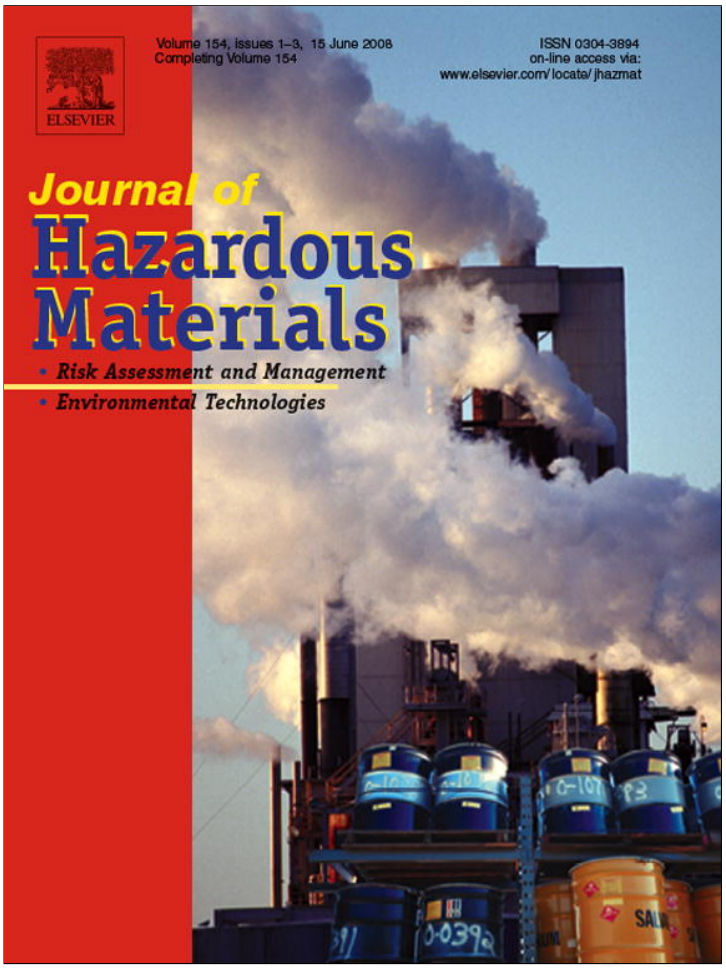

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Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier-producing microbial consortium

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Abstract

The biodegradation of a hazardous waste (bilge waste), a fuel oil-type complex residue from normal ship operations, was studied in a batch bioreactor using a microbial consortium in seawater medium. Experiments with initial concentrations of 0.18 and 0.53% (v/v) of bilge waste were carried out. In order to study the biodegradation kinetics, the mass of *n*-alkanes, resolved hydrocarbons and unresolved complex mixture (UCM) hydrocarbons were assessed by gas chromatography (GC). Emulsification was detected in both experiments, possibly linked to the *n*-alkanes depletion, with differences in emulsification start times and extents according to the initial hydrocarbon concentration. Both facts influenced the hydrocarbon biodegradation kinetics. A sequential biodegradation of *n*-alkanes and UMC was found for the higher hydrocarbon content. Being the former growth associated, while UCM biodegradation was a non-growing process showing enzymatic-type biodegradation kinetics. For the lower hydrocarbon concentration, simultaneous biodegradation of *n*-alkanes and UMC were found before emulsification. Nevertheless, certain UCM biodegradation was observed after the medium emulsification. According to the observed kinetics, three main types of hydrocarbons (*n*-alkanes, biodegradable UCM and recalcitrant UCM) were found adequate to represent the multicomponent substrate (bilge waste) for future modelling of the biodegradation process.

Keywords: Biodegradation; Bilge waste; Fuel oil residue; UCM; Emulsification

1. Introduction

In recent years, various technologies have emerged in order to manage oil residues and effluents contaminated with hydrocarbons. Bioremediation is one of the most extensively used because of its low cost and high efficiency [1,2]. The biodegradation of oil and its derived products has been the focus of many studies. Nevertheless, an important concern still remains due to the toxicity of their constituents [3], the large amounts of effluents, waters and soils that need to be remediated in compliance with environmental standards, and the need to enhance the biodegradation of the more recalcitrant petroleum compounds [4].

Oil and derived product residues are complex mixtures of thousands of compounds, with a high proportion of hydrocarbons, which posses different solubility and microbial resistance to biodegradation [3,5]. Diesel and fuel oils constitute the main fuels for cargo vehicles and ships, and they are generally blends of medium distillate and residual petroleum cuts. Gas chromatographic analysis of these fuels usually shows, in addition to the resolved compounds, an elevation of the baseline which is named unresolved complex mixture (UCM). This portion of the fuel, UCM, is mainly composed by branched and cyclic aliphatic hydrocarbons and aromatic hydrocarbons [6], which usually show the greatest resistance to biodegradation [5,6]. In agreement with this, UCM is used as an indicator of petrogenic environmental inputs due to its large persistence after accidental or chronic oil spills [7].

Aerobic petroleum biodegradation is a multiphase reaction where microorganisms, oxygen, water soluble mineral salts, and

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low-soluble substrates are generally involved [8]. Thus, mass transfer processes are critical in the hydrocarbon biodegradation kinetics [1,9]. Surfactants act by decreasing the surface and interfacial tension and by stabilizing oil–water emulsion [10,11], becoming hydrocarbon compounds bioavailable for biodegradation. Biosurfactants are preferred to chemical surfactants as they have lower toxicity and shorter persistence in the environment [10]. Toxic or inhibitory effects on hydrocarbon biodegradation have been observed with the addition of biosurfactants [2,12], but several reports also focus on the positive effect of biosurfactant addition on biodegradation in biphasic liquid systems of *n*-alkanes [13], PAHs [14], oil [15] and hydrocarbon residues [16]. However, little information exists on the effect of the simultaneous production of surfactants and/or emulsifiers on the biodegradation pattern of complex hydrocarbon mixtures [17].

