TA98-a three strains presented practically the same responses after the experiment control). The mutagenicity pattern observed was diversified in the organic extract, as observed in Fig. 3. Before the contaminated soil was submitted to the degradation experiments it showed a positive response to all the strains tested, with 42% answers in the presence of 59 and 58% in the absence of the metabolism system, and a total effect of damages equal to 4223 rev/g soil equivalents. This higher percentage of responses without a metabolism system may be associated with the implications that can be presented by compounds such as 1,6 and 1,8-pirenoquinones, as one of its transformation products the powerful carcinogen 7,12-dimethylbenzo(a)anthracene which is considered weakly carcinogenic presents percentage of responses without a metabolism system may be associated with the implications that can be presented by compounds such as 1,6 and 1,8-pirenoquinones, as one of its transformation products the powerful carcinogen 7,12-dimethylbenzo(a)anthracene (Chibwe et al., 2015). Thus, it is in the different phases of breaking down the aromatic ring that intermediaries may arise with associated mutagenicity. An effective bioremediation process will depend directly on the ability of the microorganisms to degrade target compounds until mineralization or to such low levels with the generation of products that are safe from the perspective of risk.

3.2. Mutagenic activity and PAHs

The mutagenicity pattern observed in the organic extract, as observed in Fig. 3. Before the contaminated soil was submitted to the degradation experiments it showed a positive response to all the strains tested, with 42% answers in the presence of 59 and 58% in the absence of the metabolism system, and a total effect of damages equal to 4223 rev/g soil equivalents. This higher percentage of responses without a metabolism system may be associated with compounds such as 1,6 and 1,8-pirenoquinones, indicated by Sakai et al. (1985) as highly mutagenic both in the presence and in the absence of a metabolism system against strain TA97a – which detects substances that cause a frameshift mutation – highlighting the implication that can be presented by compounds as PAHs derivatives.

In treatment 1 – experiment control– it was observed that three strains presented practically the same responses after the experiment (TA98-S9mix and + S9mix and TA100 + S9mix); for the other strains the damage observed diminished. However, there is practically the same pattern of strains with a greater effect on the soil before being submitted to biodegradation. Thus it is evidenced that in the soil in that area a natural attenuation may be occurring, indicating that the microbial community is adapted to contaminant degradation, although this is occurring at a lower rate than the one needed to inhibit the manifestation of biological effects and possible existing risks.

After the bioremediation tests in microcosms (Pohren et al., 2016), the organic extracts tested that showed a sum total of lower mutagenic effects were treatments 2SC, 4SC and 10SC ranging from 254–1184 rev/g soil equivalents.

A correlation was found between the sum total of biological effects – mutagenic power and total PAHs concentration in the soil samples (r = 0.733, p < 0.05). This mutagenic power indicates the total mutagenicity calculated by the sum total of the number of rev/g dry soil equivalent observed per treatment, for each strain and metabolization condition. For effects observed in TA98-S9 and TA100-S9, if the responses obtained for the nitrosensitive strains (YG1041 and YG1042, respectively) were higher, these values were those that represented the effect of the class of mutagens.

Thus, after performing the mutagenicity assays in the strains sensitive to differentiated effects, it was recorded that, although positive results remain after bioremediation there is an indication that the general profile of contamination has diminished. The addition of an inoculum proved essential, because one of the inoculums (2SC), contributed to the elimination of mutagenicity, while in control treatment, biodegradation did not fully reduce the biological effects.

In the case of the 4SC and 10SC soils, it is noted that the input from the PAHs pool to the soil did not inhibit the action of the inoculums added, on the contrary, the enrichment stimulated the microorganisms, favoring the reduction of mutagenic activity compared to the other treatments tested (Fig. 3).

It is emphasized that the greatest reduction of the mutagenic effects occurred in the soil bioaugmented with Inoculum 1 (2SC), since after biodegradation effects were only observed on strain TA98-S9mix, indicating mutagens that caused frameshift errors in the DNA. In the other strains tested, there was an absence of damage, thus inferring that Inoculum 1 effectively diminished the pattern of mutagenicity in soil. It could be said that the effects observed are not associated with the PAHs, since the action caused by this class of compounds would require the expression of a

Fig. 3. Sum total of the mutagenicity of different strains in the organic extracts of soils before and after biodegradation experiments.
metabolization system. Therefore, a few byproducts of PAHs biotransformation, direct mutagens or other contaminants that exist in the contaminated area could be acting on this soil. Among them, we might consider the effect of the metals, dioxins and furanes, and/or the synergistic action of PCP (pentachlorophenol) — since this is not considered mutagenic (Barbee et al., 1996). This organic contaminant was also used as a wood preservative in the study area. Currently the use of PCP is forbidden in many countries, including Brazil, since it is considered possibly carcinogenic to humans by IARC (Group 2B). Nevertheless, given the history of its use, it is still detected in environmental samples.

