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Selection of surfactants for enhancing diesel hydrocarbons-contaminated media bioremediation

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Abstract

The use of surfactants represents a valuable method to enhance the access of the microorganisms to low-soluble and recalcitrant compounds in bioremediation techniques. The choice of surfactants is the first step of feasibility studies for this application. So far, no defined procedures are present in literature to select the most suitable surfactant for the treatment of a specific contaminated site. Furthermore, the characterisation of physico-chemical parameters is important to understand the reason of successes and failures. In this paper a step procedure to select and characterise a commercial surfactant to be used in bioremediation enhancement of hydrocarbon-contaminated media was developed. Among the commercial surfactants, the procedure was applied to alkyl polyethoxylates (Brij family) and sorbitan derivates (Tween family). The selection resulted in the application of Brij 56 and Tween 80 as biodegradation-enhancer in different lab scale systems for remediation of diesel contamination. In liquid systems, Tween 80 greatly increased biodegradation of highly branched and high-molecular weight hydrocarbons, while Brij 56 enhanced degradation of highly branched hydrocarbons. Based on these results, the potential applications and the limitations of these surfactants at full scale level were estimated.

Keywords: Bioremediation; Surfactant; Environmental fate; Diesel hydrocarbon; Decontamination

1. Introduction

Bioremediation techniques could represent an economical and environmental sound tool for managing sites polluted by organic wastes. Application of bioremediation technologies for hydrocarbon-contaminated soil is often limited by presence, within the mixture of contaminants, of high recalcitrant and low bioavailable compounds. Compounds characterised by low solubility and high hydrophobicity tend to be strongly sorbed to organic matrix and to be low available to microorganisms for degradation. It has been demonstrated that slow release of these compounds from soil and free phase to water phase could represent a rate-limiting factor for bioremediation processes, leading to impossibility in reaching the target of remediation [1,2]. Extent of limitation depends on pollutant characteristics like K_{oc} value and on soil parameters like clay content, Cation Exchange Capacity (CEC) and, in particular, organic matter. Furthermore, biodegradation of complex mixtures of hydrocarbons is often characterised by preferential degradation of linear and low-molecular weight hydrocarbons leading to accumulation of recalcitrant compounds [3].

For these reasons, suitable methods to overcome metabolic and bioavailability drawbacks are needed to successfully remediate contaminated wastes. To enhance bioavailability of contaminants, surfactants may be useful due to their capability to increase water solubility and mass transfer. Many applications of surfactants are reported in literature for remediation of hydrocarbon-contaminated soil both in solid and slurry systems [4–7]. Before application, an environmental fate assessment of surfactant is strictly recommended to avoid unacceptable accumulation of surfactant in soil system [8].

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A key step in evaluation of applicability of a commercial surfactant in soil remediation is the choice of surfactant. So far, no defined procedures are present in literature for the selection of the most suitable surfactant for the remediation of a specific contaminated site.

In this work, the selection of a surfactant to enhance diesel hydrocarbon biodegradation was carried out by a step procedure considering: (i) the physico-chemical properties of surfactants; (ii) the biodegradability of surfactants; and (iii) the influence of the surfactants on the biodegradation rate and yield of contaminants in different systems. Among the commercial surfactants, non-ionic surfactants are considered less toxic and more biodegradable than anionic and cationic ones [9]; furthermore, among non-ionic surfactants, the use of alkyl phenol polyethoxylates is discouraged because their biodegradation leads to formation of alkyl phenol, more toxic and persistent than parent compounds [10]. For this reason, the selection of surfactant was carried out among alkyl polyethoxylates (Brij family) and sorbitan derivates (Tween family).

The first phase of selection estimated the environmental fate and toxicity of a wide group of surfactants belonging to the cited families to reduce the number of surfactants for laboratory characterisation. For this purpose, K_{oc} and *Daphnia magna* EC_{50} were estimated by a molecular descriptor and a QSAR model. In the second step, laboratory experiments were conducted to evaluate soil sorption and biodegradability of surfactants and Brij 56 and Tween 80 were selected. In the last part of the research, the effect of these surfactants on biodegradation of diesel fuel was studied in liquid cultures and in slurry and solid phase systems. Liquid phase experiments were carried out using a diesel hydrocarbon-degrading microorganism named Ap. The latter experiments were performed with diesel-contaminated soil in bench scale slurry phase bioreactor and solid phase columns. To assess the effect of selected surfactants on the final composition of residual hydrocarbon mixture, an elaboration of the chromatographic data obtained in the chemical analyses was developed.

