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Full Length Research Paper

Microbial community dynamics in diesel waste biodegradation using sequencing batch bioreactor operation mode (SBR)

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The dynamic of molecular microbial community during diesel waste biodegradation was investigated. The waste was treated in bioreactors operated in sequencing batch operation mode (SBR) in four cycles of 72 h, using optimized setpoints (pH, initial waste load, C:N ratio, aeration). Optimal conditions allowed the system to reach biodegradation of 53.3, 96.0, 76.2 and 75.0% at the end of cycles one, two three and four, respectively. Oxygen uptake rate (OUR) indicated increases in microbial activity from cycle one to cycle two (124.9 to 252.9 mgO_2/L/h) and decreases in cycles three and four (120.4 to 108.8 mgO_2/L/h, respectively). Investigations of microbial diversity showed changes in the microbial community members at the end of the cycle one. Significant reductions in the relative ecotoxicity were observed beginning with cycle two, and the reductions extended until the end of process. The SBR operation mode proved to be an efficient method for treating the diesel waste, and the process allowed for relevant reductions in the hydrocarbon content of the waste along with an increase in its environmental quality. Changes in the microbial members are evidence of the synergistic action of the microbiota in the process.

Key words: Microbial diversity, denaturing gradient gel electrophoresis, sequencing batch, biodegradation.

INTRODUCTION

The accumulation of petroleum wastes such as those generated by diesel fuel can cause serious damage to the environment and to living organisms (Paixao et al., 2007). Biological treatments are emerging as a “green” alternative to treat such environmental contaminants. The applicability of these treatments varies according to factors such as the characteristics of the contaminated site, the biochemical potential of microorganisms, the nature of operation generating the pollutant (e.g., storage, distribution, distillation, exploitation, shipping, pipeline transportation, etc.) and cost effectiveness (Khan et al., 2004). In addition to water and nutrients, bio-technological techniques to treat wastes usually consist of stirred bioreactors (*ex situ* treatment) or a mixture of sediments at the contaminated site (*in situ* treatment) (Nano et al., 2003). The effectiveness of treatment in a bioreactor depends on pollutant concentration, the size of inoculum, oxygen, pH, nutrients, and to a large degree on the bioreactor’s mode of operation. The *ex situ* techniques are recommended for storage tank wastes due to the need for treating wastes intermittently after

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Abbreviations: SBR, Sequential batch bioreactor; **CSTR,** continuously stirred bioreactor; **GC,** gas chromatograph; **FID,** flame ionization detector; **DGGE,** denaturing gradient gel electrophoresis; **PCR,** polymerase chain reaction; **OUR,** oxygen uptake rates; **DSS,** dodecyl sodium sulfate.

Table 1. Real and encoded values of independent variables in the 3^{4-1} factorial fractional design.

Variable	Level		
	Lower	Center point	Upper
pH	5.50	6.75	8.00
C:N ratio	50:1	425:1	800:1
Initial waste concentration (%, v/v)	10	40	70
Agitation speed (rpm)	50	150	250

every discard. As a consequence, the efficiency of such treatments requires high degradation rates. Residues with high environmental risk have been efficiently treated by *ex situ* techniques at the pilot and laboratory scale. These treatments were carried out using different reactor operation modes including a sequential batch bioreactor (SBR), continuously stirred bioreactor (CSTR) and tanks in series. In the SBR mode, the bioreactor is fed during a certain period and subsequently operated as a single batch in order to reach the desired degradation level of contaminants. A fraction of the treated residue is removed after a defined period of time, and the bioreactor is fed again with the same volume of untreated residue to complete the cycle. A significant advantage of the SBR operation mode is the ability to assess the toxicity of the treated residue during the process and to study the acclimatization of microorganisms that promote high degradation rates (Cassidy et al., 2000).

Due to the scarcity of studies on biological treatment of these classes of residues, this work is aimed at investigating the performance of a bioreactor operated in SBR mode and used to treat diesel waste collected from the bottom of storage tanks.

MATERIAS AND METHODS

Characterization of diesel waste

Diesel waste samples were collected from the storage tanks of Petrobras Oil Company in Pernambuco, Brazil. Physical-chemical analyses of flash point, density at 20/4°C, kinematic viscosity and color were previously carried out in the Petrobras oil company laboratories as recommended by Environmental Agency (2007). Identification of hydrocarbons present in the diesel waste were carried out in a gas chromatograph (capillary column DB-5: 5% phenyl methylpolysiloxane, 30 m length and 320 µm internal diameter) coupled to a mass spectrometer (Shimadzu® GC 17A, GCMS QP5050). An internal standard DRH 0085s purchased from Accustandard™ (straight-chain alkanes: C_8 to $C_{40;}$ branched alkanes: pristane and phytane) was utilized to confirm the identification of hydrocarbons. The oven temperature of chromatograph was held for 4 min at 40°C, increased at 5°C/min to 250°C, held at that temperature for 2 min, increased at 10°C/min to 280°C and finally held for 4 min. Injector temperature was maintained at 280°C. Injections of 1 µL aliquots were done automatically in triplicate. A split ratio of 1:50 was adopted. Helium was the carrier gas. Elemental analysis of carbon, nitrogen, hydrogen and sulfur (CNHS) was accomplished utilizing an Elemental Analyzer Carlo Erba GC, model EA-1110, equipped with a manual injection port, Porapak PQS gas column (length 2 m, internal diameter 4 mm, Carlo Erba, Italy). Oven temperature was maintained at 1000°C.