In the present study the biodegradation of a fuel oil-type residue (ship bilge waste produced in normal ship operations) by an emulsifier-producing microbial consortium was investigated. Bilge waste is a hazardous waste composed by a mixture of seawater and hydrocarbon residue, where *n*-alkanes and UCM are the main constituents. Knowledge of the biodegradation kinetics and understanding the effects of *in situ* emulsification on UCM biodegradation is necessary to improve the biodegradation process, e.g. by reducing the time required to biodegrade recalcitrant compounds. The aim of this study was to investigate bilge waste biodegradation kinetics and understand the effects of the emulsification produced during the biodegradation experiment on hydrocarbon bioavailability, in consequence, on bilge waste biodegradation. In addition, the study focused on the hydrocarbon biodegradation pattern, in order to identify the key types of compounds in the multicomponent hydrocarbons mixture that could be used to model bilge waste hydrocarbon biodegradation processes.

2. Materials and methods

2.1. Waste

The residue used as substrate in the biodegradation assays was obtained by gravitational separation of bilge waste downloaded from ships that moored at the port of Puerto Madryn, Argentina. The floating organic phase named bilge waste oily phase (BWOP), a partially weathered fuel oil-type residue, was previously physically and chemically characterized [18–20]. Hydrocarbon analyses were performed by dissolving BWOP in *n*-hexane and treating with silica gel (see more detail in Section 2.4). BWOP was also fractioned in alumina column to quantified aliphatic and aromatic hydrocarbons [20]. Hydrocarbon-concentrations found by GC analysis were (expressed as $g\,kg^{-1}$ BWOP): total hydrocarbons (TH), 542 (being 89% aliphatics and 11% aromatics); resolved TH (RTH), 136; and UCM, 406. TH represents the sum of RTH and UCM. RTH contained $75\,g\,kg^{-1}$ BWOP of *n*-alkanes. BWOP density at 15 °C, kinematic viscosity (KV) at 40 °C and water content were $863\,kg\,m^{-3}$, $1.995 \times 10^{-5}\,m^2\,s^{-1}$ and 15% (v/v), respectively, determined as described by Nievas et al. [19].

2.2. Microorganisms and culture medium

BWOP from a bilge waste dumping pool was used as source of microorganisms. A microbial consortium was obtained by an enrichment procedure, using a mineral medium and 1% (v/v) BWOP as the only carbon and energy source [20]. The consortium, whose emulsifier production capacity was previously tested [20], was mechanically stirred using an orbital shaker (160 rpm) at 25 °C. Cultures were transferred twice a week in fresh liquid medium (1%, v/v) and then incubated in the same conditions. Inoculums used in the present study were prepared with a 48-h culture by washing and resuspending the cell at $OD_{450} = 0.1$ [20]. Consortia used as inoculums were at least 10 transfers old since the start of the enrichment procedure.

2.3. Assay conditions

Assays were carried out in a 3-l bioreactor using 2 l of sterile seawater mineral medium (SWM), supplemented with inorganic nutrients and $0.2\,g\,l^{-1}$ yeast extract [19]. SWM was prepared with natural filtered seawater (through 0.45 μm) supplemented with $1\,g\,l^{-1}$ NH_4NO_3 and $4\,ml\,l^{-1}$ of phosphate solution ($20\,g\,l^{-1}$ $Na_2HPO_4{\cdot}7H_2O$ and $4\,g\,l^{-1}$ $NaH_2PO_4{\cdot}H_2O$). Experiments were performed with two levels of initial sterile BWOP concentration at 0.53 and 0.18% (v/v) of the SWM. BWOP was sterilized in autoclave in a sealed container (121 °C, 15 min). A control experiment without BWOP addition was also performed. The inoculum suspension was added at 1% (v/v) of the SWM in each experiment. Cultures were stirred at 250 rpm, aerated at 0.5 vvm (volume of air per volume of culture medium per minute), and the temperature was maintained at 25 °C. Experiments were carried out for 14 and 10 days for the high and the low initial BWOP concentrations, respectively. Culture pH, monitored online with an electrode, was kept in the range 7–8 with 0.5N NaOH. Duplicate culture samples were withdrawn through a liquid sampler at different times to perform biomass, turbidity and superficial tension analyses. Hydrocarbon content was measured in the batch liquid culture and in the exhaust air of the bioreactor to assess hydrocarbon biodegradation efficiency. Duplicate 20 ml liquid samples were also withdrawn using a syringe at each sampling time for hydrocarbon analysis. The exhaust air was sampled by solid phase microextraction (SPME) to determine evaporated hydrocarbons concentration as it was previously described [19,21,22]. The continuous airflow rate was controlled by means of pressure and flow controller valves and was measured by a bubble-flowmeter to quantify the hydrocarbons evaporation rate. A sterile humidifier was used before the air inlet to the bioreactor to avoid medium loss by evaporation.

