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Effect of pH modification on bilge waste biodegradation by a native microbial community

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Abstract

During laboratory biodegradation of the oily liquid residue generated in normal ship operations, bilge waste, by an adapted native microbial community, bioemulsifier production and decrease in pH were observed. When the pH of the medium was modified by adding NaOH or buffering with Tris–HCl (pH 7.8), the greatest hydrocarbon reduction and biodegradation were found with NaOH treatment. With this treatment, total hydrocarbon removal and biodegradation efficiencies of, respectively, 97% and 86% for *n*-alkanes, 40% and 30% for total aliphatic hydrocarbons and 25% and 17% for total aromatic hydrocarbons were recorded after culture for 10 days. The emulsifying activity (quantified as emulsion index E24) was highest (60%) for neutralized treatment. For the three treatments the emulsifier activity was optimal at pH 6.5–7.5. Therefore, the culture medium should be neutralized to increase the rate of biodegradation and to minimize the residence time of the waste in a treatment unit.
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Keywords: Bilge waste; Biodegradation; pH; Hydrocarbons; Bioemulsification

1. Introduction

Bilge water discharge is prohibited at Puerto Madryn (Patagonia, Argentina) to prevent the pollution of Golfo Nuevo, a special area at the southern border of Península Valdés, where projects with the aim of preventing coastal pollution (particularly hydrocarbon contamination) and preserving the Patagonian marine biological biodiversity are being carried out. Port reception facilities and bilge waste treatment plants are recognized as important preventative actions in minimizing coastal hydrocarbon pollution. Bilge wastes are liquid residues generated in normal ship operations and collect in the bottom of the hull. They are mainly composed of a seawater phase and an oily phase, which usually contains diesel and fuel oil, and other hydrocarbon residues (Karakulski et al., 1998). Regulations require that any oil or oil residue discharged into oceans when a ship is on route must have a hydrocarbon content <15 ppm. Any oil or oil residue containing a greater concentration must be retained on board and discharged to a port reception facility (IMO, 1989).

Bioremediation has become one of the most rapidly developing fields of environmental restoration (Dua et al., 2002), and bioreactor systems are attractive low-cost methods for carrying out bioremediation ex situ in comparison with other means of waste disposal (Alexander, 1999; Van Hamme et al., 2003), also having low environmental impact and public acceptance. Mixed microbial cultures have been demonstrated to be more efficient in degrading complex hydrocarbon mixtures than individual members of the mixed population, as the mixtures include a wide range of compounds of different biodegradability characteristics (Atlas, 1981; Bhatnagar

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and Fathepure, 1991; Van Hamme et al., 2003). Bilge waste varies depending on the ship, but indigenous microbiota have been shown to be capable of degrading the hydrocarbons presented in different bilge wastes (Olivera et al., 2000; Olivera et al., 2003).

Hydrocarbon-degrading microorganisms produce a variety of surfactants (Atlas, 1981; Desai and Banat, 1997; Van Hamme et al., 2003). These biosurfactants are diverse surface-active chemical compounds that emulsify or pseudosolubilize poorly water-soluble compounds, improving accessibility of these to microorganisms (Banat, 1995; Van Hamme et al., 2003). Surfactants have been shown to enhance microbial degradation of hydrocarbons (Sekelsky and Shreve, 1999; Olivera et al., 2000, Van Hamme et al., 2003).

In previous experimental studies on treating bilge waste with autochthonous microorganisms, a pronounced decrease in pH, together with an increase in turbidity and dispersion of the oily phase, was observed in the medium. These changes were attributable to biosurfactant/bioemulsifier production. Some biosurfactants have been shown to behave differently in response to pH at which they act (Cooper and Goldenberg, 1987; Zhang and Miller, 1992). For this reason, and in aiming to find operating conditions that minimize residence time in plant facilities, the effect of pH modification on hydrocarbon removal efficiency was investigated in this study, and the behavior of emulsifier as a function of pH analyzed. The understanding of the relationship between acid production, emulsifying activity and hydrocarbon degradation efficiencies is of great importance in the satisfactory operation of waste treatment reactors for hydrocarbon remediation. Knowledge of the optimal pH culture condition will allow kinetic parameters of bilge waste biodegradation to be obtained and reactor operation for waste treatment on larger scales mathematically modeled and simulated.