2. Materials and methods

2.1. Soil characterisation

In this study soil from a diesel fuel-contaminated site was used. Two kind of soil samples were collected with the same characteristics apart from the presence of contaminants: one in the contaminated zone and one in an uncontaminated part of the site. In the former, hydrocarbons in the mixture were in the range of C_{12}–C_{30} and the concentration was 1700 mg/kg_{dw}. The characterisation of the soil is already reported in a previous work [8] and here summarised. Content of organic carbon of 11.4 g/kg_{dw}; granulometric composition as follows (less than 250 μm: 3%; 250–500 μm: 13%; 500–850 μm: 31%; 850–2000 μm: 53%). Value of Cation Exchange Capacity (CEC) was determined as 7.34 ± 0.01 cmol(+)/kg. Heterotrophic bacteria were determined as 1.5×10^6 CFU/g_{dw}.

2.2. Screening of surfactants by estimation of K_{oc} and toxicity

K_{oc} values of surfactants among alkyl polyethoxylates (Brij family) and sorbitan derivates (Tween family) were evaluated by a modified first-order molecular connectivity index (MCI) [11]. Toxicity of alkyl polyethoxylate surfactants was estimated by a specific QSAR model, that estimated the toxicity of each specific molecule on the basis of the number of ethoxylate groups and the length of alkyl chain [12].

2.3. Measurement of surfactants

Brij surfactants were analysed by CTAS method [13]. Briefly, non-ionic surfactants react with aqueous cobalt thiocyanate to give a cobalt-containing product. The concentration of this product, extracted in an organic liquid, is measured spectrophotometrically.

2.4. Surfactant sorption experiments

Surfactant sorption experiments were conducted preparing 100 ml Erlenmeyer flasks filled with 1 or 2 g of soil sample (previously sterilised by addition of sodium azide) and 10 ml of saline surfactant–water solution (NaCl 1 g/l). Five flasks at different concentrations were prepared for each surfactant. Initial concentrations of surfactants were 0.5, 1, 2, 3, 4 mg/l. Flasks were left at 20 °C and 100 rpm for 24 h to reach sorption equilibrium (equilibrium time was determined by preliminary tests) and then centrifuged at 8100 rpm for 10 min. Supernatant solution was analysed for surfactant concentration as described above (Section 2.3) and sorbed concentration was calculated by difference.

2.5. Biodegradability of surfactants

Biodegradability of the surfactants was assessed by respirometric tests both in liquid culture and solid phase. Experiments were performed in Biochemical Oxygen Demand (BOD) apparatus (VELP Scientifica, Italy) and ratio between actual BOD and theoretical BOD (BOD_{Th}) was calculated over time. For experiments in liquid cultures, bottles were filled with 125 ml surfactant solutions in mineral bacteriological medium M9 [14], and 2 ml of soil bacteria inoculum at optical density at 540 nm (OD_{540}) equal to 1, as an indicator of bacterial biomass concentration. Bacterial inoculum was obtained as previously described [8]. Different concentrations of surfactants (500, 250, 100, 50 mg/l) were tested to evaluate the biodegradability in different conditions. To evaluate the biodegradation of surfactants in presence of soil bacteria and of surfactant sorption on soil, solid phase respirometric test were carried out. Bottles were filled with 5 g of soil and 2 ml of saline (NaCl 1 g/l) surfactant water solution to reach desired surfactant concentration in soil. Initial concentrations of surfactant in soil were 0.5 and 2 g/kg. Other bottles without surfactant were assembled to measure basal activity of inoculated bacteria. Bottles were incubated at 20 °C. BOD was calculated by

difference between oxygen consumption in each bottle and basal activity.

2.6. Biodegradation experiments for contaminants

To evaluate influence of selected surfactants addition on biodegradation of diesel fuel, kinetic experiments were designed in liquid, slurry and solid phase.

2.6.1. Diesel-degrading inoculum

A Gram-positive diesel fuel-degrading bacterium, named Ap, was isolated by enrichment cultures from the same diesel-contaminated soil used in this experimentation using commercial diesel fuel as carbon source. Liquid cultures were prepared in LD rich medium [14]. The cultures were incubated in a rotary shaker at 30 °C. The cells were removed by centrifugation, washed twice and resuspended in M9 medium to obtain desiderate OD_{540}.