2.4. Analytical methods

A hydrocarbon degrading bacteria count (HDC) was performed by the most probable number (MPN) procedure [23]. The liquid medium used consisted of a mineral medium (with the following composition in $g\,l^{-1}$ of distillate water: NaCl, 24.0; $MgSO_4{\cdot}7H_2O$, 1.0; KCl, 0.7;

KH_2PO_4, 2.0; Na_2HPO_4, 3.0; NH_4NO_3, 1.0) with 1 ml l^{-1} of a trace metal solution (with the following composition in mg l^{-1} of distillate water: $FeSO_4{\cdot}7H_2O$, 200; $ZnSO_4{\cdot}7H_2O$, 10; $MnCl_2{\cdot}4H_2O$, 3; $CoCl_2{\cdot}6H_2O$, 20; $CuCl_2{\cdot}2H_2O$, 1; $NiCl_2{\cdot}6H_2O$, 2; $Na_2MoO_4{\cdot}2H_2O$, 500; H_3BO_3, 30 [24]). Petroleum from Comodoro Rivadavia provided by Shell Capsa, was sterilized in autoclave (121 °C for 15 min) and used as substrate. Plates were counted after incubation between 16 and 20 °C for 3 weeks. Heterotrophic microbial counts (HC) were also performed with the same procedure as that for HDC, but using a rich culture medium that contained (in g l^{-1} of natural seawater): glucose, 3; yeast extract, 2.5; meat peptone, 5; meat extract, 3. Plates were counted at 72 h of incubation at 16–20 °C. Comparison between MPN results were performed as described by Cochran [25]. Medium turbidity was measured at 450 nm in a Hewlett Packard 8452A spectrophotometer. Samples of culture medium were separated from cells and hydrocarbons by centrifugation at 16,000 × *g* for 15 min to determine the production of extracellular biosurfactant. Surface tension measurements in the supernatant were performed using the ring method on a DuNouy tensiometer at standard room temperature. Hydrocarbons in the liquid culture medium were determined by GC as previously described [18]. Briefly, 20 ml samples were acidified to pH < 2 with 6N HCl and extracted thrice with 20 ml of *n*-hexane. Then, the hexane extracts were pooled, dried over anhydrous Na_2SO_4, and treated with activated (at 200 °C during 24 h) silica gel to remove polar compounds. The dried hexane extracts were shaken with silica gel in a magnetic stirrer by 5 min and then silica gel was removed by filtering using a filter paper Whatman No. 40. The hydrocarbon extract was concentrated under N_2 flow, and analyzed by GC-FID. Hydrocarbons were quantified using the *n*-alkanes homologous series as external standards. The masses of TH, RTH, UCM and *n*-alkanes were determined at different culture times. Hydrocarbon loss by evaporation from the bioreactor was quantified by SPME using a non-equilibrium quantitation method [21,22]. Hydrocarbon concentration in the bioreactor air outlet stream was quantified as described by Nievas et al. [19]. Evaporation rate was calculated by multiplying the hydrocarbon air concentration by the volumetric air flow rate. Total evaporated mass of the hydrocarbons (TH, RTH, UCM and *n*-alkanes) as a function of time was obtained by the integration of the evaporation rate over the experimental time.

3. Results and discussion

3.1. Microbial growth and emulsification

Heterotrophic count (HC) and culture turbidity for the control and biodegradation experiments with 0.53 and 0.18% (v/v) initial BWOP content are shown in Fig. 1. For all experiments HC reached around 10^8 MPN ml^{-1} in approximately 0.8 day. Then cultures with BWOP showed HC values between 2- and 10-fold higher than that of the control experiment without hydrocarbon substrate (Fig. 1), being significantly greater than the control between days 2 and 6 ($p < 0.05$). This fact suggested that the microbial consortium used hydrocarbons and yeast extract as carbon sources.

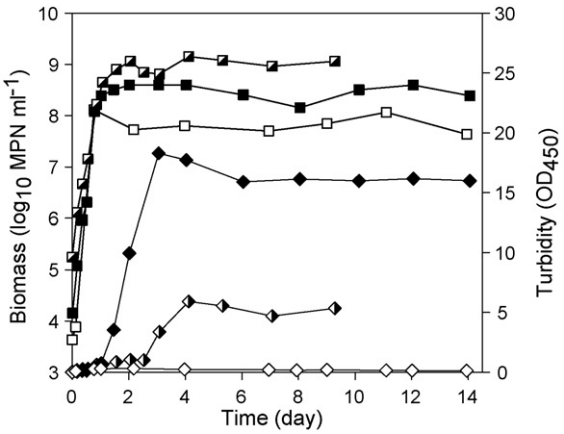

Fig. 1. Heterotrophic microbial count (HC, MPN ml^{-1}) (squares) and turbidity (OD_{450}) (diamonds) time course of the BWOP biodegradation and control assays. Empty symbols, control without BWOP; filled symbols, initial BWOP concentration 0.53% (v/v); semi-filled symbols, initial BWOP concentration 0.18% (v/v).

Time course of HDC, pH, turbidity, NaOH (used for neutralization), and hydrocarbons content for the biodegradation assays with initial concentrations of 0.53 and 0.18% (v/v) of BWOP are shown in Figs. 2 and 3, respectively. Figs. 2a and 3a indicate that HDC reached approximately 2×10^8 MPN ml^{-1} after 1 day for both cultures, showing the culture with 0.53% (v/v) initial BWOP content the highest hydrocarbon-degrading microorganism concentration between 2 and 3 days (9×10^8 MPN ml^{-1}). For the BWOP concentration of 0.18% (v/v), the maximum HDC was approximately 3×10^8 MPN ml^{-1} at 2.5 days. The highest concentration of hydrocarbon-degrading microorganisms was found for the highest BWOP concentration tested (statistically greater than the lowest BWOP concentration, at a significance level of 10%). Contrarily lower heterotrophic microorganism growth was observed for the 0.53% (v/v) BWOP experiment ($p < 0.10$ from days 4 to 9) compared with that of 0.18% (v/v) one (Fig. 1), probably due to the inability of some hydrocarbon-degrading microorganisms to grow in the rich medium used to determine HC in the assays. BWOP could also contain some toxic compounds, such us heavy metals, that could be responsible of lower heterotrophic microorganism concentrations observed for the 0.53% (v/v) BWOP experiment with respect of those of 0.18% (v/v) one. A metal analysis performed in a bilge water sample, withdrawn from the same source that BWOP used in this work, showed the presence of Cr(III), Cu, Fe, Ni and Zn. These metals come probably from the ship bilge and engine corrosion, as the exhausted lubricant oils are usually disposed in the bilge wastes.

