PROGRESS OF TOTAL PETROLEUM HYDROCARBONS (TPH$_s$) TREATED WITH BIOSOLVENT IN A SIMULATED OIL SPILL ON SANDY BEACH MICROCOSMS.

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(Received: June 1, 2011 - Accepted: September 13, 2011)

ABSTRACT

Experiments with microcosms of sand corresponding to high, mid and low intertidal area were contaminated with crude oil and treated with biosolvent. Two treatments were done. The first (treatment 1) treating the sand 5 days before the event of contamination and the second (treatment 2) treated immediately after the contamination. The degradation of total TPH after 90 days ranged from 44 to 78%. However, the first 10 days were very important in terms of degradation and it slowed down significantly until 90 days. The degradation rate for light fractions in treatment 1 was slightly slower than treatment 2. On the other hand, for heavier fractions the degradation rate of heavier fractions in treatment 1 was at least twice as faster than treatment 2. Implicating that pre-treating the samples with biosolvent some days before the event of contamination may accelerate the degradation process of heavier fraction that are more difficult to degrade. The biosolvent used was degraded in a range from 55 to 100% showing that the biosolvent employed was biodegradable.

INTRODUCTION

The increasing demand for energy has increased the use of petroleum based hydrocarbons. Due to this, transportation of crude oil in ships with enormous capacity is needed to carry this product from country to country. Some of the risks involved with such transportation include catastrophic accidents which may lead to a major environmental impact, especially if this occurs near to coastal areas; these zones are sensitive to external pressures. Most of the best known disasters were after the accidental release of tons of crude oil to the marine environment. Such incidents have included the Prestige in the coastal area of Galicia, the Exxon Valdez in Alaska and the most recent in the Gulf of Mexico.

When an oil spill occurs there are social–political implications due to the fact that the oil is visible in the shoreline and many coastal areas have important economical and cultural value[1]. In addition, shoreline and intertidal ecosystems are complex and susceptible to impacts both from oiling and response operations. If a spilled chemical is not removed or treated, it may take several years to be degraded by natural processes. Traces of oil components have been detected several years after an oil spill [2].

When an offshore spill of oil reaches the coastal area, one of the primary solutions is the physical removal of oil mixed with contaminated sand. Once on shore, cleanup operations become complex, expensive and time consuming [3]. As much as 80–90% of the cleanup costs of a major spill are attributable to shoreline cleanup [1]. Unfortunately, oil spills frequently reach shorelines and other environmentally sensitive areas and by then, the oil is usually several days old and weathered; it is usually thick, sticky, highly absorbed on rocks and organisms, often emulsified and frequently difficult to remove by physical methods [3].

Within the methods to clean contaminated sediments the use of chemical cleaners are very frequent. Chemical cleaners that are often employed are organic solvents with or without surfactants, these agents act emulsifying the oil dissolving it to then be diluted by the water [4]. These cleaners provoke a dispersion of the oil to clean areas where the contaminant did not reach initially, increasing the area affected and allowing the oil to be transported deeper in sediments. It is also known that these agents can be toxic and in most cases make the impact of an oil spill even worse [5, 6]. Other treatments involved are the use of pressurised hot water or bioremediation by local microbial activity induced by nutrients [7].

The contaminant distribution and the effect of removal will depend on the place within the intertidal area. Low and middle sediments are affected by surf washing and chemically by the use of chemical cleaners. However, high intertidal areas are reached by waves only under specific conditions such as storms or high tide (spring tides), so physical removal by waves is not the predominant way of taking away contaminants.

Biosolvents based on Fatty Acid Methyl Esters (FAMEs) dissolve the crude oil and its components forming a complex that has less density than water, the capacity to dissolve oil appears to be dependent on the type of biosolvent used,

the ratio of biosolvent to oil employed and the type of substrate cleaned [3, 8, 9]. The main effects that biosolvent produce on crude oil and derivatives are:

1 dissolve the crude oil reducing its viscosity,
2 extract the oil and reduces its adherence to coastal areas,
3 reduce the viscosity of weathered oil,
4 breaks oil emulsions,
5 form an aggregated complex with oil that floats on the water allowing to retrieve it for example using skimmers,
6 increase the biodegradation of crude oil by assisting bacteria co-metabolism.