Flask experiments – factorial fractional design

A 3^{4-1} statistical factorial fractional design was used to evaluate hydrocarbons biodegradation percentage as a function of pH, C:N ratio, initial waste load, and agitation speed. Initial waste load was reported in terms of percentage of diesel waste volume to total liquid medium volume (mineral medium + waste); agitation speed varied, as for evaluating the influence of superficial aeration; C:N ratio represented the relationship between the estimated percentage of carbon in the waste and the nitrogen concentration calculated in terms of percentage of nitrogen present in all the ammonium nitrate (NH_4NO_3) provided by the Bushnnel-Haas mineral medium. These experiments were carried out in 250 ml Erlenmeyers flasks. Concentrations of ammonium in Bushnell-Haas mineral medium (KH_2PO_4 1.0 g/L, K_2HPO_4 1.0 g/L, NH_4NO_3 1.0 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $FeCl_3$ 0.05 g/L, $CaCl_2.2H_2O$ 0.02 g/L) varied according with the desired C:N ratio. In order to calculate the ammonium nitrate concentration for each C:N ratio, the following expression was employed: mC = ρvf, where, mC was the approximated carbon mass; ρ was the waste density (0.8542 kg/cm^3); v was the waste volume and f was the percentage of carbon in the waste obtained by elemental analysis (78.87%). Ammonium nitrate mass was adjusted to compose the desired C:N ratio as follows: mNH_4NO_3= [(mC/mCd) x 80.05]/28, where, mNH_4NO_3 was the ammonium nitrate utilized mass; mC was the approximated carbon mass; mCd was the carbon desired proportion to compose the C:N ratio. Inoculum was provided directly from the diesel waste. Independent variables values are presented in Table 1. Statistical analysis was performed using Statistica v6.0 (Tulsa, Oklahoma, USA).

Sequencing batch bioreactor description

Sequencing batch operation was performed in a Biostat-B® Bioreactor (B. Braun Biotechnology International). The bioreactor consisted of a covered, 5 L glass vessel with a rounded bottom and a working volume of 4 L. Bioreactor off-gases was directed through a port in the bioreactor cover that contained a C_{18} trap (*SepPak* ®) to collect volatile compounds. Additionally, the fraction collected was recovered with methylene chloride and quantified in a gas chromatograph (GC) to estimate the losses. Two other ports supported control of the pH using sodium hydroxide and hydrochloric acid. Initial pH was set in 7.0. All connections were appropriately sealed in an attempt to minimize emissions of volatile compounds as well as liquid compounds. The bioreactor was operated batch wise before the SBR, to optimize conditions and to define the cycle duration. Conditions utilized in the batches were indicated by the experimental design responses. The SBR was performed using the following conditions: Agitation speed, 300 rpm;

aeration rate, 0.5 vvm; percentage of dissolved oxygen, 35%; temperature, 30 ± 1°C; volume, 3.0 L. The volume of waste that was added to the reactor vessel was approximately 10% (v / v), approximately 8.5 g/cm based on the density. The bioreactor was operated in four cycles of 72 h each. At the end of every cycle, the volume of organic phase was removed by pumping, and untreated waste was added to the vessel in the same volume.

Microbial growth

Microbial growth was estimated by dry weight measurements in the aqueous phase of samples at 6 h intervals. Samples were collected using a sampling system connected directly to the bioreactor. Total sample volumes were registered (from 4 to 7 ml), and the non-polar phase was separated with methylene chloride (Merck®) for chromatographic analysis. The aqueous phase volume was registered, and 1.5 ml was centrifuged for 25 min at 10,000 rpm at 10°C. The biomass was washed with sterilized water and filtered through a cellulose acetate membrane (0.22 µm). Filter membranes were dried at 80°C either for 24 h or until they achieved a constant weight.

Hydrocarbon quantitation

Hydrocarbons present in the non-aqueous phase of samples were chromatographically quantified utilizing a Hewlett-Packard/Agilent GC, model 6890N, equipped with a flame ionization detector (FID) and a capillary column HP-5 (5% phenyl methylpolysiloxane: 30 m length, 320 µm internal diameter). The injector and detector temperatures were 250 and 300°C, respectively. The GC oven temperature was held for 1 min at 50°C, increased at 10°C/min to 300°C and then held at that temperature for 4 min. Reduction of hydrocarbons was estimated in terms of the percentage of total area under the chromatographic peaks of treated waste compared to the total area under the chromatographic peaks of untreated waste. Hydrocarbons were quantified at the beginning (after each load) and at the end of cycle period (before new load).

Oxygen uptake rate

Oxygen uptake rates of acclimated microbiota were investigated by respirometric method, carried out in a BI-2000 respirometer from Bioscience® (Pennsylvania, USA). Samples were collected from the aqueous phase at the end of each cycle of SBBR and utilized as inocula at 10% v/v. Experiments were conducted using data acquisition intervals of 0.05 h, working volume of 500 ml, temperature of 30°C, pH 7.0, C:N:P ratio of 300:1:1 and initial residue concentration of 10% v / v. The total run time was 73 h.