Turbidity increased to approximately 1 absorbance unit (AU) in the first day, for the 0.53% (v/v) BWOP assay (Fig. 2a). For the lowest BWOP concentration experiment, the same turbidity level was reached after approximately 2.5 days (Fig. 3a). Then sharp OD_{450} increases occurred about 1 and 2.5 days reaching a final value of around 16 and 5 AU for initial concentrations of 0.53 and 0.18% (v/v) BWOP, respectively (Figs. 2a and 3a). On

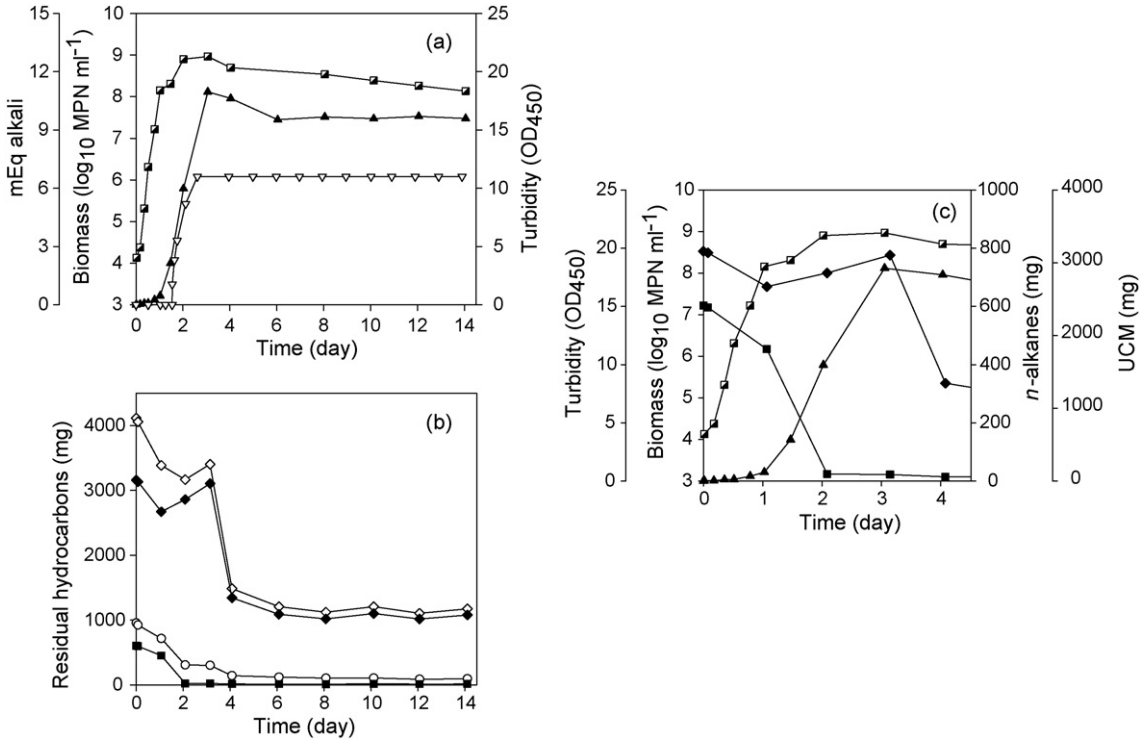

Fig. 2. Time course of the 0.53% (v/v) initial BWOP concentration biodegradation assay. (a) (◪) HDC (MPN ml^{-1}), (▲) turbidity (OD_{450}), and (▽) amount of NaOH added to maintain pH between 7 and 8 (meq alkali); (b) (◊) TH (mg), (⧫) UCM (mg), (◯) RTH (mg), and (■) *n*-alkanes (mg); (c) detail of HDC, turbidity, UCM and *n*-alkanes in the 0–4.5 days period.

