J Ind Microbiol Biotechnol (2003) 30: 542–548
DOI 10.1007/s10295-003-0078-5

ORIGINAL PAPER

N. L. Olivera · M. G. Commendatore · O. Delgado
J. L. Esteves

Microbial characterization and hydrocarbon biodegradation potential of natural bilge waste microflora

Received: 24 February 2003 / Accepted: 20 June 2003 / Published online: 1 August 2003

Abstract Shipping operations produce oily wastes that must be managed properly to avoid environmental pollution. The aim of this study was to characterize microorganisms occurring in ship bilge wastes placed in open lagoons and, particularly, to assess their potential to degrade polycyclic aromatic hydrocarbons (PAHs). A first-order kinetic was suitable for describing hydrocarbon biodegradation after 17 days of treatment. The calculated rate constants were 0.0668 and 0.0513 day^{-1} with a corresponding half-life of 10.3 and 13.5 days for the aliphatic and aromatic hydrocarbon fractions, respectively. At day 17, PAH removal percentages were: acenaphtylene 100, fluorene 95.2, phenanthrene 93.6, anthracene 70.3, and pyrene 71.5. Methyl phenanthrene removals were lower than that of their parent compound (3-methyl phenanthrene 83.6, 2-methyl phenanthrene 80.8, 1-methyl phenanthrene 77.3, 9-methyl phenanthrene 75.1, and 2,7-dimethyl phenanthrene 76.6). Neither pure cultures nor the microbial community from these wastes showed extracellular biosurfactant production suggesting that the addition of an exogenously produced biosurfactant may be important in enhancing hydrocarbon bioavailability and biodegradation. DNA analysis of bilge waste samples revealed a ubiquitous distribution of the *nahAc* genotype in the dump pools. Although almost all of the isolates grew on naphthalene as sole carbon source, only some of them yielded *nahAc* amplification under the experimental conditions used. The variety of PAHs in bilge wastes could support bacteria with multiple degradation pathways and a diversity of catabolic genes divergent from the classical *nah*-like type.

Keywords Hydrocarbons · Bilge wastes · Biodegradation · PAHs · *nahAc*

N. L. Olivera (✉) · M. G. Commendatore · J. L. Esteves
Centro Nacional Patagónico (CONICET), Blvd. Brown s/n,
9120 Puerto Madryn, Chubut, Argentina
E-mail: olivera@infovia.com.ar
Tel.: +54-2965-451024
Fax: +54-2965-451543

O. Delgado
Planta Piloto de Procesos Industriales
Microbiológicos (CONICET), Av. Belgrano y Pje. Caseros,
4000, Tucumán, Argentina

Introduction

Ship operations produce wastes that are collected in the lowest part of the hull, called the bilge area. This oil-containing bilge waste must be managed properly to avoid environmental pollution. Large vessels, for example, are equipped with oily bilge waste processing systems that allow the separation of oil from water, discharging water containing very little or no oil and retaining the residual waste on board.

Oil pollution on the Patagonian coast has been associated with ship and port operations [2]. The city of Puerto Madryn (Golfo Nuevo, Argentina) has two piers with intense fish and cargo shipping activity. This city is in an environmentally sensitive area where whales, seals, seabirds, and other coastal fauna reproduce. Local regulations state that no ship can leave the port without first unloading its bilge waste. Since 1995, bilge wastes are being collected in the port and disposed of on shore to prevent marine pollution. However, these residues must receive effective treatment and therefore studies leading to their natural biodegradation are of great interest.

Previous studies have shown that microorganisms occurring in bilge wastes placed in open lagoons have a high hydrocarbon degradation capacity, being able to biodegrade a wide range of hydrocarbons when they are cultured in a simple seawater-based medium. The use of a biosurfactant produced by a *Bacillus* strain, isolated from polluted sediments from the Patagonian coast, was also considered to improve their biodegradation activity. This biosurfactant (surfactin), used as a crude extract, increased hydrocarbon bioavailability and shortened the time required for its biodegradation [16, 18]. Moreover,

the microbial community from bilge wastes showed the capacity to degrade both aliphatic and aromatic hydrocarbons, the latter being the most dangerous fraction of hydrocarbons in these wastes at almost the same rate [18].