Evaluation of changes in microbial community

Changes in microbial community members during SBBR were investigated by employing the denaturing gradient gel electrophoresis (DGGE) molecular technique. Extraction of DNA was accomplished by utilizing a DNA extraction kit (FastDNA® SPIN Kit for Soil) purchased from BIO 101™ (California, US). Samples of 0.5 ml were filtered, and the filter membranes were cut and recoiled into Eppendorf tubes for application of the sequence of extraction reagents. The 16S rRNA gene-based primers used in the polymerase chain reaction (PCR) reactions were U968f-GC and L1401r. The PCR products were run as described by Aboim et al. (2008). Gels were photographed under ultraviolet (UV) light with the Gel Doc 2000 system (Bio-Rad Laboratories, CA, USA). The digitized images of DGGE gels were analyzed using Image Quant (ver. 5.2). For sequencing of selected DGGE fragments, bands were extracted from the gels. The excised DNA was amplified according to the 16S rRNA-based PCR reactions (Rosado, 1997). Sequence identifications were performed using the BLAST-N facility of the National Center for Biotechnology Information. The calculation of similarities was based on the Pearson (product–moment) correlation coefficient and resulted in a distance matrix. The unweighted pair-group method average (UPGMA) clustering algorithm was used to calculate dendrograms using the software package Statistica 5.5.

Ecotoxicity assays

Samples of 50 ml of treated waste extracted with hexane were added to Mariott flasks in concentrations of 10% v./v. Filtered and sterilized seawater (salinity adjusted to 28%) was utilized as dilution water and blank for the assays performed with the urchin species *Echinometra lucunter* and the oyster species *Crassostrea rhizophorae*. Distilled and sterilized water was utilized for the microalgae specie *Pseudokirchneriella subcapitata*. Treatments involved blank, untreated waste, hexane and treated waste from cycles two and four of the 72 h cycles of SBR. Volumes were homogenized for 24 h, and aqueous phases were withdrawn in order to perform the assays. Assays consisted of exposing urchin eggs in the division phase and embryos of oyster and sample media containing microalgae to various concentrations of the water-soluble fraction of the above mentioned treatments (Nascimento, 1989). The trimmed Spearman-Karber statistical method (Hamilton et al., 1977) was utilized to determine EC_{50}-24h for urchin and oyster assays and IC_{50}-96h for microalgae assays. Relative toxicity was calculated in terms of percentage of reduction of toxics units (TU), which is represented by the expression: $TU=100/EC_{50}$, where TU is the toxic units and EC_{50} is the effective concentration that causes abnormal development in tested organism. In this expression, EC_{50} was substituted by IC_{50} when the tested organism was the microalgae. All the results were reported in terms of relative ecotoxicity reduction, which was calculated as the ratio between the TU of the solvent (hexane) and the TU of treated waste and reported in percentage.

RESULTS AND DISCUSSION

Characteristics of the waste

The most representative hydrocarbons identified were straight-chain alkanes ranging from C_{10} to C_{28}. The 2,6,10,14 tetramethyl-pentadecane (pristane) and 2,6,10,14 tetramethyl-hexadecane (phytane) were the predominant branched alkanes identified. Physical chemical analysis indicated values close to those specified for the diesel oil, according to the American Society for Testing and Materials (ASTM), as described by Environmental Agency (2007). Elemental analysis results showed the predominance of carbon and hydrogen among the elements detected (78.87% w/w of carbon and 10.95% w/w of hydrogen). This percentage of carbon was utilized to calculate the C:N ratio in subsequent experiments (data not shown). The most predominant hydrocarbon was the *n*-hexadecane (*n*-C_{16}), followed by *n*-pentadecane (*n*-C_{15}). Between the two branched alkanes identified, the pristane was the most abundant, followed by phytane. Lee et al. (2005)

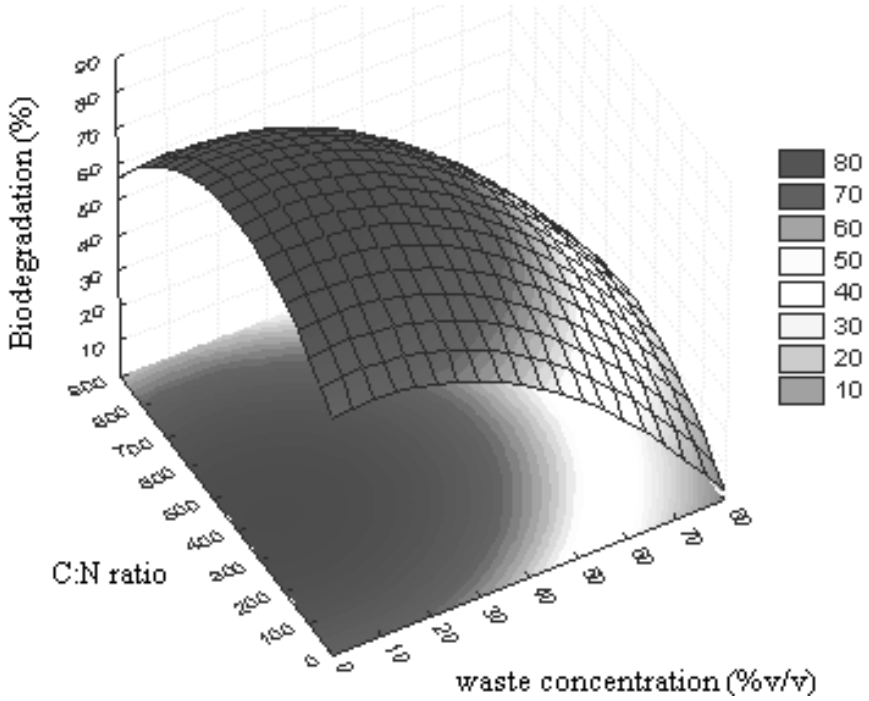

Figure 1. Surface-response of initial waste concentration and C: N ratio.

investigated the effect of synthetic mycolic acid in diesel fuel degradation and identified (by GC-MS) *n*-alkanes and branched alkanes, representing 42.7% of total hydrocarbons.