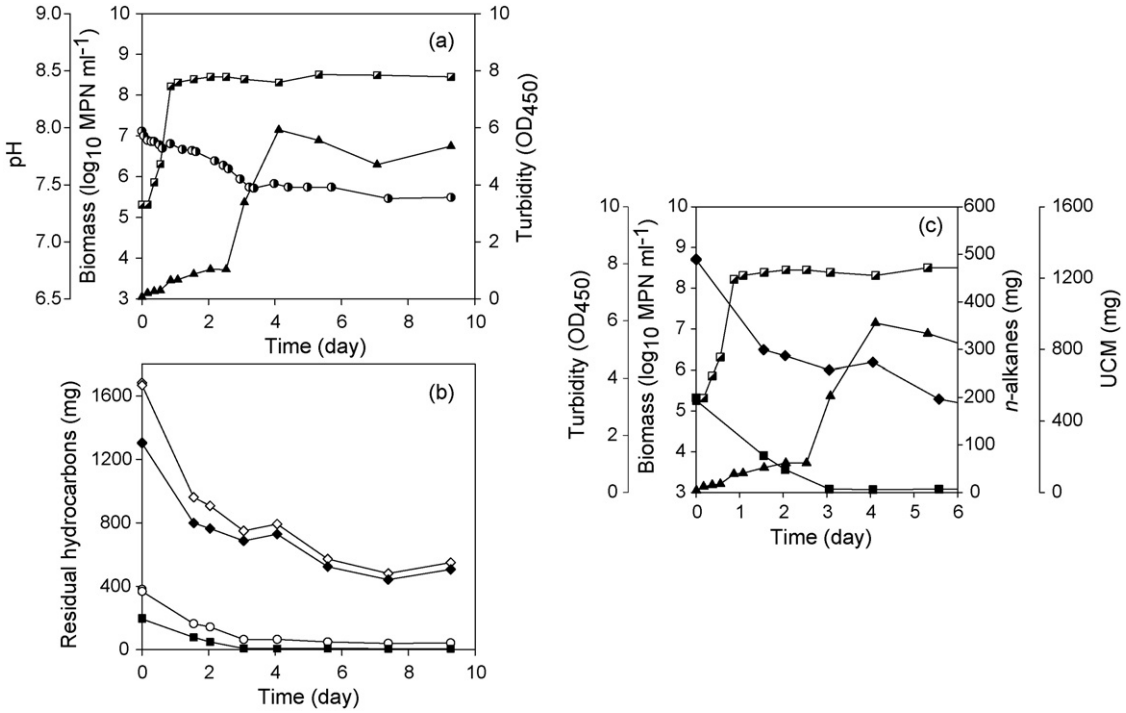

Fig. 3. Time course of the 0.18% (v/v) initial BWOP concentration biodegradation assay. (a) (◪) HDC (MPN ml^{-1}), (▲) turbidity (OD_{450}), and (◑) pH; (b) (◊) TH (mg), (⧫) UCM (mg), (◯) RTH (mg), and (■) *n*-alkanes (mg); (c) detail of HDC, turbidity, UCM and *n*-alkanes in the 0–6 days period.

the other hand, culture turbidity in the control assay increased in agreement with the microbial count to a maximum value of $OD_{450} = 0.32$ at day 2, and then remained lower than this value up to the end of the experiment at day 14 (Fig. 1). For both BWOP concentrations, HC and HDC showed initially linear correlation with OD_{450} in the range of 0–1 AU. For the cases of 0.53 and 0.18% (v/v) BWOP, good linear correlations between biomass and optical density were verified up to 1 day ($R^2 > 0.84$, $n = 6$, $p < 0.01$, being n the number of experimental points regressed in each experiment over the mentioned time period) and 2.5 days ($R^2 > 0.84$, $n = 9$, $p < 0.01$), respectively. These results indicated that, for both cultures, turbidity initially augmented due to microbial growth. However, R^2 decreased to values lower to 0.65 ($n = 14$, $p < 0.05$) for both experiments when data of the whole duration of the assays were correlated. This fact indicated that the observed sharp turbidity increases could not be associated with microbial growth, suggesting that hydrocarbons emulsification could be responsible of them, as it was found in a previous study where the same microbial consortium was used [20]. Furthermore, the final turbidity values (16 and 5 AU for the high and the low initial BWOP concentrations, Figs. 2a and 3a, respectively) were in good agreement with the initial hydrocarbons concentration of the cultures (approximately a 3:1 ratio), reinforcing the hypothesis that turbidity increases were caused by hydrocarbons emulsification. The sharp turbidity increases started after the end of the microbial exponential growth phase, in agreement with reports that state that emulsifiers are usually produced when cultures reach the stationary phase of growth [10,11]. Surface tension of the culture medium supernatant along the assays showed little change (data not shown), decreasing from the initial value of 70 to 50 and 60 mN m^{-1} (relative percentage error less than 13%) for the high and low initial BWOP concentrations, respectively, at the end of the experiments. Cultures in the present study showed a low reduction in surface tension, compared with that found when low molecular weight biosurfactants are produced by hydrocarbon-degrading microorganisms (up to 30 mN m^{-1}) [10,26]. This fact suggests that a high molecular weight emulsifier was produced during the BWOP fermentation, as these types of compounds show high emulsification capabilities without extensively lowering the surface tension [11,14]. The emulsification start point occurred at different incubation times for the BWOP concentrations tested, being later for the culture with the lowest hydrocarbons concentration (Figs. 2a and 3a). Some authors have reported that bioemulsifier production starts when a nutrient is depleted, e.g. the nitrogen source [10,27]. Although in the present study the nitrogen source was not monitored during the experiments, a previous analysis in a biodegradation experiment with similar hydrocarbons biodegradation extent and initial nutrient content (1 g l^{-1} NH_4NO_3) showed that it was not nitrogen limited [28].