2. Materials and methods

2.1. Waste

Waste oily liquid was obtained from an open pool where bilge waste of vessels that dock in Puerto Madryn port are collected. Hydrocarbon content was determined by dissolving approximately 0.39 g bilge waste oily phase (BWOP) in 60 mL *n*-hexane. This solution was dried over anhydrous Na_2SO_4, cleaned up, fractionated and analyzed as described in Section 2.5. The hydrocarbon composition, determined by high-resolution gas chromatography (HRGC), is summarized in Table 1. The hydrocarbon profile corresponding to the aliphatic fraction presented the *n*-alkane homologous series (from C9 to C27), pristane (Pr) and phytane (Ph) isoprenoids, and an unresolved complex mixture (UCM); while the presence of PAHs was detected in the aromatic hydrocarbon fraction. When compared with published reference chromatograms (Bishop, 1997), the aliphatic hydrocarbon profile was consistent with a gas oil/fuel oil mixture.

Table 1
Hydrocarbon content of BWOP

		g kg^{-1} BWOP
Aliphatics	RAlH[a]	69.6
	UCM[a]	278.1
	TAlH[a]	347.7
PAHs	Acenaphthylene	0.11
	Fluorene	0.04
	Phenanthrene	0.47
	Anthracene	0.02
	3-methyl phenanthrene	0.25
	2-methyl phenanthrene	0.29
	9-methyl phenanthrene	0.18
	1-methyl phenanthrene	0.25
	2,7-dimethyl phenanthrene	0.29
	Fluoranthene	0.06
	Pyrene	0.03
	Total	1.99
Aromatics	TArH[a]	43.2

[a]RAlH, resolved aliphatic hydrocarbons; UCM, unresolved complex mixture; TAlH, total aliphatic hydrocarbons; TArH, total aromatic hydrocarbons.

2.2. Inoculum and culture media

Microorganisms were obtained from a BWOP sample taken aseptically in a glass bottle from the waste storage pool. Enrichment was performed using 1% (v/v) BWOP, which was shaken at 160 rpm and maintained at 25 °C in 125-mL flasks containing 50 mL seawater mineral medium (SWM). A 48-h culture was harvested by centrifugation (16 000*g*, 15 °C, 20 min). Cells were washed twice, by resuspending in saline (0.85%, w/v) and harvesting by centrifugation as before. The pellet was then resuspended in SWM, adjusted to OD_{450} 0.1 and used as inoculum for the biodegradation assay. SWM was prepared with natural seawater (aged and filtered through a 0.45-μm membrane filter) supplemented NH_4NO_3 at a rate of 1 g L^{-1} and phosphate solution (20 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$ and 4 g L^{-1} $NaH_2PO_4 \cdot H_2O$) at 4 mL L^{-1}. The initial SWM pH was 7.5.

2.3. Biodegradation assay

BWOP biodegradation was conducted at 25 °C for 10 days in 125-mL Erlenmeyer flasks containing 50 mL SWM, 1% BWOP (v/v) and 1% inoculum (v/v), which were shaken at 160 rpm. Biodegradation assays were performed in duplicate for each of three treatments: A,

in which the pH of the medium was not modified during biodegradation; B, in which NaOH was added every 48 h to neutralize the medium and C, in which 10 mM Tris–HCl buffer (pH 7.8) was added initially. Biomass, pH, medium turbidity and emulsion index (E24) were monitored periodically on aliquots collected during the experiment. Sterile controls and controls without BWOP were also incubated, and for each treatment and control at zero-time and at 10 days a duplicate set of flasks was sacrificed for hydrocarbon analysis.

2.4. Analytical methods

Microbial growth was monitored by plate counts on nutrient agar containing 20 g L^{-1} nutrient agar (Merck) prepared with seawater and supplemented with (g L^{-1}) (glucose, 1; yeast extract, 2.5). The agar plates were incubated at 25 °C for 48 h. Turbidity of the liquid medium was measured in a Hewlett Packard 8452A spectrophotometer at 450 nm. Emulsifying activity was determined by a modified version of the procedure described by Cooper and Goldenberg (1987). Culture medium (2 mL) plus 3 mL gas-oil was vortexed at maximum speed for 2 min in glass tubes (105 mm × 16 mm), allowed to stand at room temperature (18–24 °C) for 24 h and the E24 then expressed as the percentage of the height of the liquid column occupied by the emulsion.

