TOXIC EFFECTS OF LINEAR ALKYLBENZENE SULFONATE, ANTHRACENE AND THEIR MIXTURE ON GROWTH OF A MICROBIAL CONSORTIUM ISOLATED FROM POLLUTED SEDIMENT

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ABSTRACT

The aim of this study was to determine the effect of linear alkylbenzene sulfonate (LAS), anthracene and a LAS-anthracene mixture on the growth of a microbial consortium isolated from polluted sediment. The microbial consortium was grown in a sterile glass bottle with mineral medium containing 1 g/L of glucose. Microbial growth inhibition produced by LAS, anthracene and combinations of LAS and anthracene was determined by viable count in nutritive agar; inhibitory concentration 50 (IC_{50}) was calculated. The concentrations evaluated were 0.16, 0.8, 1.6, 16 and 160 mg/L of LAS or anthracene. The LAS-anthracene mixtures were prepared by fixing either LAS or anthracene at 0.16 mg/L while increasing the other compound at the above concentrations. Microbial growth was sensitive to LAS at an IC_{50} of 8.22 and to anthracene at an IC_{50} of 5.2 mg/L. In the LAS-anthracene combination, if LAS concentration was fixed and anthracene concentration varied, IC_{50} (5.92 mg/L) was similar to IC_{50} for anthracene alone. In contrast, the inhibition effect was diminished when anthracene remained constant and LAS concentration was increased (IC_{50}: 70.11 mg/L). The sediment microbial populations were capable of degrading the LAS-anthracene mixture if the concentration of both compounds were at 0.16 mg/L.

RESUMEN

El objetivo de este trabajo fue determinar el efecto del sulfonato de alquilbenceno lineal (SAL), antraceno y su mezcla sobre el crecimiento de un consorcio microbiano aislado de sedimento contaminado. El crecimiento del consorcio se obtuvo en botellas de vidrio con medio mineral estéril más 1 g/L de glucosa. La inhibición del crecimiento microbiano, producida por SAL, antraceno o la combinación de ambos, fue determinada por cuenta viable en agar nutritivo y se determinó la concentración inhibitoria 50 (CI_{50}). Las concentraciones evaluadas fueron 0.16, 0.8, 1.6, 16 y 160 mg/L de SAL o antraceno. Las mezclas SAL-antraceno fueron preparadas manteniendo constante la concentración de SAL o antraceno en 0.16 mg/L mientras se aumentó la concentración del otro compuesto a las mismas concentraciones mencionadas. El

crecimiento microbiano fue sensible al SAL y antraceno a una CI_{50} de 8.22 mg/L y 5.2 mg/L respectivamente. Cuando la concentración de SAL y antraceno fue evaluada, si la concentración de SAL se mantuvo fija y la concentración de antraceno varió, la CI_{50} (5.92 mg/L) fue muy similar a la CI_{50} para antraceno solo. En contraste, el efecto de inhibición disminuyó cuando el antraceno permaneció constante con concentraciones en incremento de SAL (CI_{50}: 70.11 mg/L). Por otra parte, se observó que las poblaciones bacterianas del sedimento son capaces de biodegradar la mezcla SAL/antraceno cuando la concentración de ambos compuestos fue de 0.16 mg/L.

INTRODUCTION

Pollution of freshwater sediment ecosystems is generally due to the presence of a mixture of chemical compounds (Alexander 1997). Detergents and polycyclic aromatic hydrocarbons are organic pollutants that accumulate in freshwater sediment, constituting pollutant mixtures (Smulders and Krings 1990, Aboul-Kassim 1992). Anthracene and linear alkylbenzene sulfonate (LAS) are some of the many pollutants present in different aquatic ecosystems (Comber *et al*. 2006, Hamdi *et al*. 2006). The toxicity of chemical compounds on aquatic organisms depends on concentration in both the sediments and the water, as well as in processes related to their bioavailability. Bioconcentration, biodegradation, desorption and solubilization processes that occur in these substrata determine the quantity of free compounds that will reach toxic levels in the organs of aquatic organisms.