Acidification of the reaction medium found was dependent on hydrocarbon concentration. For the 0.53% (v/v) BWOP assay, it was necessary to add alkali to maintain the pH in the range 7–8, showing the acid production a linear correlation with turbidity for the whole experiment duration ($R^2 = 0.98$, $n = 17$, $p < 0.01$) (Fig. 2a). For the low BWOP concentration, pH decreased slightly from 8.0 to 7.5 (Fig. 3a) until the highest turbidity was reached, then remained almost constant. For this hydrocarbons concentration, neutralization was unnecessary to keep the pH in the range 7–8, while OD_{450} and pH were negatively linear correlated ($R^2 = 0.86$, $n = 14$, $p < 0.01$) for the whole experiment duration (10 days). For the control assay, without BWOP, the pH gradually decreased from 8 to around 7.0 at the end of the experiment (data not shown), the culture neutralization was not necessary. No linear correlation was found for the control experiment between pH and turbidity ($R^2 = 0.005$, $n = 12$, $p < 0.95$). These results indicate that the medium emulsification can be related to the release of acid compounds, which depends of the hydrocarbon concentration in the reactor (Figs. 2a and 3a), suggesting that the emulsifier could has an acidic structure. Experimental observation indicated that the highest acidification and turbidity were found for the highest hydrocarbons concentration. Another possible explanation for the acidification found could be related with the nutrient amendment of the culture medium. Foght et al. [29] have reported that when NH_4NO_3 was used as nitrogen source in a crude oil biodegradation experiment in a seawater medium (using a six-bacterial-strains consortium) the pH of the culture medium dropped significantly (to less than 6). These authors reported that saturate-hydrocarbon-degrading strains were responsible of the pH drop, when they grew in the presence of ammonium. In spite of the acidification mechanism was not clearly established, Foght et al. [29] stated that acidification was not linked to growth on crude oil, as it was also observed when the same consortium was grown in glucose as sole carbon source. In the present study the observed acidification could be explained by the presence of ammonium as nitrogen source, as previously was reported by these authors. However, pH drop seems to be linked to the hydrocarbons amount, due to the poorly buffered medium used was enough to keep the pH medium above 7.0 for the control and 0.15% (v/v) BWOP experiments, while 6 meq of alkali were needed to keep the pH near neutrality (7.0–8.0) for the highest BWOP content experiment (Figs. 2a and 3a). If the acidification was caused by the saturated-degrading strains growing in the presence of ammonium as reported by Foght et al. [29], the differences observed in the medium acidification between both BWOP assays could be attributable to a highest growth of this specific component of the hydrocarbon-degrading consortium for the 0.53% (v/v) BWOP experiment. This fact is in agreement with the hydrocarbon-degrading microorganism concentrations found for the 0.18 and 0.53% (v/v) BWOP assays.

3.2. Hydrocarbon biodegradation extent

For both BWOP levels, all the hydrocarbon types showed an important concentration reduction from their initial values. The extent of biodegradation followed the order *n*-alkanes > RTH > UCM (Table 1). The residual hydrocarbons, expressed as percentage of the initial hydrocarbons, were found to be similar for both experiments (Table 1). TH reduction of around 70% and TH biodegradation efficiencies in the range of 57–65% were achieved for both BWOP concentrations in less than 6 days (Table 1). Some UCM compounds remained recalcitrant to the microorganism action (approximately 30%

Table 1
Extent and efficiency of biodegradation in assays using a bioemulsifier-producing microbial consortium with two different BWOP initial concentrations

Hydrocarbon type	0.53% (v/v) BWOP initial concentration						0.18% (v/v) BWOP initial concentration					
	Initial mass[a]	Evaporated mass		Residual mass[a]		Biodegradation efficiency[b]	Initial mass[a]	Evaporated mass		Residual mass[a]		Biodegradation efficiency[b]
	mg	mg	%[c]	mg	%[c]	%[c]	mg	mg	%[c]	mg	%[c]	%[c]
n-Alkanes[d]	603	27	4.4	24	4.1	91.5 (2 days)	200	28§	14.1	7	3.6	82.4 (3 days)
RTH[d]	956	90	9.4	118	12.4	78.2 (6 days)	380	62	16.4	43	11.4	72.1 (5.7 days)
UCM[d]	3158	137	4.3	1089	34.5	61.2 (6 days)	1307	120	9.2	490	37.5	53.3 (5.7 days)
TH[d]	4115	231	5.6	1208	29.3	65.0 (6 days)	1684	182	10.8	534	31.7	57.5 (5.7 days)

Values are mean of determination in duplicate samples. Relative percentage errors for duplicate hydrocarbon masses were less than 11%, except for the § sample that was 21%.
[a] In the liquid culture medium.
[b] Values are calculated at the assay time indicated in parenthesis.
[c] Percentage referred to the initial mass of the same hydrocarbon type.
[d] Values correspond to the time indicated in the biodegradation efficiency column, except the initial mass.

of the initial TH for both experiments). BWOP is a mixture of residues from hydrocarbon products used in ships, mainly fuels and lubricant oils. Chemical composition of the BWOP used in this study matched with fuel oil #2, but the kinematic viscosity (KV) value observed was five times greater than the one expected for this fuel [19,30] indicating that BWOP included exhaust lubricant oil. Haus et al. [31] correlated physical properties of paraffinic base oils with their intrinsic biodegradability, finding a negative linear relationship between %biodegradability and log(KV). The BWOP kinematic viscosity was about 2×10^{-5} m^2 s^{-1}; according to the correlation obtained by Haus et al. [31], for this KV value 60–70% biodegradability can be expected. This result is in good agreement with the percentage of TH remaining in the present study (about 30%), suggesting that recalcitrant branched hydrocarbons from lubricant oils in BWOP were probably resistant to microbial attack.

3.3. Hydrocarbons biodegradation pattern

Hydrocarbons time course during biodegradation assays is shown in Figs. 2b and 3b. *n*-Alkanes, RTH, UCM and TH biodegradation were observed for both initial BWOP concentrations; however, differences in the biodegradation patterns were found.