2.5. Hydrocarbon analysis

The complete content of each Erlenmeyer flask was acidified to pH < 2 and extracted thrice with 20 mL fresh *n*-hexane. The extracts were pooled and dried over anhydrous Na_2SO_4, and the solvent was evaporated under a gentle nitrogen gas flow to reach a final volume of 10 mL. Clean-up and isolation of hydrocarbon fractions were carried out by column chromatography. A 1-mL aliquot of the concentrated extract was fractionated on a totally activated alumina column. The aliphatic fraction was eluted with 8 mL *n*-hexane, and the aromatic fraction with 4 mL *n*-hexane/methylene chloride (7:3, v/v) (Commendatore et al., 2000). Compounds in the fractions were identified and quantified by HRGC in a Konik 3000 instrument with a 30-m fused silica (DB-1 phase) capillary column (0.25 mm i.d.) operated as described by Commendatore et al. (2000) against external standard compounds. In the aliphatic fractions, the resolved aliphatic hydrocarbons (RAlH: *n*-alkanes plus pristane and phytane isoprenoids), the unresolved complex mixture (UCM) and the total aliphatic hydrocarbons (TAlH: RAlH plus UCM) concentrations were calculated. The nC17/pristane (C17/Pr) and nC18/phytane (C18/Ph) relationships were used as indicators of biodegradation. In the aromatic fractions, some unsubstituted PAHs recognized by the US Environmental Protection Agency (EPA) as priority pollutants (acenaphthylene, fluorene, phenanthrene, anthracene and pyrene) and substituted phenanthrenes (1-methylphenanthrene, 2-methylphenanthrene, 3-methylphenanthrene, 9-methylphenanthrene, and 2,7-dimethylphenanthrene) were identified and quantified. The total aromatic hydrocarbon (TArH) content was determined from the whole area in aromatic fraction chromatograms, multiplying by an average resolved aromatic response factor.

2.6. Effect of pH on emulsifying activity

Ten-day cultures prepared as in Section 2.3 were centrifuged for 15 min at 16 000*g* and 15 °C to separate the cells, supernatant and hydrocarbon phase. The hydrocarbon phase was carefully removed and SWM added to make up the original culture volume. After the supernatant was collected, the tube walls and cell pellet were gently washed twice, before the cells were resuspended in SWM to the final volume of the original culture. The cell suspension, supernatant and hydrocarbon dispersion phase were then diluted (1:1, v/v) with buffer solutions of different pH. For dilutions with a pH < 7, phosphate buffer (200 mM) was used, and for pH ⩾ 7 Tris–HCl (100 mM). For each dilution, E24 was assessed as in Section 2.4 using triplicate independent samples.

3. Results and discussion

3.1. Biodegradation assay

As BWOP biodegradation proceeded, the pH did not show marked change during the first 3 days. After the fourth day, it gradually fell, finally to around pH 5.5 (Fig. 1). In treatment A (without pH modification), a marked pH decrease occurred between days 4 and 6 (Fig. 1A), the acidity of the medium remaining almost constant thereafter. In treatment B, the pH decreased as in A, but NaOH addition every 48 h starting on day 6 (Fig. 1B) temporarily raised the pH to near neutrality. After each addition, the medium returned to pH 5.5. In treatment C, Tris–HCl buffer was unsuccessful in controlling the pH (final pH 5.8), probably because acid production during biodegradation exceeded the buffering capacity of the 10 mM employed and/or biodegradation of the buffer. In all three treatments (Fig. 1), the stationary phase of growth was reached after 2 days. Final heterotrophic counts were around 10^8 CFU mL^{-1} in all.

In all treatments, increases in the turbidity of the medium were found after pH fell (Fig. 1). As microbial biomass remained constant after day 2, the turbidity change could not be related to cell concentration. It