The laboratory experiments have given important information about the possible uses of FAMEs to clean contaminated areas after an oil spill. Considering these results, biosolvent effectiveness could be improved in the natural environment particularly in exposed beaches with strong wave action. Several ways of application, amounts, different sources of biosolvent in micro and mesocosms have been tested[3]. Results have demonstrated the effectiveness and potential uses of this biosolvent in the removal and the enhancement on the biodegradation of crude oils.

Biodegradation of FAMEs in the marine environment is expected to be within 4 to 28 days [10]. Experiments demonstrated that FAMEs were degraded at roughly the same rate as *n*-alkanes, and more rapidly than other hydrocarbon components[11]. It has also been demonstrated that FAMEs has a synergistic effect on the degradation of hydrocarbons by bacteria, demonstrating that FAMEs enhance the biodegradability of both diesel fuel and gasoline by means of co-metabolism [12].

When crude oil is spilled at sea, a number of weathering processes cause changes in the physical and chemical properties of the crude oil and in its behaviour at sea. The factors that are comprised in the term "weathering" are the changes that the spilled oil undergoes since its exposure to the environment.

The aim of this research was to simulate and study the progress of the degradation of crude oil through the quantification of total petroleum hydrocarbons (TPHs) on artificial sandy beaches treated with biosolvent based on FAMEs in three different areas on the intertidal zone (high, mid and low). A further objective is to determine if there are any differences between the degradation rates for areas treated immediately or pre-treated with biosolvent prior to oil coming from offshore arrives to the beach.

MATERIAL AND METHODS

The crude oil employed was provided by the national petrol company "ENAP". The type of crude oil was "*caño limon*". Due to the original oil was too light it was left for 35 days exposed to the atmosphere. After this process the oil lost about a 5% of its original weight, principally corresponding to light hydrocarbons and other volatile components (*e.g.* BETXs).

The biosolvent applied was synthesized in the laboratory through the esterification of fatty acids from natural sources with methanol in presence of sodium hydroxide at 50°C [13].

The sand employed for the experiments was collected from a local beach (Curiñanco Beach, 39°40´48.07´´ S; 73°21´56.90´´ W) that is remote from urbanised areas and considered less contaminated with petroleum hydrocarbons than beaches close to industrial or urban areas. The sand collected had a pH in the range of 7-8. The grain was not determined; however the samples of sand can be visually categorised as fine sands. The sand was collected in three boxes from three different locations on the intertidal zone (low, mid and high). The sand was taken from the first 5 cm of depth during low tide conditions (0.87 m). Before using the sand, it was cleaned by hand from major particles and major living organisms. The water content for each sample was determined by drying it for two days at 40°C up to constant weight; the water percentage was 3.0, 4.3 and 36.2% for high, mid and low intertidal areas respectively.

EXPERIMENTS

To perform the experiments, the sand from each intertidal area (600 grams) was placed in aluminum trays (12x26x4cm). The first experiment (treatment 1) was performed adding 30 ml of biosolvent to the samples taken in the three intertidal areas of the beach 5 days before contaminating the sand with 6 mL of crude oil. The second (treatment 2) the 30 mL of biosolvent were added immediately after 6 mL of crude oil contaminated the sand for the three intertidal areas. The proportion of oil to biosolvent for both experiments was 1:5. In a real situation the proportion of oil to biosolvent should be 1:1 or less, however we used the proportion 1:5 in order to be sure that most of the FAMEs in treatment 1 after 5 days can be present when the oil arrives to the shore despite of chemical, physical or biological degradation.

The samples were stirred everyday with a glass stick to homogenise the systems. Due to the oil was weathered it tended to be very sticky, and in order to ensure that samples were representative of the system, they were taken from the each corner plus the central point up to complete 5 grams. However, no experiments were done to test the homogeneity of the microcosms.