As a model of low-molecular-weight polycyclic aromatic hydrocarbons (PAHs), naphthalene biodegradation has been studied extensively. The first step in its degradation pathway is catalyzed by naphthalene dioxygenase (NahA); genes encoding different subunits of this enzyme have been studied in oil-degrading microorganisms in different environments [1, 5, 13, 23, 24]. A number of aromatic hydrocarbons such as phenanthrene, 2-methyl-naphthalene, 1-methyl-naphthalene, toluene, ethylbenzene, biphenyl, anthracene, and benzene are substrates of this enzyme [7, 9].

The aim of this study was to characterize native bilge waste microorganisms and assess their potential to degrade PAHs and to produce biosurfactants. As part of such characterization, we analyzed *nahAc* catabolic gene distribution in samples from bilge waste dump pools and in isolates recovered from the wastes. On the other hand, biodegradation experiments reported in this paper were performed in batch stirred-tank bioreactors; these systems allowed us to use larger volumes and concentrations of residues than those of previous experiments, and thus to evaluate future applications on a larger scale.

Materials and methods

Characterization of ship bilge waste hydrocarbons

In order to determine the composition of hydrocarbons contained in ship bilge wastes, composite samples were collected and solutions of 2.5 g residue in 50 ml hexane were prepared. Samples (1 ml) of the solutions were evaporated to 0.2 ml under soft flowing high purity N_2 gas at room temperature. The analysis and measurement using high-resolution gas chromatography (aliphatic hydrocarbons) and fluorescence (aromatic hydrocarbons) have been extensively described in previous papers [2, 18]. The aliphatic hydrocarbon fraction was analyzed for individual *n*-alkanes, pristane and phytane isoprenoids, total resolved *n*-alkanes, unresolved complex mixture (UCM), and total aliphatic concentrations. The UCM involves cycloalkanes, branched alkanes, and other compounds unresolved by the capillary column, which show as a "hump" below the resolved compounds. The total aliphatic fraction was defined as the sum of the identifiable alkane peaks and, if present, the UCM. Total aromatic hydrocarbons were measured by fluorescence. Excitation and emission wavelengths were 310 and 360 nm, respectively. Calibration was performed with chrysene as a standard, and results were expressed in chrysene equivalents [25].

In addition, the aromatic hydrocarbon fraction was analyzed by high resolution gas chromatography-mass spectrometry to identify and quantify individual PAHs.

Clean-up and isolation

A variety of substituted and non-substituted PAHs are found in bilge wastes. In order to optimize the clean-up and isolation of such compounds for their subsequent analysis by high-resolution gas chromatography, different column adsorption chromatography protocols were tested. The efficiency of each test was assessed using a mixture of aliphatic and aromatic hydrocarbon standards. The mixture contained 50 μg ml^{-1} of the following compounds: *n*-C20, *n*-C22, *n*-C25, *n*-C30, *n*-C38, naphthalene, acenaphthene, fluorene, anthracene, 1,2-benzanthracene, pyrene, indenopyrene and benzo(a)pyrene. Volumes of 2 ml were evaporated under high purity N_2 gas, redissolved in 0.2 ml hexane, and applied to the different adsorption columns. The test also included the analysis of bilge waste samples with addition of the standard mixture described above. The most efficient column contained 1 g 3% deactivated silica gel covered by anhydrous sodium sulfate (both pre-calcinated for 4 h at 450°C). Silica gel was activated at 200°C for 4 h, and then partially deactivated by addition of water previously extracted with methylene chloride. The mixture was agitated and allowed to equilibrate overnight. The column was first eluted with 4.5 ml hexane to collect the aliphatic fraction, and then with 4 ml of a mixture of hexane and methylene chloride (3:1), followed by 4 ml pure methylene chloride. PAHs were identified and quantified by comparison with external standard mixtures and mass spectrometry.