Anthracene is a tricyclic aromatic hydrocarbon; its molecules fate in nature is of great environmental concern due to their potential toxicity, mutagenicity and carcinogenicity (Li *et al*. 2008). Anthracene is a hydrophobic substance which has been shown to be toxic to fish and algae (Moody *et al*. 2001). An increasing number of studies have been conducted on anthracene biodegradability to examine its elimination from ecosystems. An interesting and important observation made by Cerniglia and Heitkamp (1989) was that eukaryotic microbes such as *Cunninghamella elegans* use a cytochrome P-450 monooxygenase system or lignin peroxidase to break down aromatic hydrocarbon rings into the detectable product cis-dihydrodiol (Pickard *et al*. 1999).

LAS is a surfactant produced in large amounts used around the world in detergent and personal care products (Jiménez *et al*. 1991, Schleheck *et al*. 2004). LAS has been shown to affect the flora and fauna of aquatic ecosystems. It has been observed that this compound denatures proteins in the cell membrane, altering the permeability of the membrane to nutrients and other chemical substances (Kimerle 1989). Due to its surfactant properties, LAS is adsorbed preferentially onto sediments (Sanderson *et al*. 2006).

The initial enzymatic attack in LAS biodegradation occurs by omega oxidation of the terminal carbon of the alkyl side chain. The enzymes involved in this reaction, although not yet identified, are probably associated with cell membranes. This enzymatic attack results in a carboxylated alkyl chain or sulfophenyl-carboxylate, which is further biodegraded through beta oxidation. Once beta oxidation has taken place, the molecule loses its surfactant properties because it no longer has a hydrophobic side chain. Following complete mineralization of the alkyl chain, the benzene ring is desulfonated and cleaved (White and Russell 1994, Schleheck *et al*. 2004).

Laboratory studies have demonstrated that these toxic organic compounds do not routinely biodegrade, as many of them are resistant to microbial degradation. This is due to microbial biodegradation is usually accessible only when they are dissolved in aqueous solution or at least in direct contact with water (Sundaram *et al*. 1994). Fu and Alexander (1995) found that the desorption or solubilization of petroleum hydrocarbons can be accelerated with the addition of surfactants. As a result, bioavailability and therefore biodegradation of anthracene may be increased in the presence of LAS in bodies of water polluted with these compounds.

According to Ventullo and Larson (1986), cationic surfactants can produce alterations in heterotrophic activity in limnetic microbial populations. Therefore it is possible that the chemical structure and toxicity of LAS and anthracene would have the same effect on sediment microbial populations.

The hypothesis is that the LAS-anthracene mixture can inhibit the growth of the microbial consortium in sediment. It is possible that anthracene is more toxic than LAS, and that when they are mixed, having made the anthracene more soluble by increasing the surfactant concentration, the mixture will

be more toxic than the separate compounds. On the other hand, it is expected that at a low concentration, microbial degradation of the mixture will be possible, since there will not be an inhibition effect on microbial growth.

It was determined the toxicity and kinetics of biodegradation of the LAS-anthracene mixture by microbial populations of natural ecosystems, with particular emphasis on microorganisms found in sediment, where these compounds are concentrated. The objective was to evaluate the acute toxic effect of LAS, anthracene and the mixture of the two compounds on the growth of a microbial consortium isolated from sediment, and to determine the potential of the LAS-anthracene mixture to be degraded by isolated microbial populations.

MATERIALS AND METHODS

Sampling area

The sampling zone was located in the municipality of Tlahuelilpan de Ocampo in the state of Hidalgo, México. Samples were collected in Irrigation District 63, located in the southeast part of the state to the north of México City, between 15° 44' and 20° 29' N and 98° 57' and 99° 21' W, with an average elevation of 1895 meters above sea level.