Control experiments were performed in parallel with a mixture of sands from the three different intertidal areas, the same mixture with sand only and other with 6 mL of crude oil (crude oil control).

The experiments were performed over 90 days. Within this period the initial water content was maintained by adding water regularly for the low tide sample, intermediate for the mid tide and no water for the high tide samples. The water added was not removed from the system, keeping the oil and biosolvent in the system to represent an extreme case where no oil was removed by tides. The first 5 days the water added was a solution made from 10% of sea salt and then only distilled water to avoid salt saturation.

No replicates were performed for each treatment. However, the variability of the samples (n=6) at time 0 for all the experiments performed showed a standard deviation of 15%. Consequently, this value was assumed as a standard error in all the determinations performed.

Sample extraction and preparation

Five grams of sample were taken at 0, 5, 10, 20, 30, 45, 60, 75 and 90 days. The samples were placed in glass flasks and then 2 grams of sodium sulphate anhydrous were added to absorb the residual water, then 50 mL of a mixture 1:1 of hexane: dichloromethane was added to extract the FAMES and petroleum hydrocarbons.

The extraction procedure was assisted by ultrasonic bath for 30 minutes at room temperature. After the extraction, the extracts were filtered through a filter paper (Whatman 41) containing sodium sulphate anhydrous. The extracts were rotoevaporated and concentrated up to approximately 2 mL and kept refrigerated at 4°C prior analysis.

Before the analysis, all samples were filled up to 2 mL and an aliquot of 10 µL of the sample was added to a clean vial, containing 100 µL of the internal standard (1-chlorooctadecane 5µg/mL⁻¹, SUPELCO) and filled up to 2 mL with a mixture 1:1 of hexane: dichloromethane.

Sample analysis

The analysis of the samples for total petroleum hydrocarbons (TPHs) and FAMEs was performed simultaneously using a gas chromatograph (FOCUS GC, Thermo Scientific) coupled to a mass detector (DSQ II, Thermo Scientific). One µL of sample was injected with the following conditions: The program temperature used was 70°C held for 4 minutes, then a ramp up to 300°C at a rate of 6°C min⁻¹, then maintained for 18 minutes at 300°C. The transfer line was kept up to 250°C, the carrier gas flow was 1.5 mL min⁻¹ and the injector temperature was 250°C. The injection was done with a split ratio of 10. The ionization energy was 70 eV. The column employed was a Rtx®-5MS (RESTEK, USA) 30 m long, 0.25 mm ID, 0.25 µm of film thickness. The samples were run in a scan mode from 40 to 450 m/z.

Identification and quantification

The identification and quantification of hydrocarbons was performed using a standard of petroleum hydrocarbons from n-C$_{10}$-C$_{40}$ even and uneven plus pristine and phytane (S-4110-100-CY) and the internal standard 1-chlorooctadecane (Supelco 10.000 µg mL⁻¹ in CH$_2$Cl$_2$). The identification and quantification of FAMEs was performed using a standard (Supelco 37 compounds FAME mix 10 mg mL⁻¹ in CH$_2$Cl$_2$).

RESULTS AND DISCUSSION

Weathering Index (WI)

The weathering index was calculated for all the samples. It can be used to describe the weathering process and to evaluate the degree of loss including physical weathering of the studied samples. The weathering index formula was taken from Mudge and Muller, 1997 [9] and modified due to the detection and quantification of hydrocarbons lower than 10 carbon atoms was not possible due to a quick degradation in most of the samples after 10 days, the formula employed was the follow:

$$WI = (n\text{-}C_{12} + n\text{-}C_{14} + n\text{-}C_{16} + n\text{-}C_{18}) / (n\text{-}C_{22} + n\text{-}C_{24} + n\text{-}C_{26} + n\text{-}C_{28})$$

This formula represents the loss of low-molecular weight n-alkanes and relative increase in the concentration of less volatile high-molecular weight n-alkanes[14]. Values at time "0" (just before the oil spill occurs) should be high and decrease in number while evaporation and degradation affects the light components of the crude oil preferentially. However, the oil employed for the experiments had a previous weathering process (evaporation of light fractions) before using it to contaminate the sands (35 days exposed to the atmosphere). This procedure is done to avoid report evaporation as degradation (chemical or biological) during the degradation process.