For the 0.53% initial BWOP experiment TH (UCM + RTH) decreased in two stages (Fig. 2b), influenced firstly by the *n*-alkanes and secondly by UCM biodegradation patterns. *n*-Alkanes disappeared in around 2 days, while UCM did not change markedly during the first 3 days. UCM was quickly degraded after medium emulsification reached its maximum value (Fig. 2a and b), showing a reduction of 57% of the initial UCM between days 3 and 4. Subsequent reduction after day 6 was almost negligible, remaining undegraded 34.5% of the initial UCM. For this initial BWOP concentration *n*-alkanes degradation agreed with hydrocarbon-degrading microbial growth, that showed its maximum concentration (9×10^8 MPN ml^{-1}) by the time that *n*-alkanes were almost extinguished. This fact suggests that *n*-alkanes were used as substrate for hydrocarbon-degrading microbial growth (Fig. 2c). On the other hand, UCM biodegradation produced between days 3 and 4, that constitute a hydrocarbon mass three times greater than *n*-alkanes degraded, did not cause an increase in the microbial concentration. (Fig. 2c). UCM showed non-growth associated degradation kinetics [1]. This fact suggests that UCM was degraded following a different mechanism from *n*-alkanes, being degraded probably after emulsification increases the hydrocarbons bioavailability. Surfactants and emulsifiers have frequently enhanced hydrocarbon aqueous solubility and biodegradation rates of individual hydrocarbon compounds, usually producing changes in hydrocarbons bioavailability and in microorganism adhesion properties to hydrophobic interfaces [1,14,26]. Three mechanisms are accepted for microbial hydrocarbon biodegradation: through utilization of (a) soluble organic molecules, (b) pseudosolubilized hydrocarbons by surfactants (substrates within droplets less than 1 μm) and (c) stabilized emulsions [1,11,14,26]. As the surface tension showed a low reduction during fermentation, the last mechanism seems to be adequate to describe the observed UCM biodegradation. Increasing apparent solubility mechanism by high molecular weight bioemulsifiers is not fully understood [14,32]. However, it was shown that it was different from the micellar mechanism described for low molecular weight biosurfactants, and that, probably, multimolecular interactions could occur between hydrophobic regions of the bioemulsifier molecules and the hydrophobic substrates [14,32]. Except for the *n*-alkanes, the rest of the RTH were biodegraded with similar pattern as UCM (Fig. 2b). Fig. 4 shows, for the 0.53% BWOP assay, the TH chromatographic profiles obtained from liquid and air samples at different times. *n*-Alkanes biodegradation occurred in the first 2 days (Fig. 4a and b), while the highest UCM reduction was found after day 3 (Fig. 4b and c). The evaporated compounds, detected in the outlet air stream, comprised resolved and UCM hydrocarbons from C9 to C16, showing complementary profiles with those of the liquid samples (compare right and left graphs of Fig. 4).

UCM biodegradation, with less preference and with a time delay with respect to *n*-alkanes, has been reported in other biodegradation studies of different hydrocarbon mixtures such as diesel oil [33–35]. This behavior was usually attributed to the intrinsic difficulty of microorganisms to biodegrade these compounds. Geerdink et al. [33] consider that the probable cause for