The sediments are in continuous contact with domestic, agricultural and probably industrial wastewaters. These wastewaters did not receive any prior treatment; thus microorganisms present in the sediments are in direct contact with a great variety of chemical pollutants. The water is subsequently used for irrigating agricultural crops; however, this water contains toxic hydrocarbons and other by-products associated to their breakdown by the microorganisms contained in the sediments, which will pollute the crops.

Screening and maintenance of the microbial consortium

The microbial consortium was isolated from sediment obtained at the same time as the wastewaters were sampled. One kilogram of sediment was collected and placed in a sterile carrier bag. The sediment was stored at 4 ºC until required.

To maintain the microbial population, the ISO-9439 system was employed (Pineda-Flores *et al*. 2004). One gram of sediment was placed in a 250 mL sterile glass bottle, which contained 100 mL of mineral medium (composition in mg/L: KH_2PO_4 0.085, K_2HPO_4 0.22, $Na_2HPO_4•2H_2O$ 0.33, NH_4Cl 0.05, $MgSO_4•7H_2O$

0.023, $CaCl_2$ 0.028, $FeCl_3•6H_2O$ $2.5×10^4$) plus 1 g of glucose as a carbon and energy source.

The microorganisms that constitute the microbial population were identified as *Pseudomonas mendocina*, *Flavobacterium breve* and *Corynebacterium flavescens* by microscopy, colony morphology and various biochemical tests following the schemes of Weaver and King (Weyant *et al*. 1996) and Bergey's manual (Sneath *et al*. 1986).

The culture was maintained at 25 ºC and agitated by magnetic stirring (150 rpm). The microbial population was maintained by introducing weekly subcultures of 1 mL of the microbial population into 100 mL of fresh sterile mineral medium containing 1 g of glucose. Aseptic technique was used for all transfers.

Toxic effects of LAS and anthracene on microbial growth

Once the microbial population had reached the exponential phase (with an absorbance of 0.09 at 650 nm), 1 mL of culture was serially diluted from 10^{-3} to 10^{-6} in assay tubes with 9 mL of sterile distilled water (Espigares *et al*. 1990). Each tube was added with 0, 0.16, 0.8, 1.6, 16 or 160 mg/L of LAS or anthracene (Aldrich Chemical Co., 98 and 96 % purity respectively, both sterilized by 0.45 μm membrane filtration). The samples were stirred for 5 seconds, then a 0.2 mL aliquot was taken and streaked onto nutritive agar obtained from Becton Dickinson and Company, Cockeysville, MD. Each set of plates was incubated at 25 ºC for 48 hours and the colonies enumerated. The experiment was replicated five times.

To establish the interval of concentrations for the tests, growth of the consortium was evaluated with 1, 4 and 16 mg/L of each compound as described above. With 16 mg/L of each compound, a reduction in the growth of the consortium was observed (data not shown). For this reason, the choice of the range of concentrations was based on decimal reductions and decimal increases of 16 mg/L (0.16, 1.6, 16 and 160 mg/L). The 0.8 g/L concentration was included to complete the five concentrations needed as minimums to determine IC_{50}.