Microbial analysis

Microorganism isolation from ship bilge wastes

Bilge waste samples were serially diluted in sterile seawater and plated on seawater medium (SWM) containing, per liter of seawater: 1 g NH_4NO_3, 4 ml phosphate solution (25 g l^{-1} $Na_2HPO_4{\cdot}12H_2O$ and 3.6 g l^{-1} NaH_2PO_4), 0.2 g yeast extract, and 12 g agar-agar, with sterile bilge waste (6 ml l^{-1}) as carbon source. Culture medium and bilge wastes were autoclaved separately for 20 min at 120°C. The agar plates were incubated at 25°C for 5 days. Individual colonies were selected and purified by streaking them repeatedly under identical growth conditions. In order to characterize the isolates, morphology, Gram-staining, oxidase, and API 20 NE tests (bioMérieux, Marcy L'Etoile, France) were carried out.

Molecular analysis

DNA was extracted from isolates and purified as described by Moeseneder et al. [15]. Total DNA from ship bilge waste samples was extracted using the Ultra Clean Soil DNA kit (Mo Bio Laboratories, Solana Beach, Calif.). Purified DNA from both isolates and waste samples were used as templates for *nahAc* PCR amplification using degenerate primers: Ac149f (5′-CCCYGGCGAC TATGT-3′) and Ac1014r (5′-CTCRGGCATGTCT TTTTC-3′) [5]. PCR assays were performed in a GeneAmp PCR 9700 System (Applied Biosystems, Foster City, Calif.). The reactions were carried out in a final volume of 25 μl containing 5 ng DNA as template, 100 nM each primer, 2.5 μl 10× STR buffer, and 1 U *Pfu* DNA Polymerase (Promega, Madison, Wis.). In order to check amplifications, 10 μl PCR amplified products were electrophoresed on 2% (w/v) agarose gels [20]. DNA sequencing on both strands was performed by the dideoxy chain termination method using the commercial services of GATC Biotech (Constance, Germany). Isolate sequence data is available in the GenBank database under accession numbers AY196827–AY196830.

To correlate *nahAc* detection with the capacity to grow with naphthalene as the only carbon source, isolates were cultured in a medium containing, per liter of distilled water: NaCl 24 g; $MgSO_4{\cdot}7H_2O$ 1 g; KCl 0.7 g; KH_2PO_4 2 g; Na_2HPO_4 3 g; NO_3NH_4 1 g [14] and 2 g crystalline naphthalene. Cultures were incubated for 72 h at 25°C and 200 rpm. Turbidity of the culture medium in comparison with sterile controls was considered as a growth positive result.

Biosurfactant production

Isolates, as well as the whole microbial community from bilge wastes, were cultured in liquid SWM medium by incubating for 72 h at 25°C in an orbital shaker at 200 rpm. Samples of culture (15 ml) were then centrifuged at 12,000 *g* for 20 min. The aqueous phase was carefully withdrawn and used to measure surface tension at 25°C with a Du-