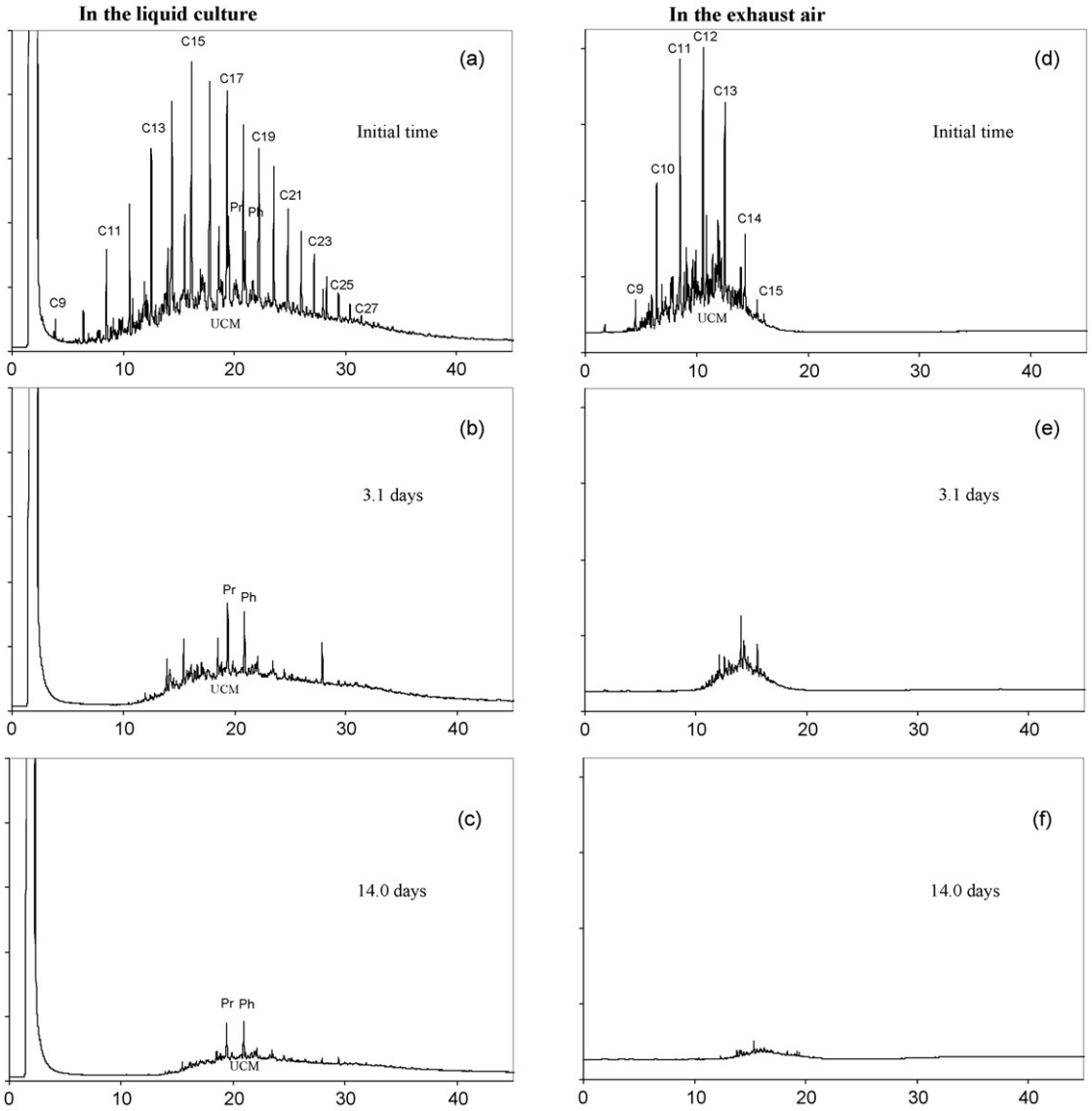

Fig. 4. TH chromatographic profiles from the biodegradation assay with 0.53% (v/v) BWOP initial concentration at different times. *X*-axis, time in minutes. Left block (a–c) corresponds to the same amount of liquid samples, being the *y*-axis scales the same so they can be directly compared. Right block (d–f) corresponds to samples in the exhaust air of the bioreactor, being the *y*-axis scales the same so they can be directly compared. First file (a and d), 0.08 day (initial time); second file (b and e), 3.1 days; and third file (c and f), 14.0 days. Only qualitative comparisons can be done between air and liquid samples. C_x: *n*-alkane of *x* carbon number; Pr: pristane; Ph: phytane; UCM: unresolved complex mixture.

the delay in branched-alkanes degradation is the higher affinity of linear *n*-alkanes for one or more of the enzymes in the system involved in diesel oil fermentation by a microbial consortium, dismissing the hypothesis of diauxic utilization. For the 0.53% (v/v) BWOP experiment, the higher UCM biodegradation occurred after emulsifier production (Fig. 2). This fact suggests that bioavailability, and not intrinsic biodegradation resistance, was responsible for the observed UCM biodegradation delay.

Hydrocarbons in the experiment carried out with the lowest BWOP concentration (0.18%, v/v) showed simultaneous *n*-alkanes and UCM biodegradation during the 0–3 days time period (Fig. 3b), in contrast with the sequential biodegradation observed for the 0.53% (v/v) BWOP concentration. However after emulsification the UCM degradation rate increases, probably following a mechanism mediated by the emulsifier which enhances the UCM bioavailability. Kim et al. [27] have reported that the bioemulsifier MEL-SY16, produced at expenses of hydrophobic substrate, reached higher concentration in the culture according the higher hydrophobic substrate concentration added to the reaction medium. In addition secondary metabolites (as bioemulsifiers) are usually produced proportionally to the biomass [36]. In the experiments carried out with 0.18% (v/v) BWOP both initial hydrocarbons concentration and hydrocarbon-degrading microorganisms were lower than those of the 0.53% (v/v) BWOP experiment, suggesting that the emulsifier was produced in less concentration. This also agreed with

the lower turbidity found in the 0.18% (v/v) BWOP experiment. These facts could be responsible for the delay observed in emulsification for the lower hydrocarbon content experiment, and in consequence on the hydrocarbons degradation kinetics.

Despite of the differences found on *n*-alkanes and UCM biodegradation pattern for both BWOP concentrations tested, experimental observations indicated that the start of the emulsification in the cultures (turbidity increases) occurred simultaneously with *n*-alkanes depletion (Figs. 2c and 3c). This fact suggests that depletion of *n*-alkanes triggered the emulsifier production. *Acinetobacter calcaoceticus* has been reported to produce the bioemulsifier *Emulsan*, a cell bound minicapsule, when growing on *n*-alkanes attached to the oil interface droplets [11]. When hydrocarbons droplets became exhausted of *n*-alkanes, cells detached from interface, releasing the biopolymer which remains attached to the hydrocarbons and acts as a potent emulsifier. The *Emulsan* production was a consequence of *n*-alkane biodegradation. A similar situation could explain the pattern observed for *n*-alkanes biodegradation and emulsification in BWOP fermentations.

4. Conclusions

Surfactant and emulsifier addition has often been used to enhanced bioavailability and biodegradation of hydrophobic compounds; however little is known about the effect of simultaneous emulsifier production on biodegradation of complex hydrocarbon mixtures. A emulsifier-producing consortium used for biodegradation of BWOP showed differences in the emulsification start time and extent, according to the initial hydrocarbon concentration used. Both facts influenced the hydrocarbon biodegradation kinetics. Emulsification appears to be linked to the *n*-alkanes depletion and occurred earlier for the higher hydrocarbon concentration tested. For 0.53% (v/v) BWOP initial concentration, a sequential biodegradation of *n*-alkanes and UMC was found, being the former a growth associated process and the later a non-growth associated one, likely following a emulsifier mediated mechanism where biodegradation was limited by hydrocarbon accessibility. For the lower concentration experiment (0.18%, v/v, BWOP) simultaneous biodegradation of *n*-alkanes and UMC were found before emulsification, although a final and important UCM biodegradation was observed after this step. These results are useful to mathematically model BWOP biodegradation, in order to design and operate an effluent treatment bioreactor. BWOP hydrocarbons can be characterized by three compound types according to the observed biodegradation kinetics: *n*-alkanes, biodegradable UCM, and UCM that remained undegraded. In view of the obtained result, further investigations are needed to explore the concentration range over which the biodegradation kinetic patterns for low and high initial hydrocarbon concentrations are observed.

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