The effect of the LAS-anthracene mixture was quantified following the method described above, except for the following difference: each assay tube contained either a fixed concentration of LAS (0.16 mg/L) plus 0.16, 0.8, 1.6, 16 or 160 mg/L of anthracene or 0.16 mg/L anthracene plus 0.16, 0.8, 1.6, 16 or 160 mg/L of LAS. This enabled the determination of the toxicity of the LAS-anthracene mixtures. Five repetitions were performed for each concentration.
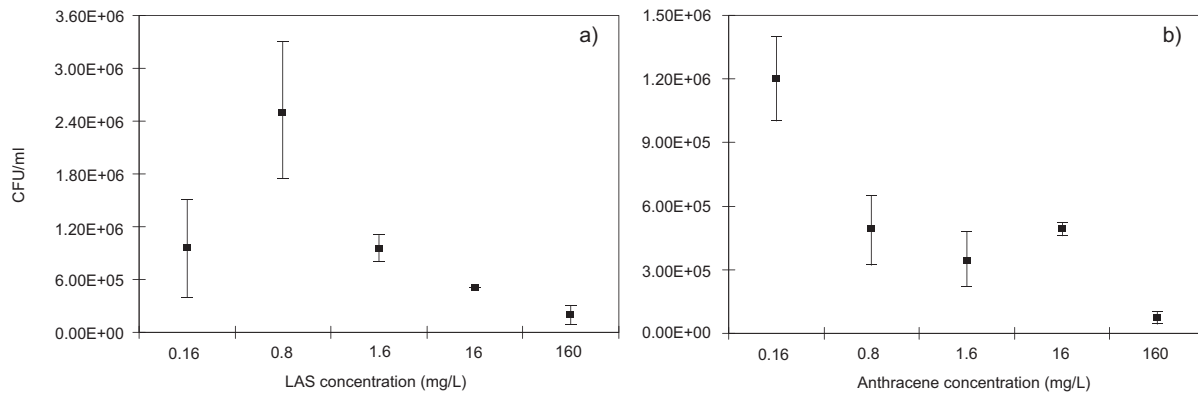
Fig. 1. Toxic effect of LAS (a), and anthracene (b) on the growth of isolated microbial consortium. CFU of microbial consortium without LAS or anthracene concentration was $1.25 \times 10^6 \pm 2.12 \times 10^5$ CFU/mL. The bars represent standard deviation of five repetitions

IC_{50} for LAS, anthracene and the mixture was obtained using linear regression by plotting the number of colony-forming units against the logarithmic concentration of LAS or anthracene.
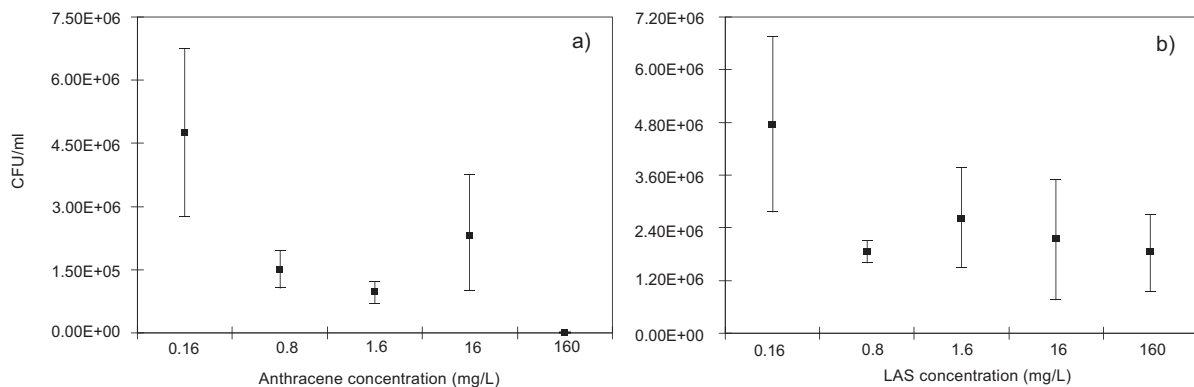
Microbial degradation of LAS-anthracene mixture

The production of carbon dioxide by microbial degradation of the LAS-anthracene mixture was evaluated using the device and methodology described by Pineda-Flores *et al.* (2004). A sterile 250 mL glass bottle containing 100 mL of mineral medium was inoculated with 1 mL of microbial consortium adjusted to $Abs_{652} = 0.09$. The concentrations of LAS and anthracene used in the mixture were as described above. Carbon dioxide was measured every 12 hours for 48 h. The concentrations used for the LAS and anthracene mixture were 10-0.1, 12.58-1, 15.84-10, 19.95-100 and 23.98-200 mg/L. Three repetitions were performed for each treatment. In order to characterize degradation of the LAS-anthracene mixture, the maximum reaction

velocity (Vmax), Michaelis constant (Km) and affinity constant (AC) were calculated from the Michaelis-Menten equation (Conn *et al.* 1987).