2.6.2. Liquid phase experiments

Liquid phase kinetic experiments were conducted by preparing five 100 ml Erlenmeyer flasks (one for each kinetic time) filled with 20 ml liquid culture of diesel hydrocarbon-degrading bacterium strain Ap at OD_{540} equal to 1 and diesel hydrocarbon concentration equal to 1 g/l. Experiments with 1 g/l of Brij 56 and Brij 76 were carried out and compared with a control experiment without surfactant. The cultures were incubated in a rotary shaker at 30 °C and, at fixed times, OD_{540} was measured; one flask was sacrificed to determine the residual diesel hydrocarbon concentration.

2.6.3. Slurry phase kinetic experiments

Slurry phase experiments were conducted with different additions of surfactants and inoculum. When added, initial surfactant concentration was 1.73 g/l for Brij 56, 4 g/l for Tween 80 and initial optical density of inoculum was equal to 1. The slurry reactor consisted of a 1000 ml glass vessel equipped with a thermostatic jacket, temperature maintained at 30 °C, and the agitation rate controlled by a stirring velocity regulator set at 200 rpm. Oxygenation was provided, through a porous air-diffuser, by injection of 50 ml/min oxygen–nitrogen mixture (20% and 80% in volume, respectively). At the outlet, the outgoing flow passed through a bottle containing 100 ml of 0.1 M NaOH solution to trap the carbon dioxide. In the reactor 200 g of contaminated soil (initial concentration of diesel hydrocarbon = 1700 mg/kg) were mixed with M9 mineral medium (weight:volume ratio = 1:3). At fixed times 3 ml samples were collected from the slurry reactor and centrifuged. Residual diesel hydrocarbon concentration (measured in duplicate) and total aerobic heterotrophic bacteria contents were determined; the NaOH trap was replaced and analysed for CO_2 content by titration.

2.6.4. Solid phase experiments

The system consisted of a glass column filled with 400 g of contaminated soil (initial concentration of diesel hydrocarbon = 1700 mg/kg) maintained at room temperature, with a 20 ml/min injection, from the bottom to the top, of moist CO_2-free air. The outgoing flow passed through two bottles filled with 100 ml of a 0.1 M NaOH solution to trap carbon dioxide. The soil was amended with NH_4Cl (745 mg/kg) and KH_2PO_4 (470 mg/kg) as source of inorganic nutrients to avoid nutrient limitation. In surfactant-added experiments the initial concentration of Brij 56 and Tween 80 was 0.5 and 1.0 g/kg, respectively. The surfactant was added dissolved in water solution. In experiments with inoculum the Ap strain culture was added in physiological solution to a final value of diesel hydrocarbon-degrading bacteria of 2.0×10^7 MPN/g_{dw}.

At fixed times 1 g soil was collected from the column evaluate diesel hydrocarbon concentration (measured in duplicate) and heterotrophic bacteria, and to verify that the moisture content remained constant; traps were replaced and analysed for CO_2 content by titration. CO_2 data can be used to evaluate mineralisation yield of contaminants and surfactants.

2.6.5. Microbiological analyses

The slurry and soil samples were serially diluted in mineral M9 medium until approximately 10^{-7}. The 0.1 ml parts of diluted solutions were pipetted into 10 ml three-tube MPN system. The tubes were incubated at 30 °C for 48 h for total heterotrophic bacteria and 10 days for diesel hydrocarbon-degrading bacteria. Each tube was considered positive if visible growth was detected after incubation. The MPN value per ml of slurry or g of soil was then determined with the suitable table. The media used were LD rich medium for heterotrophic bacteria evaluation and mineral M9 medium with diesel fuel (1 g/l) added as the only carbon source for the hydrocarbon degrading-bacteria count.

2.6.6. Diesel hydrocarbon quantification

Liquid–liquid extraction was carried out for liquid phase samples using 20 ml of *n*-hexane. Centrifuged slurry and soil samples were added to 30 ml *n*-hexane. The bottle was sealed with a teflon stopper and held for 10 min in an ultrasonic bath at 47 kHz frequency; the extract was filtered on anhydrous sodium sulphate (CODEX Carlo Erba, 97%) to remove residual water, dried, dissolved in hexane, analysed and quantified by external calibration (baseline–baseline integration).