Comparing results in the current paper to those obtained by Marshall et al. (2003), who utilized more complex analytical conditions and obtained about 2,000 to 3,000 compounds, it is possible to infer that the sequence of hydrocarbons identified represents a typical compositional profile of diesel fuel. According to the literature, acyclic hydrocarbons are more susceptible to biodegradation than the cyclic ones, and the straight-chain alkanes are generally more biodegradable than the branched alkanes (De Jong et al., 1997). In some cases, however, preferential biodegradation of branched alkanes in the presence of straight-chain alkanes is observed. Therefore, the chromatographic profile presented here, indicates the presence of low-recalcitrance hydrocarbons in the diesel waste.

Factors influencing biodegradation

Analysis of the factorial fractional design results indicates that the main factors influencing biodegradation are initial waste load (a linear negative effect) followed by agitation (a quadratic positive effect). It is also apparent that the carbon-nitrogen ratio (C:N*)* plays an important role on the biodegradation. The influences of these factors on the bioprocess were all statistically significant (p-level < 0.1) as calculated from the Statistica version 6.0 (Tulsa, Oklahoma, USA). Figure 1 illustrates the combined effect of C:N ratio and waste initial concentration on the biodegradation efficiency. It seems that (C:N) ratios near 350:1 and 450:1, and initial waste load of about 10% v/v were preferable. Although, many works have described different optimum values of C:N and C:N:P ratios, the best range is specific for the process and waste studied. Alexander (1994) refers to many studies describing different C: N: P ratios varying from 50:1 to 800:1 when different residues undergo biotreatments. This is probably due to the bioavailability of compounds, characteristics that depend on their structure and chemical nature of the compounds. Initial waste concentration directly influences biodegradation, since the organic charge (especially in case of hydrocarbon mixtures) is responsible for the toxicity of waste. In the present work, the optimum initial waste concentration indicated by the experiments in flasks ranged from five to fifteen percent (w/w) (Figure 1).

SBR evaluation

Values of variables identified as significant in the shake flask experiments were scaled-up in bioreactors operated batch wise for 200 h in order to check the operational conditions and to determine cycle intervals in subsequent SBR experiments. According to the values obtained in batch wise mode, the biodegradation reached values of 75.5% (Table 2).

Cassidy et al. (2000) obtained 75.0% degradation in a continuous process and 96.0% using SBR at the end of

Table 2. Total peak area of chromatogram, biomass and biodegradation percentage (deducting abiotic loss by C_{18} filter) in the batch wise operated bioreactor.

Time (h)	Chromatogram area	Biomass (g/L)	Biodegradation
10	-	0.20	0,00
20	-	2.33	-
30	-	3.53	-
40	-	3.73	-
50	-	4.40	-
60	-	5.00	-
70	-	6.29	-
80	-	6.40	-
90	-	7.46	-
100	$1.1x10^4$	7.93	61,10
110	-	8.13	-
120	-	8.46	-
130	-	8.73	-
140	-	8.63	-
150	-	9.20	-
160	-	11.06	-
170	-	11.16	-
180	-	11.80	-
190	-	11.83	-
200	1.3×10^3	12.00	75,49

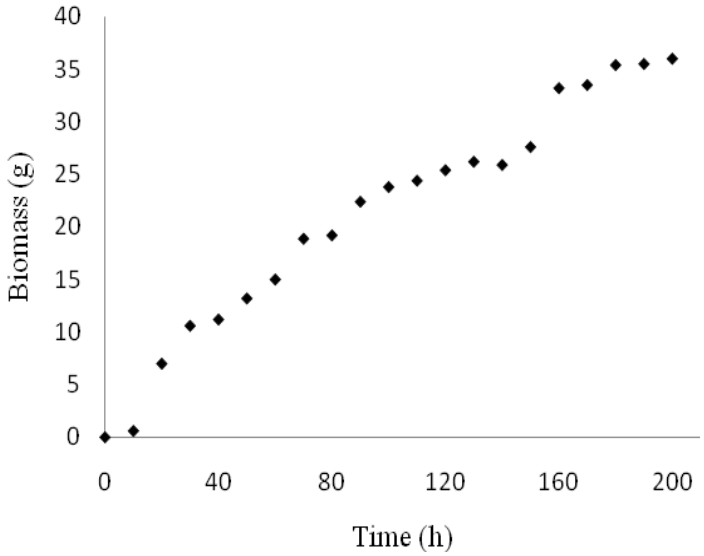

Figure 2. Microbial growth profile in the batch wise bioreactor.

80 days. It is important to observe that although, the group of identified compounds is comparable to those found by cited authors, the matrix in which the compounds were bound (sludge) makes direct comparison of the results difficult. However, we can estimate the global efficiency of biodegradation as a function of total time of the process (in the current work, 75.5% after 200 h, and in the cited work, 96.0% after 80 days).