RESULTS

Figure 1 shows the toxic effect on the growth of the microbial population exposed to different concentrations of LAS and anthracene. The growth of the isolated microbial population was not inhibited with 0.16 mg/L of LAS or anthracene alone. When the LAS and anthracene concentrations were increased, toxic effects on the microbial population also did. CFU/mL declined as LAS and anthracene concentrations were increased from 0.8 to 160 mg/L. The LAS-anthracene mixtures produced a notable growth inhibition effect, as seen when **figure 2** is compared to **figure 1**. The decline in CFU/mL was also correlated with an increase in LAS and anthracene concentration. The greatest

Fig. 2. Toxic effect of LAS-anthracene mixture increasing the anthracene concentration from 0.16 to 160 mg/L (a), and increasing the LAS concentration from 0.16 to 160 mg/L (b). CFU of microbial consortium without LAS-anthracene mixture was $4.48 \times 10^6 \pm 3.88 \times 10^5$ CFU/mL. The bars represent standard deviation of five repetitions

inhibition, with 100 % of the microbial population killed, was observed with the mixture containing 0.16 mg/L of LAS and 160 mg/L of anthracene.

Table I shows that IC_{50} for anthracene was 5.2 mg/L, 3 mg/L lower than the IC_{50} for LAS. The IC_{50} of anthracene was similar to the IC_{50} of the LAS-anthracene mixture, where anthracene concentrations increased from 5.2 to 5.92 mg/L. However, the difference was much more pronounced than for the IC_{50} of LAS-anthracene mixture, whose 70.11 mg/L was almost 8.5 times higher than the IC_{50} of LAS without anthracene (8.22 mg/L).

TABLE I. DETERMINATION OF INHIBITING CONCENTRATION 50 (IC_{50}) FOR LAS, ANTHRACENE AND THEIR MIXTURE ON GROWTH OF ISOLATED MICROBIAL CONSORTIUM

Compound(s)	IC_{50} (mg/L)
LAS	8.22
Anthracene	5.2
LAS-anthracene (increasing anthracene concentration)[1]	5.92
LAS-anthracene (increasing LAS concentration)[2]	70.11

[1] LAS concentration was kept constant at 0.16 mg/L, anthracene concentration was increased from 0.16 to 160 mg/L
[2] Anthracene concentration was kept constant at 0.16 mg/L, LAS concentration was increased from 0.16 to 160 mg/L

The kinetics of mineralization by the LAS-anthracene mixture is presented in **figure 3**. The maximum concentration of carbon dioxide produced in the system was observed at 24 hours. After this
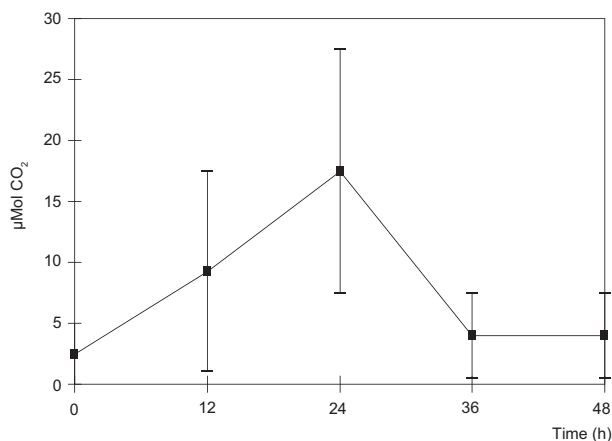
Fig. 3. Mineralization kinetics of LAS-anthracene mixture for the microbial consortium isolated from sediment. LAS and anthracene concentrations were both 0.16 mg/L. The bars represent standard deviation of three repetitions

time, the mineralization fell abruptly and remained constant up to 48 hours.