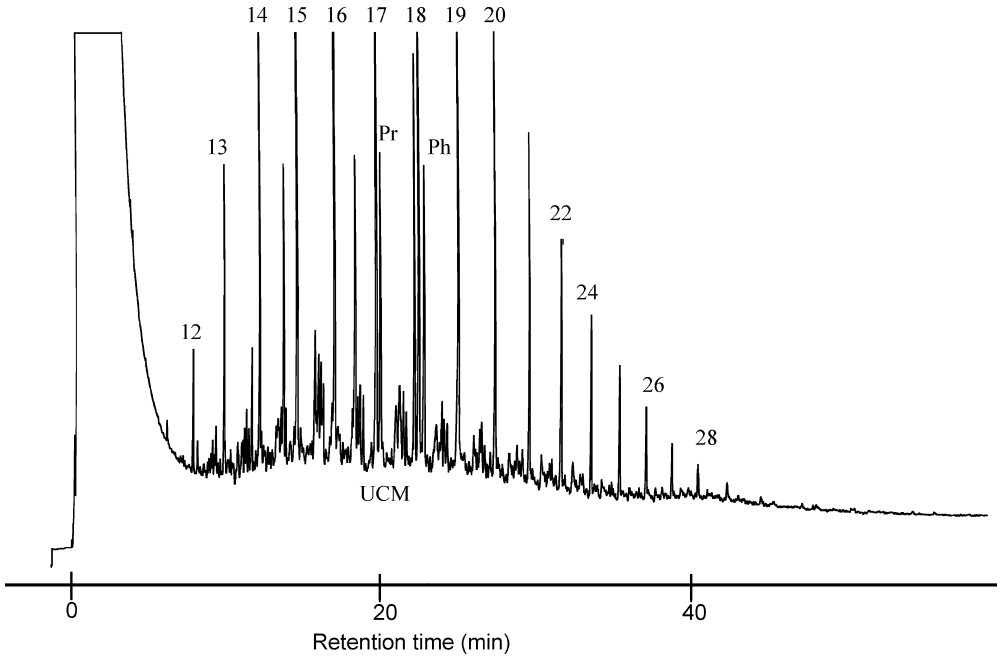

Fig. 1 Gas chromatographic profile of the aliphatic hydrocarbon fraction of ship bilge wastes. *Pr* Pristane, *Ph* phytane, *UCM*, unresolved complex mixture, *13–26* *n*-alkanes

Nouy ring tensiometer (CSC Scientific, Fairfax, Va.) as an indication of extracellular biosurfactant production [4]. The pellets were resuspended in the same medium and surface tension was determined to detect cell-associated biosurfactant activity.

Ship waste hydrocarbon biodegradation

A biodegradation experiment was conducted in two batch stirred-tanks (80-l capacity). Each reactor was filled with 47.5 l seawater and 2.5 l bilge waste. One of the reactors (Culture) was supplemented with (in g l^{-1}): NH_4NO_3, 1; yeast extract, 0,2; and 4 ml phosphate solution (25 g l^{-1} $Na_2HPO_4{\cdot}12H_2O$; 3.6 g l^{-1} NaH_2PO_4). This reactor also contained 6 mg l^{-1} *B. subtilis* O9 surfactin in the form of a crude extract [16] and 10 ml l^{-1} native waste microorganism inoculum, both prepared as previously described [18]. After 7 days of incubation, 0.25 and 0.02 g l^{-1} KNO_3 and Na_2HPO_4, respectively, were added to this reactor.

In order to quantify the abiotic loss of hydrocarbons, the second tank (Control) contained cefotaxime (20 mg l^{-1}) and nystatin (30 mg l^{-1}) to inhibit microbial growth. Neither nutrients nor inoculum were added to this reactor.

During the operation process, the reactors were maintained at room temperature (12–22°C), and the pH ranged from 7.05 to 7.62. Tanks were aerated by continuous air supply and stirred at 200 rpm, and the dissolved oxygen level was always over 60% saturation.

Microbial growth

Samples were collected periodically and viable counts were determined by the spread plate method as previously described [18].

Hydrocarbon analysis

Two composite samples from each reactor were collected at 0, 2, 9, and 17 days. Extraction, clean-up, isolation of hydrocarbon fractions (aliphatic and aromatic), and their analysis were performed as described above. The aliphatic fraction was measured by high-resolution gas chromatography and the total aromatic fraction was determined by fluorescence. First-order constant biodegradation rates were obtained from the plot of the natural logarithm of the substrate concentration, corrected for abiotic loss, vs time [21]. In the Culture reactor, the concentrations of individual PAHs were measured at the beginning and at the end of the experiment.

Results

Chemical characterization of bilge waste

In the aliphatic hydrocarbon fraction, the *n*-alkane homologous series from *n*-C11 to *n*-C30 was identified. In addition, pristane and phytane isoprenoids, and a UCM were detected. Figure 1 shows the chromatographic profile of the bilge waste used in the biodegradation experiments, where amounts of resolved aliphatic hydrocarbons and UCM were 83.2 ± 0.8 and 187.5 ± 13.8 mg (g waste)$^{-1}$, respectively. The UCM shape corresponds to a unimodal form related to low molecular weight compounds. UCM is normally present in pethrogenic hydrocarbon chromatographic profiles.