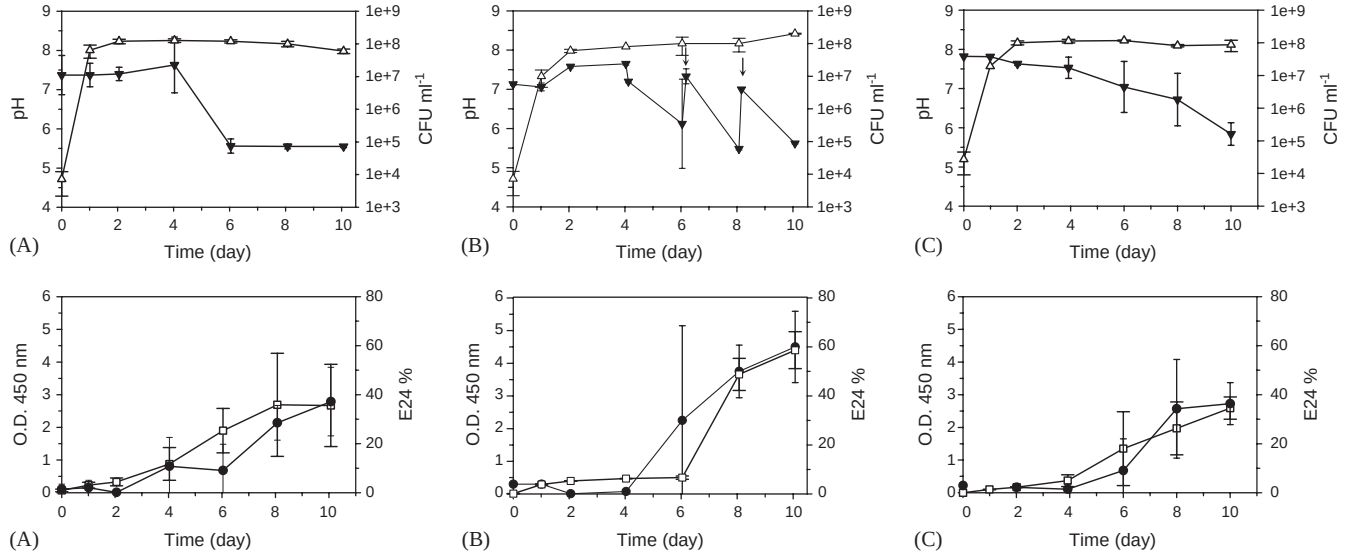

Fig. 1. Time course of biodegradation assay: (A) without modification of medium pH; (B) with addition of NaOH (the addition points are indicated by arrows); (C) with Tris 10 mM at zero time. ▼, pH; △, biomass (CFU mL^{-1}); □, O.D. 450 nm (A.U.); ●, E24 (%). Values are means of duplicate independent samples.

could be attributed to hydrocarbon emulsification, however. Turbidity has been used as a measure of hydrocarbon dispersion caused by bioemulsifier production when hydrocarbons were the carbon source (Rosenberg et al., 1992). E24 increase agreed with turbidity increase after day 4, indicating that a bioemulsifier was continuously produced in the late stationary growth phase. Production of an acid emulsifier and/or acid metabolic byproduct that appeared simultaneously could be responsible for the pH reduction, and the turbidity may have increased at the expense of a microemulsion of the hydrocarbons.

The greatest emulsifying activity and turbidity at the end of the experiments were observed in treatment B, the discontinuously NaOH-amended treatment, suggesting that pH decrease could cause inhibition of emulsifier production or activity, as has been described for surfactin production (Cosby et al., 1998). Decrease in pH was also observed by Rosenberg et al. (1979, 1992) and Marin et al. (1995) when a bioemulsifier was produced and the producer microorganisms were growing on hydrocarbons and on soluble substrates (Cooper and Goldenberg, 1987; Cosby et al., 1998).

Sterile controls showed no change in any of the four parameters measured. At the end of the experiment, inoculated SWM controls without BWOP had a final heterotrophic count of 2×10^6 CFU mL^{-1} and a turbidity of 0.08 A.U. without pH change. SWM 10 mM Tris–HCl (pH 7.8) control yielded a higher count (approximately 2×10^7 CFU mL^{-1}) than SWM alone, a maximum turbidity of 0.2 A.U. and no pH change (data not shown). None of the controls had emulsifying activity. Collectively, these results indicate that changes in the pH of the medium, turbidity and emulsifying activity found in the biodegradation assay were due to the presence of hydrocarbons in the culture flasks. The microbial growth supported by the SWM might be attributable to a small amount of hydrocarbons that could have attached strongly to microorganisms in the inoculum (Gutnick and Rosenberg, 1977; Atlas, 1981), and a higher growth on SWM 10 mM Tris–HCl (pH 7.8) than on SWM alone may suggest that the microbial community could to some extent grow on Tris.