The analyses were performed with a HP 5890 gas chromatograph coupled to a FID detector with DB5 column (30 m length, 0.25 mm i.d., 0.25 μm film thickness). The temperature program was 2 min at 40 °C, then 12 °C/min to 320 °C, and 15 min at 320 °C. Injector and detector temperatures were set to 280 and 320 °C, respectively. For peak identification of the diesel fuel components, standard solutions of *n*-alkanes (from *n*-C_{12} to *n*-C_{20}) and phytane, as a compound representative of branched aliphatic hydrocarbons in diesel fuel, were also analysed.

2.7. Analysis of chromatographic profiles

To better investigate the biodegradation of the hydrocarbon mixture, the chromatographic profiles of residual hydrocarbon at different times were compared. Reduction in peak height of *n*-alkanes and phytane were calculated. Furthermore, an analysis

was developed to semiquantitatively evaluate the preferential degradation of light hydrocarbons by microorganisms. Chromatographic signals were exported to a spreadsheet format (MS Excel), background signals were subtracted and the obtained data were elaborated as follows. The sum of the peak areas of different diesel fuel constituents within 2 min-intervals of retention time were calculated and the percentage of these values with respect to the total signal was computed for each interval. In the bar-graph that represents the computed data, each bar indicated the fraction of the hydrocarbon mixture eluted in each retention time interval. Roughly assuming a correlation between chromatographic retention time and molecular weight of hydrocarbons, bar-graphs at longer kinetic times were characterised by a relative enrichment of high-molecular weight hydrocarbons and a relative decreasing of low-molecular weight hydrocarbons. In fact, at the end of the kinetic study, the percentage of signals was lower than at the beginning for the first retention time-intervals while it was higher for the last intervals. Each experiment was so characterised by a specific retention time at which the shift between the relative decreasing and the relative enrichment of signal occurred. This time can be considered proportional to the ability of the system to degrade high-molecular weight hydrocarbons in diesel fuel mixture.

3. Results and discussion

3.1. Screening of surfactants

Environmental fate of surfactants in soil systems is affected both by chemical–physical properties of the molecules and by the characteristics of the soil. The sorption characteristics, the biodegradability of the surfactants are the most important properties that affect the mobility and the residence time of the surfactant within the soil. To be effective in biodegradation enhancement and environmental friendly, added surfactant has to be short persistent and low toxic; furthermore, for *in situ* application, surfactant has to be not too affine to soil, allowing to be easily distributed along the soil profile. Among the wide

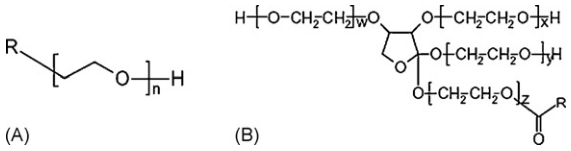

Fig. 1. General formula (A) polyethoxylates (Brij); (B) sorbitan derivates (Tween).

number of commercial surfactants available on the market, the first screening was carried out by the examination of literature information. Non-ionic surfactants are supposed to be less toxic and more biodegradable than anionic and cationic ones. Furthermore, phenol polyethoxylates are known to release toxic intermediates during biodegradation [10]. For these reasons, for this step-procedure selection, the families of alkyl polyethoxylates (Brij) and sorbitan derivates (Tween) were used. In alkyl polyethoxylates family (Fig. 1A), surfactants differ for the length of alkyl chain (*R*) and for the number of ethoxylate groups (*n*) present in the molecule. Molecules of Tween family (Fig. 1B) are derivate esters of sorbitanol.

The first screening of surfactants between the selected Tween and Brij families was carried out by model estimation of soil sorption properties and toxicity. K_{oc} parameter was estimated by a modified MCI index [11], while, for Brij surfactants, log EC_{50} on *D. magna* was calculated by a QSAR model [12]. In Table 1 results of estimation are shown.

As reported above, sorption properties of surfactant affect the possibility of distribution of the surfactant along the soil profile in *in situ* application. For these reasons, among Brij family, only surfactants with intermediate value of log K_{oc} were considered. Molecules with log K_{oc} between 2 and 4 can assure the possibility of spreading the surfactant deeply in the soil, without too high leachability. Among the surfactants with these values, Brij 56 and Brij 76 were selected for laboratory experimentation, having the highest estimated values of EC_{50} on *D. magna*. As K_{oc} values estimated for surfactants of Tween family were all similar (Table 1), Tween 80 was selected for following experimentation, because of its low toxicity and because its environmental fate properties were previously studied [8].