Microbial growth in the batch wise operation mode determined the cycle duration in the SBR. After 72 h of process in batch wise operation, the microbial growth rate changed and biodegradation did not show significant changes (Figure 2 and Table 2).

According to the microbial growth profile in the SBR, the first 40 h suggest a lag phase (Figure 3). As shown, the growth rate changed after every cycle period and increased considerably after cycle two, indicating the influence of acclimatization of the microorganisms. On the other hand, the biodegradation percentage from cycle one to cycle four (53.3, 96.0, 76.2 and 75.0%, respectively) did not accompany the increase in microbial specific growth rate (Figures 3 and 4).

Oxygen uptake rates (OUR) profile shows increases in the microbial activities in cycle two, and decreases in the following cycles (concurrent with the biodegradation evolution along the process), suggesting that the activity diminished possibly due to accumulations of unviable cells. It is remarkable that OUR increased when the inoculum provided from cycle one was used, contrary to what has been observed in the assay with the untreated waste. In this same assay (inoculum form cycle one), after 20 h, OUR reached a maximum value (124.95 mg/L/h), decreased, and then, after 40 h increased again to reach another maximum value of 84.19 mg/L/h at 55 h. Members of the microbial genera identified, that is *Pseudomonas* sp. are well known to be able to biodegrade and completely mineralize alkanes of varying chain lengths. Certain species are just better at degrading

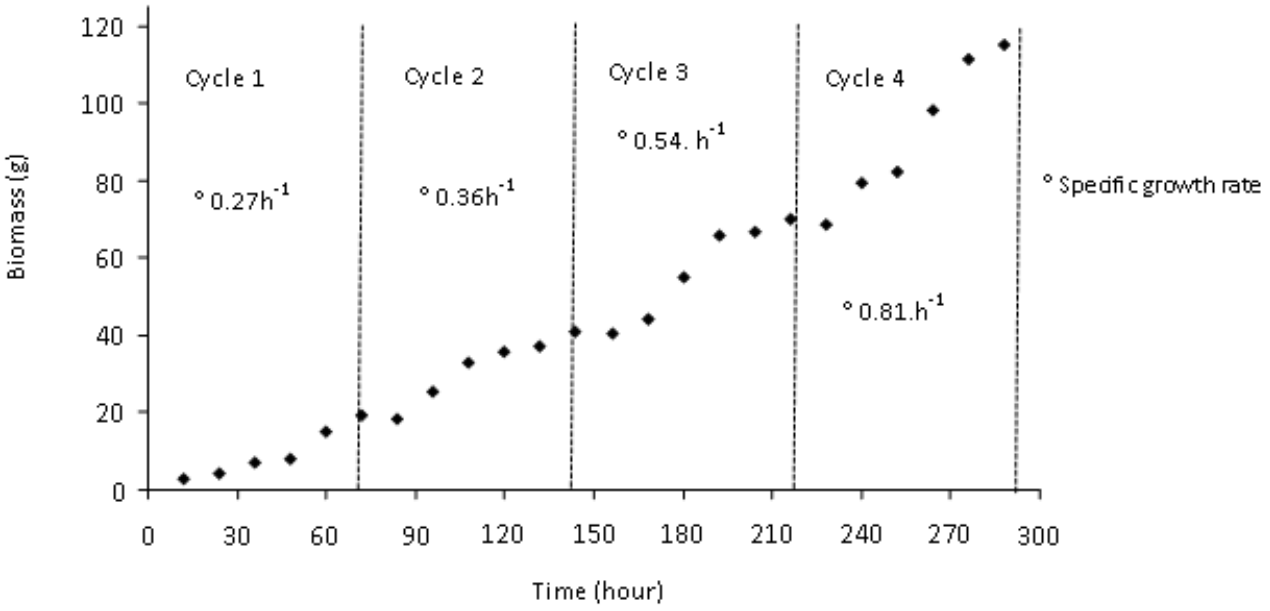

Figure 3. Microbial growth profile in the SBR.