In this study, the contaminant PCP presented a concentration equal to 1.1 mg/kg before the bioremediation tests, a value considered above the limit foreseen by legislation for prevention (0.16 mg/kg) — CONAMA Brazilian Resolution N° 420 (2009). However, it is emphasized that the action of the inoculums was observed on the contaminant PCP compared to the initial concentration before the biodegradation experiment evidenced by the response to treatments 8SC (0.7 mg/kg) and 9SC (0.82 mg/kg). This degradation of PCP would be associated with the increase in nutrients indicated by reductions of their concentration in these treatments, which received, respectively, the input of the fertilizer nutrients added and of the biostimulation and enrichment of Inoculum 2. It should be pointed out that the values detected in the treatments are below the guiding value of 3 mg/kg of soil, considered for investigation in an industrial area, according to the above mentioned Resolution.

When the treatments tested with the inoculums compared to SC before bioremediation are analyzed singly (Fig. 4), the differences in the action of the consortiums prepared are clearly noted (Pohren et al., 2016) compared to the strains tested. Thus there is a visible reduction of the mutagenic activity in the treatment with bioaugmented Inoculum 1 (2SC). Similarly, in a study by Wu et al. (2008) evaluating genotoxicity with Salmonella typhimurium in a microplate test, a lower genotoxicity factor was found for a soil sample in a microcosm bioaugmented with fungi, and also a greater reduction in the concentration of PAHs analyzed than for bioaugmented soil.

Considering treatment 9, after the action of microorganisms of Inoculum 2 (bioaugmented and enriched), a decrease in mutations was observed for all strains tested except in TA97 + S9mix and TA98-S9mix, showing values between 146–189 rev/g soil equivalent. Under these conditions, a change can be recorded in the type of biological responses identified by the strains compared to the soil before bioremediation. Thus, it emphasized that especially the effects augmented in TA97a may be associated with damage caused by the action of PAHs derivatives, as observed by Sakai et al. (1985), and strain TA97 in the presence of S9, may be more sensitive than TA98 or TA100 to the action of compounds such as 1-methylphenanthrene, fluoranthene, pyrene, benzo(a)pyrene, benzo(e) pyrene and perylene. Some studies demonstrate that compounds such as pyrene — although non carcinogenic — can present mutagenicity during their metabolic route, since pyrenoquinoines are formed with a toxic character. Besides, according to Levin et al. (1982) after metabolic activation, the PAH benzo(a)pyrene may induce damage, both of the frameshift type and base pair substitution, strain TA97a being the most sensitive to the action of aromatic hydrocarbons. This information agrees with the increase observed for the strain in treatment 9SC, where the concentration for a derivative of PAHs may have increased during the experiment. Thus, although there was an addition of the bioaugmented and biostimulated inoculum, this soil maintained high mutagenic power, indicating that there was no integral biodegradation.

This differentiated pattern was also found by Hughes et al., (1998) (Hughes et al., 1998) when evaluating creosote-contaminated soil before and after the bioremediation process, where an increase in genotoxicity was observed although its origin was not determined. It is highlighted that biological responses depend on aspects such as the effects of the soil matrix, the contaminant sorption/desorption behaviors, bioavailability and possible chemical interactions (Chibwe et al., 2015), and may involve the manifestation of variable effects that are difficult to measure.

It is considered that the dissolved form of the contaminants becomes bioavailable and the bonded compounds do not have direct biological effects (Megharaj et al., 2011). If the extract of treatment 9SC with Inoculum 2 has presented a high mutagenic potential, it can be inferred that perhaps the microbial action did not manage to access all the pollutants and did not cause integral biodegradation, or due to the increased bioavailability of the initially existing whose toxicity was not expressed before the experiment (Barbee et al., 1996; Lundstedt et al., 2007; Sayles et al., 1999). Moreover, intermediate products with associated toxicity may have been generated, which are responsible for increasing

![Fig. 4. Mutagenic power in soil before bioremediation, and after this in control SC and soil with inoculums against the strains tested.](image-url)
According to the responses found in some of the strains although there were significant reductions in the treatments performed as a bioremediation process, as for instance for 8SC 95% biodegradation of the total PAHs, and for 9SC with 94% (Pohren et al., 2016), when the soil samples were submitted to the biological tests they presented considerable mutagenic effects. Due to this increase of the mutagenic potential after the biodegradation process — as in 9SC, it is highlighted that the degradation process may be contributing to the rise and/or maintenance of contaminants that present a risk.

Another possibility would be that the stage evaluated by the tests performed in this study took into account intermediate phases of the degradation process of the contaminants present, and the responses observed may be part of one of the routes where the breakdown processes of compounds with the generation of byproducts have not yet ended. Thus there would be the presence of mutagenicity representative of the still persisting risk. In a study by Chibwe et al. (2015), with a raw soil extract obtained post-remediation, a reduction rate of 45% was found for the 16 PAHs, however a significant increase in toxicity evaluated with a cell strain, and compounds formed in the transformation of PAHs such as hydroxylated, carboxylated and quinone derivatives are considered responsible for this increase.