Table 1
Estimation of K_{oc} and log EC_{50} on *D. magna*

Family	Commercial name	Formula[a]	log K_{oc}	log EC50 *D. magna* (μmol/l)
Alkyl polyethoxylates	Brij 35	C12PO23	10.0	nd
Alkyl polyethoxylates	Brij 58	C16PO20	10.0	0.15
Alkyl polyethoxylates	Brij 30	C12PO4	1.0	0.07
Alkyl polyethoxylates	Brij 78	C18PO20	10.0	−0.61
Alkyl polyethoxylates	Brij 98	C18PO20	10.0	−0.61
Alkyl polyethoxylates	Brij 56	C16PO10	2.7	−0.85
Alkyl polyethoxylates	Brij 76	C18PO10	3.2	−1.61
Alkyl polyethoxylates	Brij 52	C16PO2	2.6	−1.65
Alkyl polyethoxylates	Brij 72	C18PO2	3.2	−2.41
Alkyl polyethoxylates	Brij 92	C18PO2	3.2	−2.41
Sorbitan derivates	Tween 20	S PO20 C12	9.3	nd
Sorbitan derivates	Tween 40	S PO20 C16	10.0	nd
Sorbitan derivates	Tween 60	S PO20 C20	10.0	nd
Sorbitan derivates	Tween 80	S PO20 C18	10.0	nd

[a] C: carbon atoms in the alkyl chain; PO: number of ethoxylate groups; S: sorbitanol.

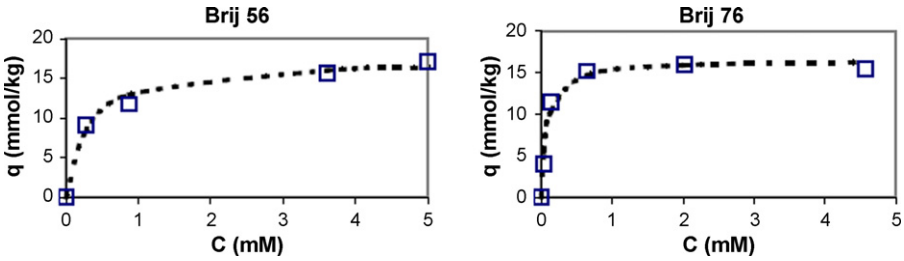

Fig. 2. Experimental data for soil sorption of Brij 56 and Brij 76 and calculated Langmuir isotherms.

Table 2
Model isotherms and estimated parameters

	Langmuir $q = KbC/(1+KC)$		
	K	b	R^2
Brij 56	3.38	17.35	0.96
Brij 76	16.57	12.27	0.96

3.2. Soil sorption

To confirm estimated values and to evaluate selected surfactants on site specific soil samples, soil sorption experiments were carried out with Brij surfactants.

Data from sorption experiments were fitted with common sorption isotherm equations; Brij surfactant data are best fitted by Langmuir equation showing similar behaviour between them, with medium affinity and saturation of sorption sites. In Fig. 2 experimental sorption isotherms of Brij surfactants are shown together with calculated model, while in Table 2 fitted parameters for sorption isotherms of Brij surfactants are reported. This kind of shape, with the saturation of sorption sites leads to a maximum concentration of sorbed surfactants onto soil of around 15 mmol/kg of soil for both surfactants. Further surfactant addition is not sequestered by the soil and can remain active. Thouren et al. [15] and Liu et al. [16] found the same shape of sorption isotherm of different ethoxylated non-ionic surfactants to soil and (un)coated silica.

These surfactants show different behaviour than Tween 80. Using soil with same characteristics, Franzetti et al. [8] found that sorption data of Tween 80 was fitted with Freundlich equation and presented very high affinity and cooperative behaviour, i.e. surfactant molecules showed more affinity for sorbed molecules of surfactant than for soil. That meant that there was not a saturation of the sorption sites. From an applicative point of view a continuous addition leads to a continuous sorption of surfactants. These data confirms estimations calculated for K_{oc} of Tween 80 in Table 1.

Therefore, Brij 56 and Brij 76 present the suitable sorption behaviour for optimal distribution in soil, even at low depths, while Tween 80 can find application in liquid phase and in media with low sorption capacity such as low carbon content soil restoration.

3.3. Biodegradability of surfactants

To evaluate the persistence of surfactants, biodegradability experiments were carried out with Brij surfactants. Table 3 shows the BOD/BOD_{Th} ratios for all tested surfactant concentrations for liquid and solid phase experiments after 10 and 26 days of experiment, respectively.