Table II shows the results of the LAS-anthracene mixture degradation. It is to be noted that 20.45 % of the mixture was biodegraded by the microbial population within 24 hours.

TABLE II. CHARACTERIZATION OF LAS-ANTHRACENE MIXTURE DEGRADATION

Variable	Magnitude
Vmax	4.99 µMol CO_2
Km	0.5235 µMol CO_2/h
Affinity constant	0.2 1/µMol CO_2
Biodegradation percentage[1]	20.45 %

[1] The biodegradation percentage was calculated 24 hours after inoculation

DISCUSSION

There have been few studies of the effect of organic pollutants on sediment microbial populations. Toxicity data of chemical pollutants on microorganisms are scarce due to the considerable ability of microbes to resist or biodegrade organic chemicals. Some microbial populations are very sensitive to low concentration of LAS. Brandt *et al*. (2001) showed that ammonia-oxidizing bacteria isolated from soil are inhibited by 5-9 mg/L of LAS; inhibition was shown by its effect on microbial growth, specific growth rate and CO_2 fixation. García *et al*. (2006) demonstrated that for LAS, an EC_{50} of 14 mg/L can be considered a toxic concentration for anaerobic microorganisms, and that the addition of LAS homologues to anaerobic digesters at surfactant concentrations higher than 5-10 g/kg of dry sludge gave rise to partial or total inhibition of methanogenic activity.

The microbial population isolated from sediment was previously exposed to high concentrations of LAS contained in the polluted water. Therefore it could be assumed that the polluted water would selectively isolate microbial populations which could grow in environments with a high LAS concentration. However, in contrast to the prediction, the microorganisms in this study were sensitive to low concentrations of LAS and the LAS-anthracene mixture, with IC_{50} values of 8.22 and 5.92 mg/L, respectively. Therefore, in this case prior exposure to polluted water did not contribute to an increase in microbial resistance. Jensen *et al*. (2007) demonstrated that concentrations of LAS in untreated sludge can range from 400 to 14,000 mg/kg dw.

The dose-response curves (**Figs. 2** and **3**) indicated that microbial growth was not inhibited at very low concentrations of LAS, anthracene and LAS-anthracene mixtures (0.16 mg/L). Increasing the concentration of all compounds and mixtures decreased microbial growth. This may be due to the LAS concentrations evaluated being less than the critical micelle concentration reported (410 mg/L, Brandt *et al*. 2001); therefore, it is not possible to suggest that there were surfactant-micelle interactions. The results suggest that the LAS toxicity may have been due to direct interactions of LAS monomers with the cell structure, causing an increase in membrane permeability, dissipation of ion gradients and membrane potential or leakage of essential cell constituents. Sartoros *et al*. (2005) reported that 20 mg/L of the surfactant Tergitol NP-10 may disrupt cell membranes by interacting with lipid structural components of bacterial cells isolated from soil polluted with polycyclic aromatic hydrocarbons.

Anthracene has a high octanol-water partition coefficient (log K_{ow} = 4.1), and readily partitions into organic phases such as phospholipids, which are also found in bacterial membranes. This interaction provokes a hydrophobic region inside the bacterial membrane, which can act as a reservoir for anthracene accumulation (Bugg *et al*. 2000). It is suggested that the toxic effect of anthracene on isolated microbial populations in this study is due to the accumulation and mutagenic activity of anthracene, similar to the effect produced by polycyclic aromatic hydrocarbons on *Salmonella* strain YG1041 (Kummrow *et al*. 2006).

Evaluation of the effect of the mixtures showed clearly that there was no synergy between the compounds, and that the toxicity of the mixture decreases as the concentration of LAS is increased, contrary to the initial hypothesis. Martínez-Tabche *et al*. (1997) report a similar phenomenon with a mixture of crude petroleum and sodium dodecyl sulfonate: when the concentration of the latter was varied, toxicity of the petroleum on *Moina macrocopa* acetylcholinesterase activity was reduced approximately 100-fold (antagonistic effect).