Effects were observed specifically in the strains sensitive for the diagnosis of nitrocompounds: YG1041 (derived from TA98) and YG1042 (derived from TA100). Damage was found in both strains in soil before bioremediation with values of 747 and 567rev/g soil equivalent, respectively (Fig. 5).

Treatment 1SC, considered soil that is naturally attenuated, since it did not receive any input to carry out the experiment, maintained significant effects in both strains, indicating the permanence and broadening the visualization of damage caused by nitroarenes. Under the action of the Inoculums, it is found that in the microcosm with Inoculum 1 there was a complete decrease of the effects of strain YG1042; on the other hand, with Inoculum 2, the effects were maintained only in YG1042. This inverse pattern could be understood from the strains that originated it: treatment 2SC with Inoculum 1 presented damage only in strain TA98, which agrees with their manifestation of effects only in YG1041 also, indicating exposure to compounds that cause the same type of genetic damage, frameshift error of DNA. This highlights the mutagenic effect of the direct action of some nitrated polycyclic hydrocarbons in some bacterial strains of Salmonella typhimurium (Möller, 1994; Rosenkranz and Mermelstein, 1983).

As to 9SC, damage was observed both in TA98 and in TA100, with effects in TA98 predominating — which would result in a greater mutagenic potential against YG 1041. The different behavior observed indicates that the responses obtained previously against strains TA98 are being caused by different compounds from the nitro-PAHs class. However, with the result in YG1042 superior to TA100, the presence of these nitrocompounds acting on the soil sample is confirmed.

The values of treatment 6SC can also be observed. Their derivative strains express specific damages indicating the action of the nitroderivatives according to the values found when the mutagenic powers are evaluated against strains TA98 and TA100. Since the pattern of responses is similar to those of soil before bioremediation, it is indicated that the mutagenicity detected would not be a result of the fertilizer added.

As to 8SC, a total reduction of the derivative strains was found. It could be considered that in this soil, the remainders responsible for mutagenicity are not the nitro-PAHs, maintaining the idea of the action of the PAHs themselves, or other contaminants of the area in a synergistic effect such as PCP or the potentially toxic metals.

The indicative of the presence of this class of substances by observation of the effects on sensitive strains - YG1041 and 1042 — indicates persistent risks in the soil samples, even after they presented an excellent reduction in the concentration of PAHs (Fig. 1-a). Nitrated PAHs are a great risk among the environmental contaminants, since, besides mutagenic effects — observed here — they present carcinogenic properties (Rosenkranz and Mermelstein, 1983; Sayara et al., 2011). In this way there is a clear need to evaluate post-remediation efficiency considering also toxicity and mutagenicity in the minimization of risk from contaminated soils.

Fig. 5. Mutagenic potency in the strains TA98 and TA100 their derivatives.
4. Conclusions

The results of the study show that evaluation of the bioremediation processes using genotoxicity tests increases the information regarding process efficiency, since differentiated effects are considered through this type of biological test. This integrated evaluation, enables visualizing the damage due to the persistence of some contaminants throughout the stages of decontamination and/or formation of byproducts associated with greater toxicity, even after the biodegradation period.

It is underscored that the choice of the Salmonella/microsome assay as a tool for this integrated evaluation was based on studies showing the feasibility and applicability of different strains as biomarkers for PAHs and their nitroderivatives, enabling the definition of a dose-response curve and quantifying the effects, indicating that the use of this type of test amplifies and qualifies the risk assessment for soils of contaminated areas, according to recent propositions by other authors (Kuppusamy et al., 2017).

It was found that the inoculation of cultures from contaminated soils may have been responsible for the high rates of PAHs reduction and contributed (in the case of Inoculum 1) to the decay of its mutagenic potential. However, for the inoculum (SSC), there was a considerable increase and maintenance of mutagenic responses in this soil even after bioremediation. Thus, even if a high rate of degradation occurs for the PAHs in this soil, the mutagenicity detected indicates that other chemical and/or intermediate species of PAHs with biological effects may have been generated.

The responses found in some of the strains showed that there might be an increased mutagenic potential of the soil samples analyzed after the partial biodegradation process. It is thus underscored that the degradation process may contribute to the rise and/or maintenance of contaminants with an associated risk — whose conditions for attenuation are still unknown. This indicates that the analysis of contaminants and their relationship with mutagenic effects is an essential stage in the actual evaluation of the efficiency of bioremediation processes before considering the soil really remediated and free from the risks that may result from contamination.

Acknowledgments

To Márcia Kaffer for help in performing the statistical analyses. This study was developed with a doctoral fellowship granted by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) within the Postgraduate Program of Ecology at Universidade Federal do Rio Grande do Sul (UFRGS).

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