In liquid phase respirometric tests, BOD/BOD_{Th} ratio reached values up to 35% already after 5 days of experiment for Brij 56; the lag time (the initial time without any degradation) was proportional to concentration of surfactant (from 3 to 7 days), probably due to a toxic effect of surfactant. For Brij 76, the BOD/BOD_{Th} ratio did not reach the normal "plateau" phase, due to the long lag phases (more than 8 days) that occurred for all the tested concentrations.

In soil respirometric tests, no degradation was observed for Brij 76, while for Brij 56, value of 35% of BOD/BOD_{Th} was reached after 15 days only when the surfactant was added with a concentration of 0.5 g/kg; at the highest concentration (2 g/kg), no degradation was observed. In Tween 80 experiments in 14 days BOD/BOD_{Th} reached 30% at the concentration of 0.5 g/kg and 23% at the concentration of 1 g/kg [8].

Summarising the biodegradability results, Tween 80 is more biodegradable than selected Brij surfactants, both in liquid and in solid phase [8]. Among selected Brij surfactants, Brij 56 seems to be more biodegradable in both systems than Brij 76. On the basis

Table 3
Results of respirometric test for Brij 56 and Brij 76 in liquid and in solid phase

Liquid phase			Solid phase		
Surfactant concentration (mg/l)	% BOD/BOD_{Th} after 10 days		Surfactant concentration (g/kg)	% BOD/BOD_{Th} after 26 days	
	Brij 56	Brij 76		Brij 56	Brij 76
50	29	35	0.5	35	4
100	35	29			
250	32	27	2	3	4
500	33	29			

Table 4
Degradation of hydrocarbons after 7 days in Ap liquid cultures

Experiment	Mean residual *n*-alkanes (%)	Residual phytane (%)	Residual total hydrocarbon (%)
Ap	9.4	83	37
Ap + Brij 56	0	22	36
Ap + Tween 80	0	11	9

of these considerations enhanced-biodegradation experiments on diesel hydrocarbons were carried out with Brij 56 and Tween 80.

3.4. Biodegradation experiments for diesel hydrocarbons

After characterisation of surfactants, we evaluated influence of Tween 80 and Brij 56 on biodegradation of diesel fuel in liquid, slurry and solid systems. Experiments were carried out with the addition of a specialised diesel hydrocarbon-degrading bacterium isolated from the same site. We used a Gram-positive strain Ap, able to grow on diesel fuel as sole carbon and energy source. Furthermore, after proper time intervals, the analysis of gas-chromatogram of residual hydrocarbon was carried out.

For liquid phase experiments, results of analyses of *n*-alkanes and phytane are reported in Table 4 as percentage of residual hydrocarbons after 7 days. Addition of Tween 80 results in the higher enhancement of degradation of both linear and branched hydrocarbons even with high molecular weight. This capability of Tween 80 to stimulate the degradation of the whole range of hydrocarbons in diesel range is confirmed by chromatographic profile analyses. Observing the chromatogram (Fig. 3A) of the final kinetic time (7 days), it is clear that the addition of Tween 80 allowed the selected microorganism to degrade also the unresolved complex mixture (UCM) that is normally present in residual hydrocarbon mixture [3]. On the contrary, the chromatographic profile of the final time sample in the experiments with Brij 56 addition resulted to be very similar to the profile of the sample in the experiments with only Ap strain. Furthermore, Excel-exported signals (Fig. 3B) show that in Ap and Ap + Brij 56 experiments there is a relative enrichment of high-molecular weight hydrocarbon in the mixture after 7 days. In fact, the percentage of signal is higher than the beginning of the study for retention time intervals after 12 min. On the contrary, in Ap + Tween 80 experiments, the relative enrichment occurs after 18 min confirming the ability of Tween 80 to stimulate the degradation of high-molecular weight hydrocarbons.

To assess effect of surfactant addition on diesel hydrocarbon degradation in presence of soil and the soil complex bacterial community, different kinetic experiments were conducted in slurry and solid systems. Experiments were carried out adding to soil Brij 56 or Tween 80 and inoculum and all the results were compared to control experiments. Experiments with the only addition of the surfactants aimed to evaluate the effect of surfactant on degradation capabilities of autochthonous soil bacteria.