3.2. Hydrocarbon degradation

The *n*-alkane homologous series C9–C27 and an UCM were present in the initial BWOP aliphatic hydrocarbon fraction (Fig. 2a). A reduction of the lightest hydrocarbons (*n*-alkanes up to C13 and UCM that eluted with these compounds) was noted in a sterile control experiment as abiotic loss (Fig. 2b). Figs. 2c–e show the chromatographic profiles at the end of the experiment for the three treatments, respectively. The greatest hydrocarbon reduction was found in the NaOH-amended treatment B, with almost complete disappearance of *n*-alkanes (>96%) and reduction in UCM, demonstrated by the 40% decrease of the TAlH (Fig. 3). For treatment A, *n*-alkanes and TAlH reductions were 77% and 28%, respectively, both lower than those in treatment B. The Tris-amended treatment C showed the least reduction in *n*-alkanes (74%) and the TAlH reduction was not appreciable (Fig. 3).

Preferential degradation of *n*-alkanes with respect to isoprenoid compounds was indicated by decrease in the *n*-C17/Pr and *n*-C18/Ph ratios (Table 2). These values have been used as good indicators of hydrocarbon biodegradation in ambient processes in water,

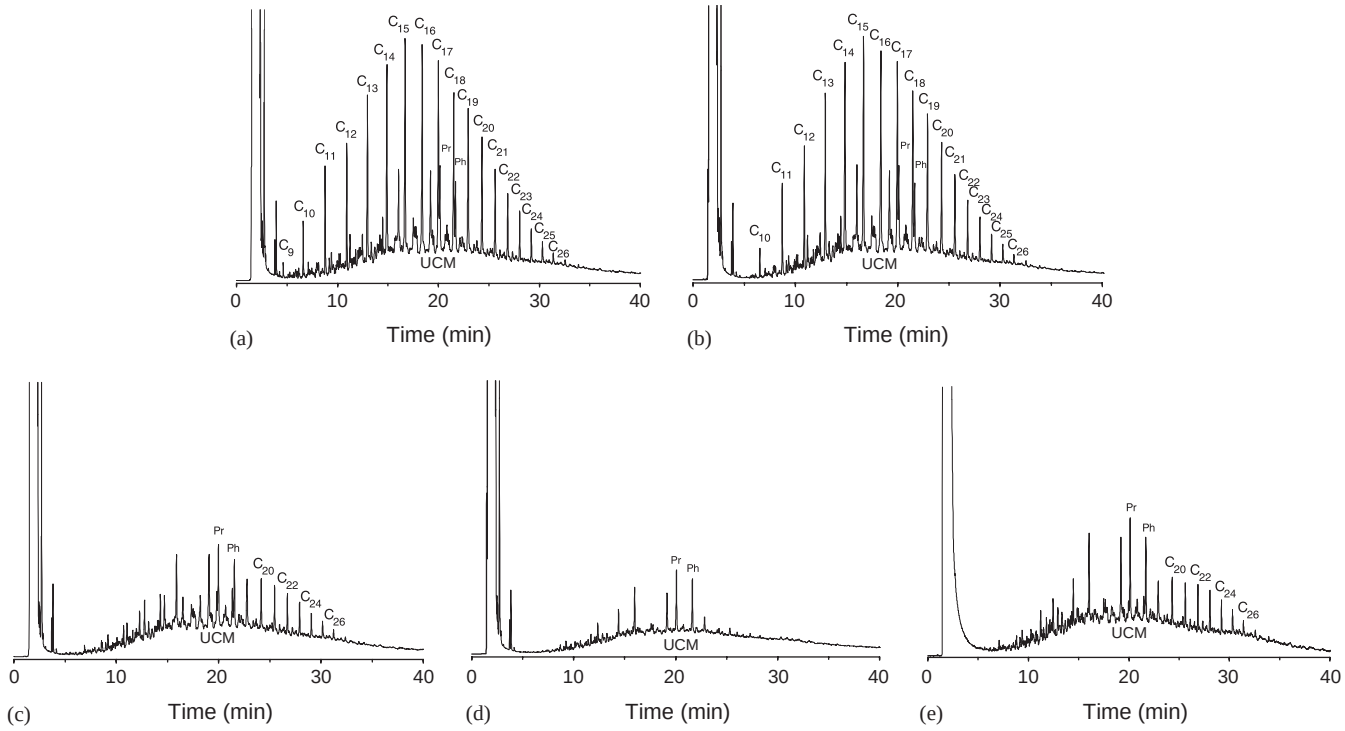

Fig. 2. Chromatographic profiles of aliphatic hydrocarbons for biodegradation and control assays. (a) Initial assay time; (b) abiotic control, at 10 days; (c) biodegradation assay without modification of medium pH, at 10 days; (d) biodegradation assay with addition of NaOH, at 10 days; (e) biodegradation assay with 10 mM Tris (pH 7.8), at 10 days. C_x: *n*-alkane of *x* carbon number; Pr, pristane; Ph, phytane; UCM, unresolved complex mixture. Chromatograms correspond to analysis using the same amount of sample. The *y*-axis scale is the same in (a)–(e).