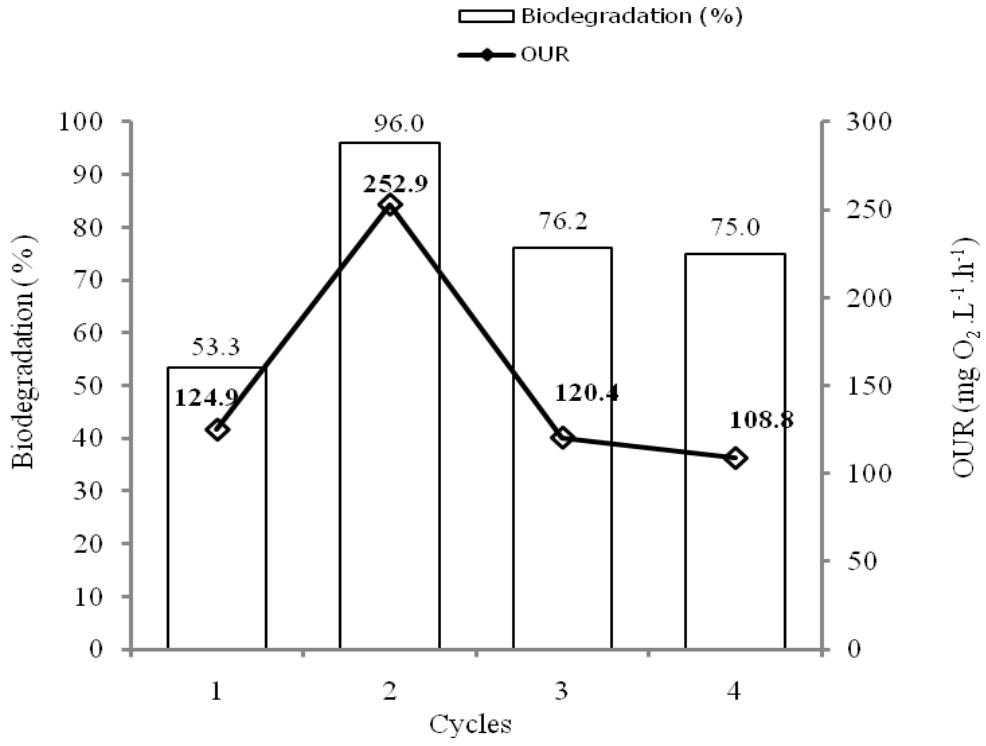

Figure 4. Biodegradation percentage and oxygen uptake rate along the cycles in the SBR.

hydrocarbons (such alkanes) than others, and thus may-out compete the others with continued incubation resulting in an apparent shift in community. Alexander (1994) discussed all aspects related to this phenomenon. According to the OUR profile (Figure 5), the microbial community of the diesel waste demon-strates a high degrading potential when acclimatized, which was demonstrated by significant decreases in the initial time of maximum oxygen uptake rate along the cycles (20 h in cycle one; 5 h in cycle 2; 5 h in cycle 3, and 8 h in cycle 4)

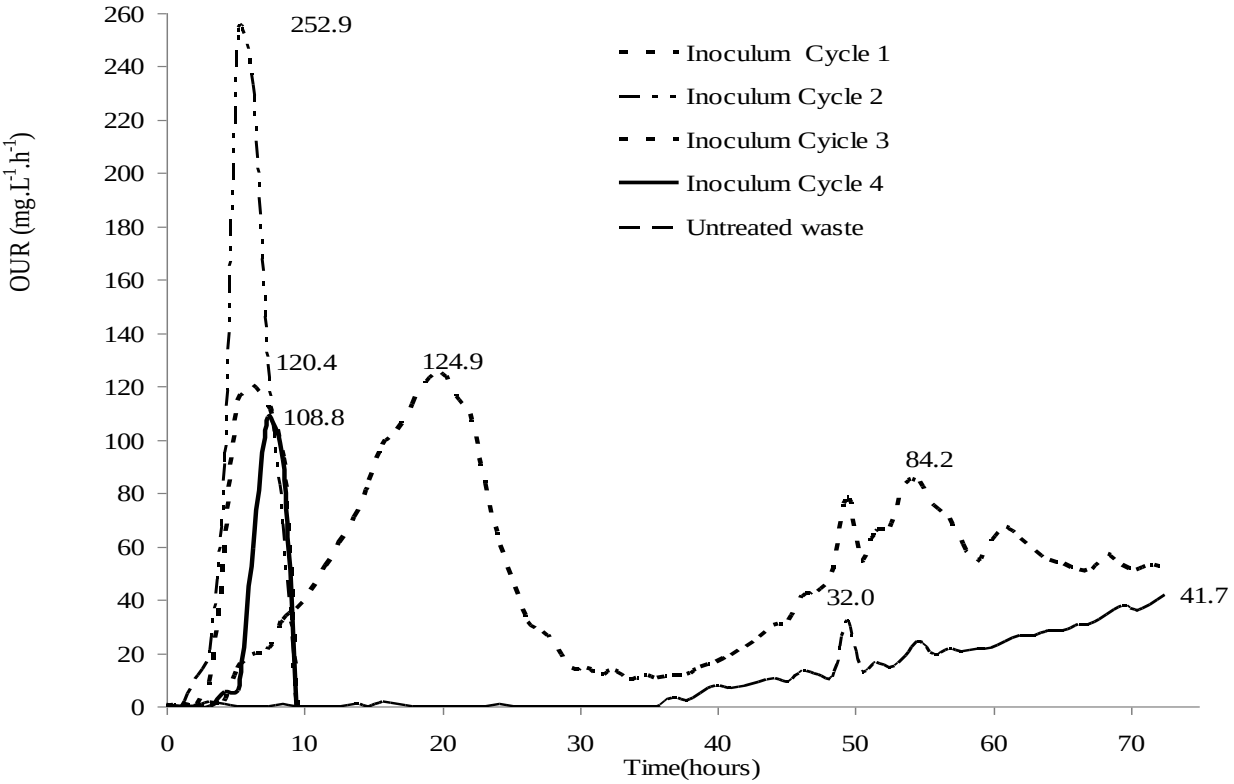

Figure 5. Oxygen uptake rate profile of the SBBR microbial community.

as opposed to the untreated diesel waste. These results also reinforce the occurrence of acclimatization of the microbial community, demonstra-ting the importance of prior exposure of the microbiota to the organic pollutants.

Pornsunthorntawee et al. (2008) investigated the production of biosurfactant by *Pseudomonas aeruginosa* during SBR and verified that the time required for the system to reach the stabilization of the chemical oxygen demand (COD) increased when the duration of cycles increased.