In slurry experiments (Table 5), nearly in all treatment conditions, final concentrations of diesel hydrocarbons reached values around 15% of initial concentration after 7 days.

Some differences are evident for initial rate of biodegradation. After 2 days, the addition of both surfactants led to higher degradation both in presence and in absence of inoculum. Particularly, Tween 80 addition allowed to reach a residual hydrocarbon concentration after 2 days of 18% and 25% in absence and in presence of inoculum, respectively. The effect of inoculum addition seems to be less important; after 2 days, the residual concentration of hydrocarbons is 65%. In Table 5 the abundance of total and diesel hydrocarbon-degrading bacteria after 7 days are also reported. It is clear that nearly all tested systems result in a great enhancement of both total and diesel hydrocarbon-degrading bacteria compared with initial values. It is likely that in bioreactor the limiting factor of biodegradation rate is bioavailability of hydrocarbon and addition of further biomass does not affect rate of removal. In Table 5 the shift time, computed by chromatogram analyses (see Section 2.7), is reported showing no significant differences between treatments.

In solid phase system (Table 6) we arranged two experiments, one with addition of Brij 56 and inoculum and the other with Tween 80 and inoculum; control experiments were without addition and with inoculum addition. Neither addition of inoculum nor surfactants seemed to significantly increase degradation of total diesel hydrocarbons. Ap addition was actually effective in degrading *n*-alkanes and phytane, due to its own catabolic capabilities towards this class of compounds. Furthermore, no differences were detected between inoculum plus surfactants amended experiments and only inoculum amended experiments (around 50% of residual concentration after 28 days). The lack-

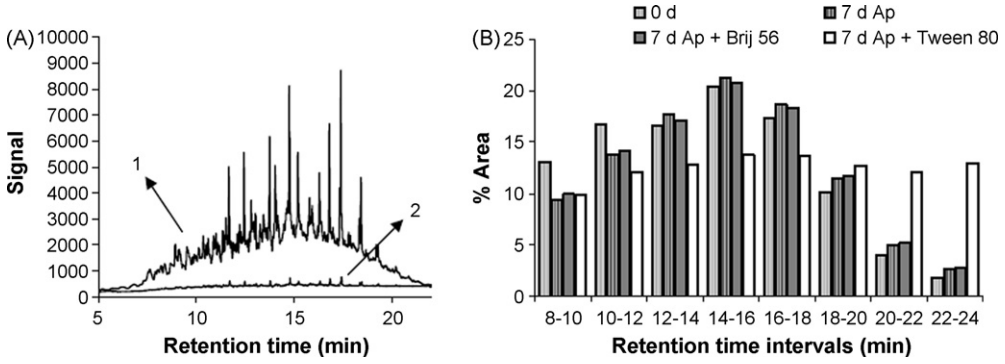

Fig. 3. (A) Chromatograms of liquid cultures at 7 days (1: Ap; 2: Ap + Tween 80) and (B) bar-graphs of chromatogram analysis.

Table 5
Degradation of hydrocarbons and growth of microorganisms after 7 days in slurry experiments

Experiment	Residual hydrocarbons (%), 2 days	Residual hydrocarbons (%), 7 days	Residual alkanes (%), 7 days	Residual phytane (%), 7 days	Total heterotrophic bacteria, 7 days (MPN/ml)	Diesel hydrocarbon-degrading bacteria, 7 days (MPN/ml)	Shift time (min)
Without addition[a]	79	9	0	34	2.4×10^8	2.4×10^7	18
Tween 80[a]	18	8	0	31	4.9×10^9	$>2.4 \times 10^9$	18
Brij 56[a]	33	11	0	35	2.4×10^7	$>2.4 \times 10^7$	18
Ap[b]	65	14	0	37	ND	ND	18
Ap + Tween 80[b]	25	13	0	30	2.0×10^{10}	4.6×10^9	16
Ap + Brij 56[b]	59	37	0	31	2.4×10^{10}	2.3×10^6	16

[a] Initial total heterotrophic bacteria: 2.4×10^7 MPN/ml; initial diesel degrading bacteria: 4.6×10^6 MPN/ml.
[b] Initial total heterotrophic bacteria: 4.6×10^7 MPN/ml; initial diesel degrading bacteria: 4.6×10^7 MPN/ml.