The values were calculated for all the experiments and compared with the control of crude oil without biosolvent (see table 1). The value calculated for the control of crude oil after 90 days was 16.7% (This value was calculated with the weathering index value at day "0" and day "90" according to the following formula W% = ((WI$_{day\ 0}$ - WI$_{day\ 90}$)/WI$_{day\ 0}$)*100). This value is lower than the values calculated for the rest of the experiments. Consequently, values higher than this indicate a quicker degradation of the light fractions over heavier fractions, demonstrating not only evaporation as well biodegradation of the hydrocarbons.

Values for high and low tides were similar for both treatments. However, the values for mid tide for treatment 2 were at least twice the value of treatment 1. A possible explanation, as discussed later on, might be that for both treatments the degradation of light hydrocarbons was similar. However, with previous application of biosolvent the degradation of heavier fractions was higher than with immediate application. That implies that when the calculation of weathering index is done the values for treatment 1 will be at least half of the values of treatment 2. Nevertheless, these values are only an indicative of the degradation process and is important to consider the whole suit of hydrocarbons detected in order to make stronger conclusions.

Table 1. Weathering index (WI) for the samples and the control of crude oil.

	Days				
	0	**30**	**60**	**90**	
		WI			**WI%[3]**
Control Crude oil	3.60	3.32	3.62	3.00	**16.7**
High[1]	3.25	1.26	1.38	0.85	**73.8**
Middle[1]	3.40	2.99	2.60	1.83	**46.2**
Low[1]	3.67	1.86	1.89	1.15	**68.6**
High[2]	3.25	1.11	1.00	0.91	**71.8**
Middle[2]	3.40	1.65	0.80	0.43	**87.3**
Low[2]	3.67	1.12	1.18	1.49	**59.4**

[1]: Treatment 1. Sand pre-treated with biosolvent 5 days before contamination with crude oil

[2]: Treatment 2. Sand treated with biosolvent immediately after the contamination with crude oil

[3]: This value was calculated with the weathering index value at day "0" and day "90" according to the following formula W% = ((WI$_{day\ 0}$ - WI$_{day\ 90}$)/WI$_{day\ 0}$)*100

Total Petroleum Pydrocarbons (TPHs)

The total petroleum hydrocarbons (TPHs) were determined considering the sum of linear hydrocarbons from n-C_{10} to n-C_{31}. Longer chain hydrocarbons were not resolved with the chromatographic conditions employed. The sand samples were analysed for TPHs before initiating the experiments. The results showed concentrations of 13.0, 13.1 and 20.4 µg Kg^{-1} for high, mid and low intertidal areas respectively. These results demonstrated that the presence of petroleum hydrocarbons was much smaller than the level of crude oil added to perform the experiments in the order of mg Kg^{-1}.

Results showed that the attenuation of Total Petroleum hydrocarbons (TPH_s) in the control of crude oil was small, with TPH concentrations of 717, 616, 728 and 584 mg Kg^{-1} for 0, 30, 60 and 90 days respectively. That represents about an 18.5% of loss after 90 days, which is almost coincident with the WI% value of 16.7%. This value principally represents the loss of light hydrocarbons by evaporation and probably none or little biodegradation occurred during this period with the control of crude oil.