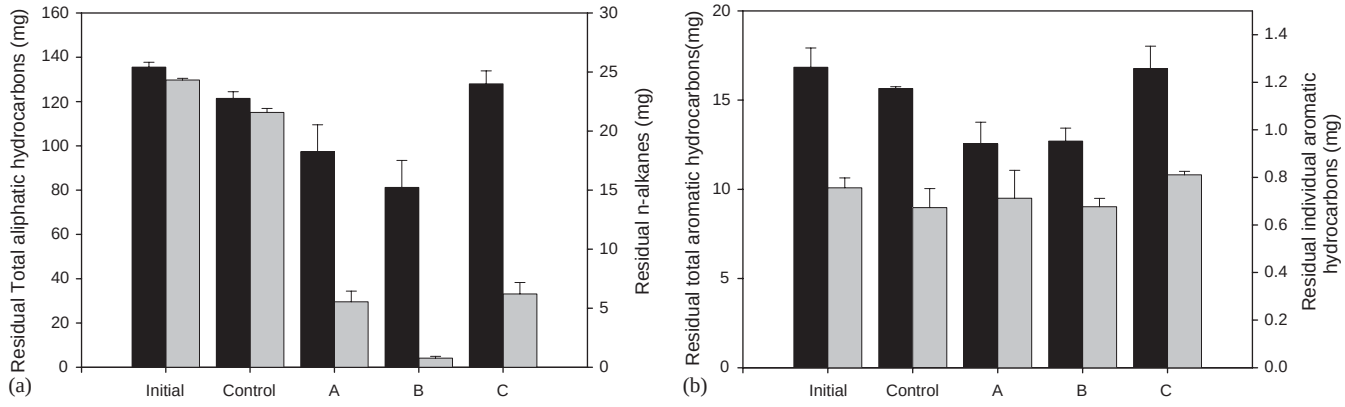

Fig. 3. Initial and final hydrocarbon content in control and treatments A, B and C: (a) total aliphatic hydrocarbons (black) and *n*-alkane hydrocarbons (grey); (b) total aromatic hydrocarbons (black); total of individual aromatic hydrocarbons acenaphthylene + fluorene + phenanthrene + methylphenanthrenes + dimethylphenanthrene + anthracene + pyrene (grey). Values are means of duplicate independent samples.

groundwater and soil environments (Atlas, 1981; Pitchard and Costa, 1991; Wade, 2001). In initial and sterile control experiments, the calculated index values were similar to those found in pure fuels (Wade, 2001). For treatment B, at the end of the experiment, *n*-C17/Pr and *n*-C18/Ph indices were lowest (0.01), agreeing with the highest *n*-alkane biodegradation (85.6%) observed (Table 2). In treatments A and B, isoprenoids were degraded to a similar degree, so that the two indices reflect the differences in *n*-alkane degradation. Treatment C showed similar *n*-alkane degradation to treatment A, but little isoprenoid degradation, so higher index values were found in the former (Table 2).

TArH reduction was similar in treatments A and B (Fig. 3b). Taking into account abiotic losses, the two treatments showed biodegradation efficiencies of approximately 18.3% and 17.5%, respectively (Table 2). There was no detectable biodegradation of

Table 2
Biodegradation efficiencies and indices in the degradation assay

	Initial	Control[a]	Treatment A[b]	Treatment B[b]	Treatment C[b]
Total aliphatic hydrocarbons (%)[c]	—	10.4	17.8	29.7	0
Total *n*-alkanes (%)	—	11.3	66.0	85.6	63.2
n-C17/Pr	2.22±0.12	2.27±0.07	0.29±0.06	0.01±0.00	0.10±0.01
n-C18/Ph	2.22±0.08	2.29±0.07	0.43±0.17	0.01±0.00	0.19±0.03
Total aromatic hydrocarbons (%)[b]	—	7.1	18.3	17.5	0

All values are means of duplicate samples.
[a]Abiotic loss at 10 days biodegradation assay.
[b]Biodegradation efficiencies at 10 days, calculated subtracting abiotic loss.
[c]Resolved, plus UCM compounds.