Table 6
Degradation of hydrocarbons and growth of microorganisms after 28 days in soil column experiments

Experiments	Residual hydrocarbons (%), 28 days	Residual alkanes (%), 28 days	Residual phytane (%), 28 days	Total heterotrophic bacteria (MPN/g_{dw})	Diesel hydrocarbon-degrading bacteria (MPN/g_{dw})	Shift time (min)
Without addition[a]	69	59	64	1.7×10^7	1.0×10^2	13
Ap[b]	51	22	47	1.0×10^7	1.0×10^7	16
Ap + Tween 80[b]	57	58	64	4.0×10^{10}	2.0×10^{10}	18
Ap + Brij 56[b]	61	57	77	1.0×10^7	6.3×10^7	18

[a] Initial total heterotrophic bacteria: 2.5×10^6 MPN/g_{dw}; initial diesel degrading bacteria: 1.0×10^2 MPN/g_{dw}.
[b] Initial total heterotrophic bacteria: 4.0×10^8 MPN/g_{dw}; initial diesel degrading bacteria: 2.0×10^7 MPN/g_{dw}.

ing effect of Tween 80 could be due to its high affinity for soil that can lead to low concentration of surfactant in pore water, on the other hand, even Brij 56, which has the suitable sorption behaviour, did not succeed in increasing biodegradation rate in solid systems. The reasons of this behaviour are probably that surfactants do not enhance and sometimes inhibit degradation as already reported [17,18]. This can be due the effect of the surfactant micelles that seize the contaminants into their core or because the surfactant itself can act as competitive source of carbon for bacteria [19]. Data of CO_2 production confirmed that the surfactants added to both solid and slurry phase systems were degraded together with the contaminants. On the other hand, the higher shift times (Table 6) seem to indicate that, even if there is no enhancement in total degradation, the systems with addition of inoculum and surfactants allowed a degradation of higher-molecular weight hydrocarbons compared with the system without addition.

4. Conclusions

Summarising the results of degradation experiments, Tween 80 increased bioavailability and consequently biodegradation rate in liquid and slurry systems. This happened in the systems in which there are high water contents and, probably because of sorption onto soil, had no effect in the experiments with the presence of soil. Tween 80 in particular, if used in liquid systems like hydrocarbon-contaminated liquid waste treatment plant, can be useful in enhancing the biodegradation of diesel range hydrocarbons allowing the degradation of some of highly branched and high-molecular weight hydrocarbons. On the other hand, neither Tween 80 nor Brij 56 showed capability to improve the degradation in soil-containing systems, probably due to different reasons. Tween 80 has high affinity for soil and can be removed from liquid phase by soil, while Brij 56 is probably not suitable as it might increase the solubility of hydrocarbons but reduce their bioavailability [17]. In conclusion, the proposed selection of surfactants for bioremediation application is based both on parameter estimation and on experimental data taking in consideration physico-chemical characteristics, toxicity and effect on biodegradation. This approach allows to find the most suitable surfactants for specific application and can be usefully applied for different classes of surfactants and different soils.

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Glossary

BOD/BOD$_{Th}$: Ratio between the actual Biological Oxygen Demand (BOD) and the theoretical Biological Oxygen Demand (BOD_{Th}) of a compound calculated by the stoichiometric coefficients of oxidation reaction.

Cation Exchange Capacity (CEC): Capacity of a soil for ion exchange of positively charged ions between the soil and the soil solution. It is normally expressed by cmol(+)/kg of soil, i.e. the centimoles of positive charge per kilogram of dry soil.

CFU/g$_{dw}$: Colony Forming Units per gram of soil (dry weight). Unit of measurement for the quantification of microorganisms by plate method on solid medium.

log EC$_{50}$: The logarithm of the half maximal effective concentration. It refers to the concentration of a toxic compound which induces a response halfway between the baseline and maximum. It is commonly used as a measure of toxicity.

MPN: Most Probable Number. Unit of measurement for the quantification of microorganisms by replicate tubes in liquid medium. It could be referred both to volume (MPN/ml) and to dry weight (MPN/g_{dw}) of the sample.

Quantitative structure-activity relationship (QSAR): Process by which the chemical structure of a molecule is quantitatively correlated with a well defined property, such as biological activity or chemical reactivity.

Soil organic carbon-water partitioning coefficient (K_{oc}): Ratio of the mass of a chemical adsorbed on soil per unit mass of soil organic carbon divided by the equilibrium concentration of a chemical in aqueous solution. It is the "distribution coefficient" (K_d) normalized to total organic carbon content of soil.