For both treatments and for the different intertidal areas studied the linear hydrocarbons were degraded through the time in different proportions (see figure 1a and 1b). The degradation can be attributed to both, biological and physical degradation. The degradation percentage of TPH for treatment 1 for high, mid and low tide sediments were 53±6, 66.7±8 and 78.5±9 % respectively. The degradation of TPH for treatment 2 for high, mid and low tide sediments were 67±8, 72±8 and 43±5 %. Results showed that the degradation of total TPH in all samples was higher than the degradation of the control of crude oil after 90 days (18.5%), implicating that biosolvent added to the samples previous or during the event of contamination facilitated the degradation process. The results also showed that the degradation of TPH with samples pre-treated with biosolvent was higher than the samples treated immediately for low intertidal area. That can be explained by an increment in microbial population due to biosolvent act as a source of carbon increasing the biomass that is able to biodegrade both the biosolvent and the petroleum hydrocarbons and/or facilitating the co-metabolism of the later [14, 15].

According to figure 1a and 1b no significant differences were found in both treatments regarding the different intertidal areas studied. The average degradation for all intertidal areas after 90 days for treatment 1 was 66±8% and for treatment 2 was 61±7% indicating that the overall degradation process is slightly higher for samples with previous application. However, as seen in figure 1c the overall kinetic for all intertidal areas for both treatments seems to be practically the same.

The degradation percentage for treatment 1 (figure 1a) for all intertidal areas studied were: for high tide was about a 41% in 10 days, to then slowed down for another 12% until 90 days. For mid tide was 65% in 10 days and a 2% until 90 days. For high tide was 61% in 10 days and an 18% until 90 days. The degradation percentage for treatment 2 (figure 1b) for high tide was about a 57% in 10 days, to then slowed down for another 10% until 90 days. For medium tide the degradation percentage was 67% in 10 days and a 5% until 90 days. For high tide the degradation percentage was 36% in 10 days and a 7% until 90 days. This shows that the first 10 days are very important in terms of degradation and it slowed down significantly until 90 days. This situation can be very different if tidal effect is considered, due to part of the oil-biosolvent complex is washed to the sea for recovery or left for natural degradation.

1a   1b

1c
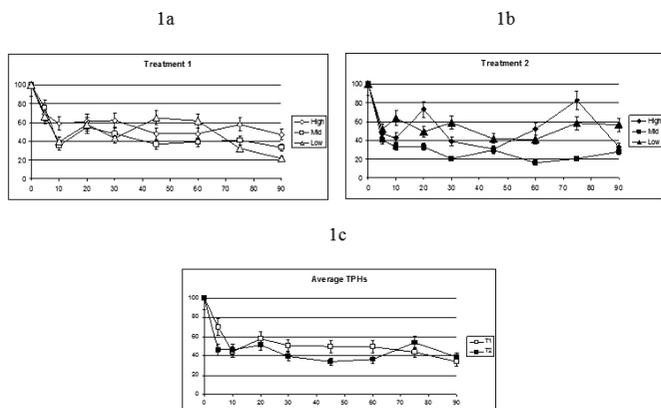
Figure 1. % of remaining THPs in the systems against the time (days) of treatment for 1a: Treatment 1; 1b: Treatment 2 and 1c: Average of remaining TPHs for all intertidal areas for each treatment.

The principal component analysis (PCA) for both treatments (Figure 2) showed two groups that are comprised by light (n-C_{10} to n-C_{15}) and heavy hydrocarbons (n-C_{16} to nC_{31}); these grouping can be attributed to the faster degradation of light hydrocarbons over the heavier fractions[16] as seen in figures 3a and 3b. Under this criterion a further data analysis was performed to see the differences in degradation for those groups within treatments.

As seen in figure 3a the degradation rate for light fractions in treatment 1 was slightly slower than treatment 2. On the other hand, for heavier fractions the degradation rate of heavier fractions in treatment 1 was at least twice as faster than treatment 2 (see figure 3b). Implicating that pre-treating the samples with biosolvent some days before the event of contamination may accelerate the degradation process of heavier fraction that are more difficult to degrade. The reasons for this behaviour are largely unknown and have hardly been explored. Some hypotheses are that (a) the additional carbon source increases the population size, (b) a large mass of cometabolising cells is produced, (c) growth factors are excreted by the population acting on the second compound, (d) the microbial enzymes necessary for the degradation of the first compound are produced [12].