Table 3
Effect of pH on the emulsifying activity of hydrocarbon phase from 10-day cultures

	Treatment A		Treatment B		Treatment C	
Buffer pH[a]	pH[b]	E24%[b]	pH[b]	E24%[b]	pH[b]	E24%[b]
5.0	5.0	19.1±2.1	5.0	13.2±2.1	4.9	16.6±6.8
6.0	6.0	22.1±6.2	5.7	23.5±4.2	5.7	13.0±4.7
6.5	6.4	29.1±0.4	6.3	27.9±2.1	6.2	25.0±4.7
7.0	7.3	25.0±6.2	7.2	36.8±2.1	6.9	34.7±4.7
7.5	7.7	19.1±2.1	7.8	35.3±0.0	7.3	35.7±5.5
8.0	7.8	7.4±2.1	8.2	28.8±3.3	7.8	16.8±7.3
9.0	9.0	10.3±2.1	9.0	27.9±2.1	8.7	9.5±4.9

[a]Used to perform dilutions. See Section 2.6 for buffer composition.
[b]Measured in the hydrocarbon dispersed phase diluted with buffer (1:1 v/v).

the individual PAHs quantified. Treatment C showed no reduction in either TArH or individual compounds.

Lack of biodegradation of TAlH and TArH in treatment C could be attributed to partial degradation of the buffer, which could have acted as an alternative carbon source for microbial growth, limiting biodegradation of the more recalcitrant compounds.

The degree of biodegradation of the different hydrocarbon classes in the bilge waste (Table 2, Fig. 3) followed the order expected from biodegradability of these compounds, i.e., in decreasing order, *n*-alkanes, branch-aliphatics (isoprenoids), monocyclic aromatics and PAHs (Van Hamme et al., 2003).

For hydrophobic substances with extremely low water solubility, uptake of dissolved compounds is believed to play a minor role in cellular growth, while evidence suggests that biosurfactant/bioemulsifiers are involved in both uptake via direct cellular contact with hydrocarbon droplets larger than the cell and interaction of cells with pseudosolubilized or micellar phase hydrocarbons (Sekelsky and Shreve, 1999; Prabhu and Phale, 2003). As revealed by *n*-alkanes, TAlH and TArH biodegradation, *n*-alkane/isoprenoid index values and hydrocarbon profiles, treatment B offered the best performance in biodegrading hydrocarbons present in BWOP. Therefore, this superior performance of treatment B could be based on the high emulsifier activity indicated by the E24 values at the end of the experiment. These results suggest that conditions near neutrality would improve hydrocarbon degradation in BWOP.

3.3. Effect of pH in emulsifying activity

When a 10-day culture was separated into its three component phases (cells, supernatant and hydrocarbons) and E24 used as a test for emulsifying activity, the cells and supernatant did not show any emulsifying activity over the pH range tested (data not shown). On the other hand, the oily phase that was easily dispersed in SWM showed evidence of emulsifying activity in all treatments. The maximum E24 values for treatments B and C were found at pH 6.5–7.5 (Table 3).

Owing to the dilution involved, the emulsifying index values were, as expected, lower than those recorded for the entire culture (Fig. 1). The occurrence of emulsifying activity in the hydrocarbon phase could be the result of extraction of the surface-active compounds produced during the degradation from the cellular membranes or supernatant by the hydrocarbons (Cooper and Goldenberg, 1987). On the other hand, several hydrocarbon-degrading microorganisms produce fatty acids and phospholipid surfactants during growth on *n*-alkanes

(Desai and Banat, 1997). Depending on the length of the hydrocarbon chain, in such cases the emulsifier would be expected in the hydrocarbon phase.

As a function of the pH of the medium, the E24 profiles suggest that the bioemulsifier possesses higher activity at neutral pH. Consequentially, the better performance of hydrocarbon biodegradation in treatment B could be related to promotion of emulsifying activity by periodical adjustment of pH.

In summary, during biodegradation of bilge waste by a native microbial community, bioemulsifier production and decrease in pH were observed, and periodical neutralization with NaOH improved biodegradative performance and emulsification of the oily matter in the culture medium. These results suggest that, in order to minimize the residence time of the waste in a treatment unit, the culture medium should be neutralized at least periodically to increase the rate of biodegradation.

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