Sundaram *et al*. (1994) note that adding a tensoactive agent to a polyaromatic hydrocarbon can delay biodegradation as the micelles of the former "protect" the latter by delaying or preventing its breakdown.

Laha and Luthy (1992) report that mineralization of phenanthrene is completely inhibited by the addition of 0.2 % of various surfactants. Similarly, Fu and Alexander (1995) state that mineralization of phenanthrene by soil microorganisms was inhibited after the addition of the anionic surfactant Neodol 25-35. Considering these reports, the increase in IC_{50} of the LAS-anthracene mixture when LAS concentration was increased was attributed to a reduction of anthracene bioavailability promoted by its adsorption of LAS. Because LAS concentration was below the critical micelle concentration, the LAS molecules did not form micelles. However, it is suggested that the LAS molecules interact with anthracene molecules, producing an interaction so strong that it reduces the bioavailability of anthracene, thus avoiding direct contact with the microorganisms and causing its toxicity to diminish. Johnsen and Karlson (2004) demonstrated that *Novosphingobium subartica* LH128 and *Mycobaterium* spp. VM572 only express their biological response to phenanthrene, fluorene, fluoranthene and pyrene when they are directly attached to crystals of these polycyclic aromatic hydrocarbons. A similar process has been described by Stelmack *et al*. (1999): when *Mycobacterium* and *Pseudomonas* strains are in direct contact with a nonionic surfactant-hydrocarbon mixture, they do not establish contact with the mixture and avoid its toxic effect. Jiang *et al*. (2005) demonstrated that 200 mg/L of LAS inhibited mineralization of phenanthrene (by 7 to 12 %) and its toxic effect on phenanthrene-degrading microorganisms in a water-lava-plant-air model ecosystem.

Since the degradation of the LAS-anthracene mixture is at a maximum at 24 hours (**Fig. 3**), it allowed the establishment of the optimum time at which samples should be taken during this study. Sampling was therefore performed 24 hours after inoculation. According to Ringelberg *et al*. (2001), the microbial populations present in sediment polluted with polycyclic aromatic hydrocarbons (PAH) are capable of degrading anthracene and other three-ring PAH in a bioslurry treatment system. The three-ring PAH were biodegraded from 115 ± 5.7 mg/kg to 56 ± 3.8 mg/kg in four months by the microbial populations present in the system. The microbial population isolated in this study had a greater capacity for breaking down the LAS-anthracene mixture, with degradation within 48 hours; however, the concentrations evaluated were low (0.16 mg/L of both compounds). In contrast, microorganisms isolated from soil or fresh water were able to degrade the single compounds up to 92 % for anthracene (Moody *et al*. 2001) and up to 90 % for LAS (Nishihara *et al*. 1997, Ying 2007); nevertheless, these results refer exclusively to the separate compounds.

Mineralization of the LAS-anthracene mixture was achieved by the microorganisms present in the polluted sediment. The capacity of LAS and anthracene to mineralize microbial populations is well known (Marchesi *et al*. 1994, Mutnuri *et al*. 2005, Perales *et al*. 2007). Still, it is important to consider that there can be non-cultivable microbial populations in the polluted sediment that make an important contribution to mineralization of the mixture.

CONCLUSIONS

The results showed that growth of the microbial population isolated from sediment was sensitive to LAS, anthracene and a mixture of the two. The toxicity of the LAS-anthracene mixture, expressed as IC_{50}, diminished as the LAS concentration was increased, indicating that direct contact between microorganisms and the chemical is important to the toxic effect. The isolated microbial population also has the capacity to degrade the LAS-anthracene mixture if the concentrations of both compounds are low. When analyzing pollutant interactions in sediments, it is important to consider the chemical structure and concentration of the pollutants involved, as the mixture formed may be toxic or capable of being eliminated by the microbial populations present in the aquatic ecosystems.

ACKNOWLEDGEMENT

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