Nano et al. (2003) verified increases in degradation rates in direct proportion to the initial diesel concentration reintroduced at the beginning of each cycle period. The authors verified increases of 20% in cell concentration and 10% in the final degradation rate after cycling, demonstrating the effect of the acclimatization period from the second cycle of the sequencing batch.

Changes in microbial community members

The sequences from most evidenced DGGE bands along the process were deposited in the GenBank database under Accession Numbers: FJ006889; EU852413; EU282110 and AB079372, belonging to the *Pseudomonas* and *Burkholderia* genus. Theses genera are well-known in the literature as hydrocarbon degrading microrganisms. In recent works, Andreoni et al. (2004) identified *Pseudomonas, Burkholderia*, *Methylobacterium*, *Alcaligenes*, *Rhizobium*, *Aquamicrobium, Stenotrophomonas* and *Ralstonia*, in polycyclic aromatic hydrocarbon contaminated soil sam-ples, using the same molecular techniques. Following the same procedures, Díaz-Ramirez et al. (2007) identified 16SrDNA sequence from *Pseudomonas*, Pizzul et al. (2006) and Das and Mukherjee (2007) identified among others, the *Bacillus* genus.

The dendrogram (Figure 6) shows the main shift in the microbial community members at the end of cycle one and at the beginning of cycle two, by means of the linkage distance illustrations. This shift is coincidental with the change in the biodegradation rates and OUR maximum value in the cycle two, indicating the selective pressure in the microbiota promoted by the carbon source (hydrocarbons). The secondary shift in the microbial community members is observed between two main groups: one including cycle three (12 h and final) and cycle four (final), and another including cycle two (12 h) and cycle one (final). The tendency of reestablishment of community members present in cycle two, is evidenced by the proximal linkage distance between the members in cycle four (final) and members in cycles one (final) and two (12 h).

Evans et al. (2004) studied the impact of oil on a

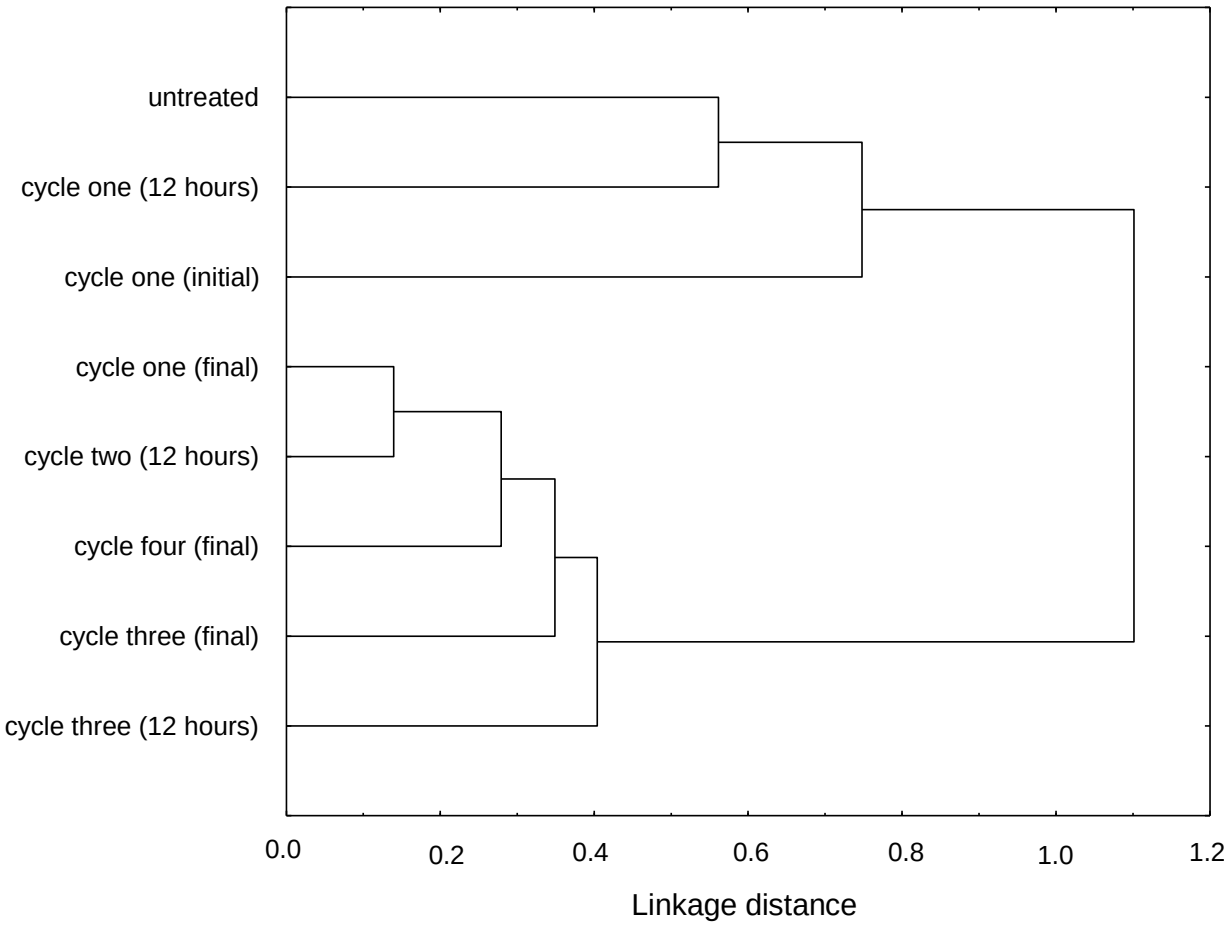

Figure 6. Dendrogram indicating linkage distance among the dominant members of microbial community along the SBR.

bacterial community in soil and observed changes in its members when oil was added to soil samples. Li et al. (2007) studying the dynamics of changes in microbial community structure during petroleum degradation, verified that the numbers of DGGE bands decreased from 40 to 25 when oil concentrations increased to 5,000 mg/kg of soil. These results confirmed the selective pressure caused by the presence of recalcitrant compounds.