The aromatic fraction presented a high proportion of two- to four-ring compounds, many of which are methyl-substituted (Fig. 2). This fraction contains the most hazardous hydrocarbons because of their toxic effects on organisms and the environment. The identified compounds were: 2,3,5-trimethyl naphthalene, fluorene, phenanthrene, anthracene, 3-methyl phenanthrene, 2-methyl phenanthrene, 9-methyl phenanthrene, 1-methyl phenanthrene, pyrene, and chrysene.

Microbial analysis

Fourteen microorganisms with dissimilar API profiles were isolated from bilge wastes, all of them Gram-negative bacteria belonging mainly to the *Pseudomonas*

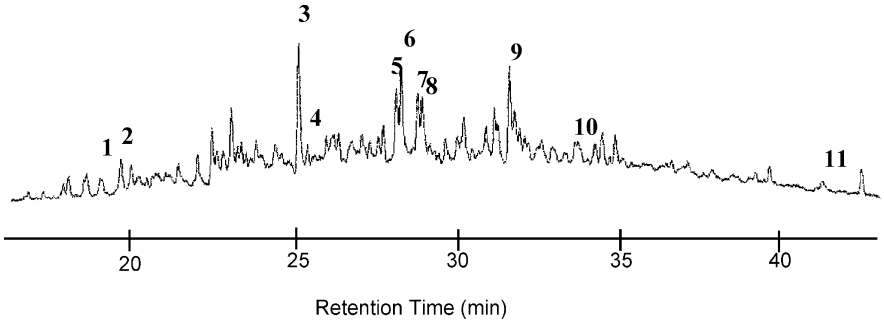

Fig. 2 Gas chromatographic profile of the aromatic hydrocarbon fraction of ship bilge wastes. *1* 2,3,5-Trimethyl naphthalene, *2* fluorene, *3* phenanthrene, *4* anthracene, *5* 3-methyl phenanthrene, *6* 2-methyl phenanthrene, *7* 9-methyl phenanthrene, *8* 1-methyl phenanthrene, *9* 2,7-dimethyl phenanthrene, *10* pyrene, *11* chrysene

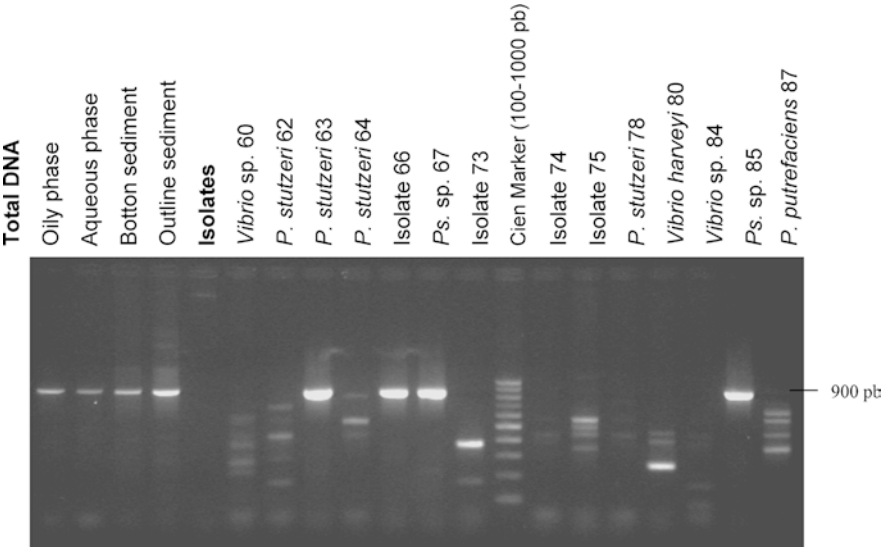

Fig. 3 Detection of the naphthalene dioxygenase gene *nahAc* in isolates and total DNA samples from bilge waste dump pools

genus. No eukaryotic microorganisms were isolated, in agreement with previous analyses of the bilge waste microbial community where the FAME (fatty acid methyl ester) profile corresponded to prokaryotic microorganisms and studies using PCR universal primers to this domain (unpublished data).