We hypothesized that this difference might be caused by an increment in the biomass and the facilitation of hydrocarbon uptake by the microbial population due to the chemical composition of biosolvent is similar to the biosurfactants produced by cells to incorporate and use fractions that are more hydrophobic such as high molecular weight hydrocarbons [16].
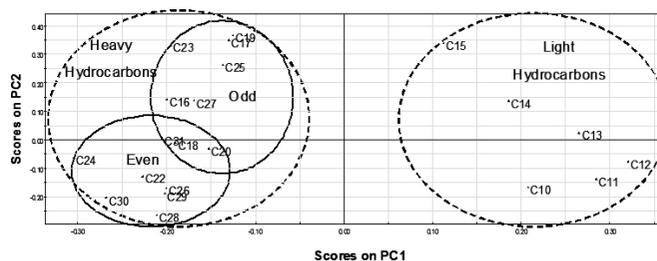
Figure 2. Score plot of TPHs for both treatments representing the different grouping during the degradation process.

3a   3b
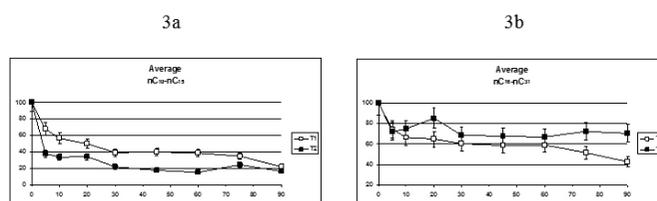
Figure 3. Average % of remaining THPs in the systems against the time of treatment for 2a: Average % for light hydrocarbons (n-C_{10-15}) for treatment 1 and 2. 2b: Average % for heavy hydrocarbons (n-C_{16-31}) for treatment 1 and 2.

Biosolvent

The main fames detected from the synthesis of biosolvent were: tetradecanoic acid methyl ester (14:0Me), hexadecanoic acid methyl ester (16:0Me), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (18:2n6Me), 9-octadecenoic acid (Z)-, methyl ester (18:1n9Me), octadecanoic acid methyl ester (18:0Me), eicosanoic acid methyl ester, (20:0Me), docosanoic acid methyl ester (22:0Me) and tetracosanoic acid methyl ester (24:0Me). With proportions in the mixture of 0.02, 5.92, **39.81**, **51.63**, 2.22, 0.10, 0.24 and 0.06% respectively.

All FAMES were degraded during the 90 days of experiment with degradation percentages ranging from 55% up to 100%. The degradation products detected were lighter fames such as pentanoic acid methyl ester, octanoic acid methyl ester and some epoxides.

The results of FAMES for all samples were analysed using principal component analysis (PCA) to detect any group of samples that can be characterised by having similar properties or behaviour. The values of concentration were standardised using proportions. Before plotting the results the values were log transformed to obtain a normal distribution.

The results are shown in figure 4. This figure shows two grouping. Group

1: comprised by samples corresponding to low and mid tide area. This group presented less or non presence of degradation products (especially epoxides), and the degradation rates of FAMEs in general were lower than in group 2. This group can be sub-divided into two groups (A: Treatment 1 and B: Treatment 2), that are different due to the degradation of the main fames such as 16:0Me, 18:2n6Me, 18:1n9Me and 18:0Me degraded in higher proportions than in treatment 1. The degradation process of these samples can be attributed principally due to bacterial degradation as the pattern of degradation followed was polyunsaturated/ monounsaturated/ saturated/ branched FAMES typical of bacterial degradation[17]. Group 2: comprised principally by samples for both treatments taken under high tide conditions. This group was characterised for presenting more degradation products formed due to oxidation processes *e.g.* epoxides. It also shows that the degradation of FAMES increased with the time for both treatments equally.
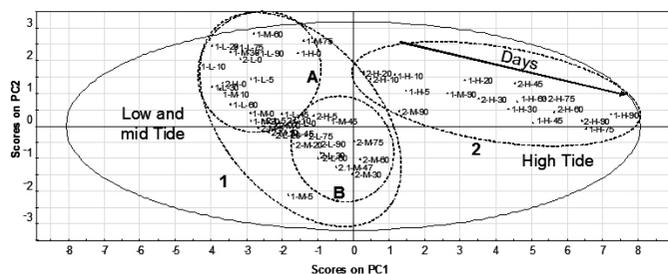
Figure 4. Score plot derived from the PCA analysis for all FAMEs detected in both treatments through the time (the nomenclature used is: treatment-intertidal area-day).