Ecotoxicological assays

Results of ecotoxicological assays revealed changes in the toxic characteristics of treated waste during the process. Reductions in the ecotoxicity were about 70% at the end of cycle two, reaching undetectable levels in cycle four (assays with *E. lucunter*), and were 86% at the end of cycle four (assays with *C. rhizophorae*). The assays with *P. subcaptata* showed no detectable levels of toxicity form cycle two onwards (Figure 7).

The values of EC_{50} observed for *C. rhizophorae* (data not shown), when exposed to blank reference dodecyl sodium sulfate (DSS), showed similar results as those observed by Paixao et al. (2007), who tested different gasoline formulations with the same organism. This approach indicates the reliability of the assays. Reductions in ecotoxicity are expected once the alkanes present in the aqueous phase were biodegraded.

The primary biological effect resulting from the absorption of the water soluble fraction is the exposition of cells and the incorporation of sub-lethal quantities of hydrocarbons, which reduces the organism's tolerance to other stress factors. As shown by Paixao et al. (2007), the toxic effects of water soluble fraction of gasoline formulations proved to be greater to oyster embryos than to algae. This tendency is also evidenced in the present work.

There is far less data available regarding the chronic effects of hydrocarbons' water soluble fraction (Paixao et al., 2005; Ramos et al., 2005). Chronic effects can include long-term noticeable alterations in plankton, benthic or pelagic communities, which may be preceded by primary biochemical defense responses. Although, the literature is scarce in reports showing application of biotreatments in the reduction of ecotoxicity of treated oil wastes, the results obtained in the present work showed

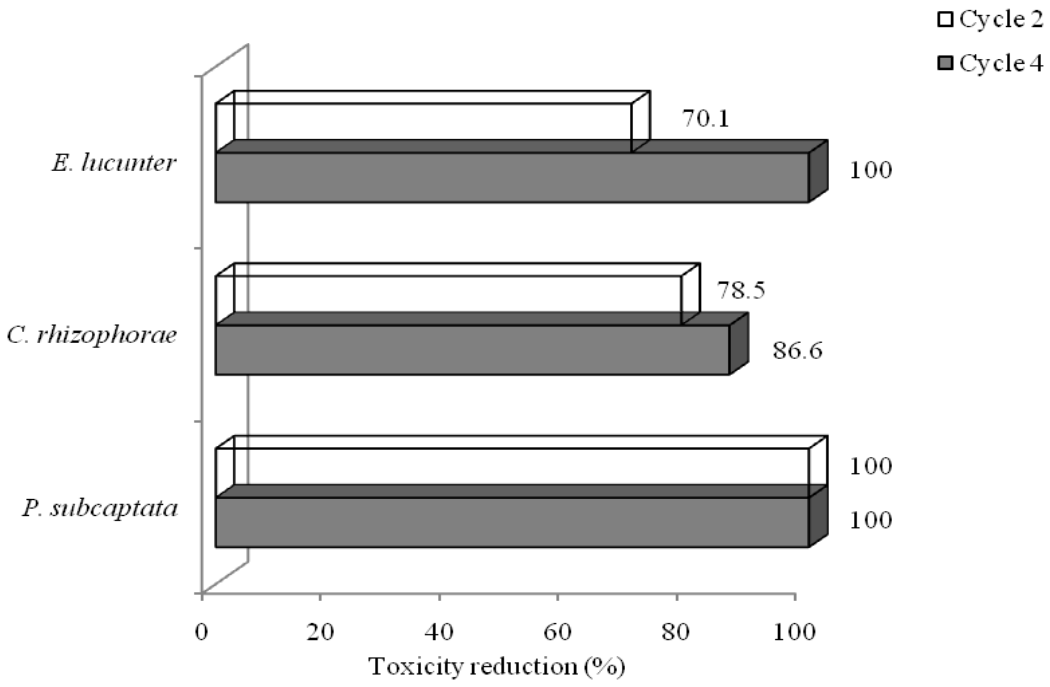

Figure 7. Reduction in toxicity in the SBR, 100% represents non detectable levels of toxicity.

the high treatability of diesel waste.

Conclusions

The SBBR operation mode employed to treat the studied diesel waste was shown to be efficient for degrading and attenuating the waste's toxicity to the environment. Results lead to infer that, improvements in effluent environmental quality were achieved by means of consecutive cycling. The effectiveness of the process in terms of biodegradation rate is not directly associated to the accumulated biomass. On the other hand, such rates seem to be more directly linked to the presence of a "competent" microbial community, whose changes in the predominance of its members is more likely to be responsible for the variations in the biodegradation rates. Microbial community adapted to the recalcitrant compounds was capable of reducing hydrocarbons in the waste by acting in different steps, as demonstrated by the changes in microbial community members.

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