CONCLUSIONS

Results showed that:

The degradation of total TPH_S in all samples was higher than the degradation of the control of crude oil after 90 days (18.5%), implicating that biosolvent added to the samples previous or during the event of contamination contributed with the degradation process.

The first 10 days are very important in terms of degradation and it slowed down significantly until 90 days.

The average degradation for all intertidal areas in both treatments for total petroleum hydrocarbons showed slight differences.

The degradation rate for light fractions in treatment 1 was slightly slower than treatment 2. On the other hand, for heavier fractions the degradation rate of heavier fractions in treatment 1 was at least twice as faster than treatment 2. Implicating that pre-treating the samples with biosolvent some days before the event of contamination may accelerate the degradation process of heavier fraction that are more difficult to degrade.

The FAMEs in the samples were degraded in a range from 55 to 100% showing that the biosolvent employed is biodegradable.

ACKNOWLEDMENTS

REFERENCES

1. Etkin, D. S., Financial Costs of Oil Spills Worldwide. ed.; Arlington, Massachutts, 1998.
2. Owens, E. H.; Taylor, E.; Humphrey, B., Mar. Pollut. Bull. 56, 14, (2008).
3. Pereira, M. G.; Mudge, S. M., Chemosphere 54, 297, (2004).
4. Fuller, C., Bonner, J., Dellamea, S., Ussery, P., Tissot, P., Louchouarn, P. In Proceedings of the Twenty-third Arctic and Marine Oil Spill Programme, Technical Seminar, Environment Canada, Vancouver, 2000; pp. 795-803.
5. Wu, R. S. S., Mar. Environ. Res. 5, 157, (1981).
6. Greenwood, P. J., Aquatic Toxicology 4, 15, (1983).
7. Gallego, J. R.; Fernandez, J. R.; Diez-Sanz, F.; Ordonez, S.; Sastre, H.; Gonzalez-Rojas, E.; Pelaez, A. I.; Sanchez, J., Environ. Eng. Sci. 24, 493, (2007).
8. Fernandez-Alvarez, P.; Vila, J.; Garrido, J. M.; Grifoll, M.; Feijoo, G.; Lema, J. M., J. Hazard. Mater. 147, 914, (2007).
9. Miller, N. J.; Mudge, S. M., Spill Sci. Technol. Bull. 4, 17, (1997).
10. Von Wedel, R., Spill Sci. Technol. Bull. 6, 357, (2000).
11. DeMello, J. A.; Carmichael, C. A.; Peacock, E. E.; Nelson, R. K.; Arey, J. S.; Reddy, C. M., Mar. Pollut. Bull. 54, 894, (2007).
12. Pasqualino, J. C.; Montane, D.; Salvado, J., Biomass & Bioenergy 30, 874, (2006).
13. Zhang, Y.; Dube, M. A.; McLean, D. D.; Kates, M., Biores. Technol. 89, 1, (2003).
14. Mudge, S. M.; Pereira, G., Spill Sci. Technol. Bull. 5, 353, (1999).
15. Owsianiak, M.; Chrzanowski, L.; Szulc, A.; Staniewski, J.; Olszanowski, A.; Olejnik-Schmidt, A. K.; Heipieper, H. J., Biores. Technol. 100, 1497, (2009).
16. Bouchez-Naitali, M.; Vandecasteele, J.-P., World Journal of Microbiology & Biotechnology 24, 1901, (2008).
17. Mfilinge, P. L.; Meziane, T.; Bachok, Z.; Tsuchiya, M., Mar. Ecol.-Prog. Ser. 265, 97, (2003).