With the exception of *Pseudomonas stutzeri* 78, the isolates showed the capacity to grow with naphthalene as sole carbon source. Previous studies showed that *P. stutzeri* 78 degraded *n*-alkanes [17], and that bilge wastes have an aliphatic hydrocarbon fraction—composed of *n*-alkanes, isoprenoids, and a UCM—that supports development of this isolate.

The *nahAc* gene was detected in the entire DNA samples from communities, showing that microorganisms with such genotype are found in oily and aqueous phases of the waste as well as in the sediments of the dump pools. This genotype could be amplified from only some of the indigenous bacteria (Fig. 3).

The *nahAc* partial gene sequences of *P. stutzeri* 63, 67 and 85 have been compared with homologous genes encoding isofunctional proteins from other *Pseudomonas* naphthalene-degradation upper pathways. Sequence analysis of these fragments revealed a high degree of sequence similarity (99%) with the *nahAc* nucleotide sequence of *P. stutzeri* AN10 (AF039533). On the other hand, isolate 66 has 96% identity to the nucleotide sequences of the archetypical *Pseudomonas putida* G7 (M83949) and *Pseudomonas fluorescens* (AY048759) strains.

Cooper [3] defined a microorganism as a good biosurfactant producer if it is able to reduce the surface tension of the medium to 40 dyn cm^{-1} or less. Neither the microorganisms from the community inoculum nor the isolates had the ability to produce extracellular biosurfactants, as indicated by the high values of supernatant surface tension. On the other hand, a decrease in the surface tension of the fraction containing the microbial cells was detected for isolates 74, 80 (identified as *Vibrio harveyi*), and 85 (*Pseudomonas* sp.), suggesting a surface-activity associated with these cells (Table 1). This activity has been explained in terms of surface components that contribute to high cell surface hydrophobicity, which allows the cell to adhere to water-insoluble hydrocarbons [19].

Hydrocarbon biodegradation

Viable counts in the Culture reactor increased from 2.0 E+06 to 1.3 E+08 cfu ml^{-1} during the first 24 h. Thereafter, counts continued to increase gradually, reaching 8.8 E+08 cfu ml^{-1} at day 17 (Fig. 4). In the Control, no viable microorganisms were detected during the first 4 days of incubation. Later, possibly due to the presence of antibiotic-resistant strains, a microbial

Table 1 Surface tension (*ST*) in supernatants and resuspended pellets from pure and whole microbial community cultures. Results are the average values of two independent replicas

Cultures	Supernatant ST (dyn cm^{-1})	Resuspended pellet ST (dyn cm^{-1})
Sterile controls	66.4 ± 0.56	66.4 ± 0.56
Vibrio sp. 60	51.9 ± 1.16	46.8 ± 4.97
Pseudomonas stutzeri 62	53.8 ± 0.32	45.5 ± 2.71
P. stutzeri 63	50.0 ± 0.96	48.4 ± 8.31
P. stutzeri 64	57.9 ± 2.63	46.9 ± 0.35
Isolate 66	56.5 ± 0.23	59.8 ± 0.89
Pseudomonas sp. 67	52.6 ± 0.69	53.9 ± 0.74
Isolate 73	55.9 ± 0.33	46.0 ± 0.53
Isolate 74	62.4 ± 0.71	39.3 ± 1.18
Isolate 75	52.3 ± 0.33	45.5 ± 0.71
P. stutzeri 78	52.3 ± 1.20	55.2 ± 4.52
Vibrio harveyi 80	60.6 ± 1.04	38.4 ± 1.10
Vibrio sp. 84	55.1 ± 1.97	58.5 ± 1.35
Pseudomonas sp. 85	50.8 ± 0.85	35.3 ± 1.43
Pseudomonas putrefaciens 87	51.0 ± 1.24	50.7 ± 4.76
Bilge waste microbial community	48.7 ± 0.88	43.4 ± 1.17

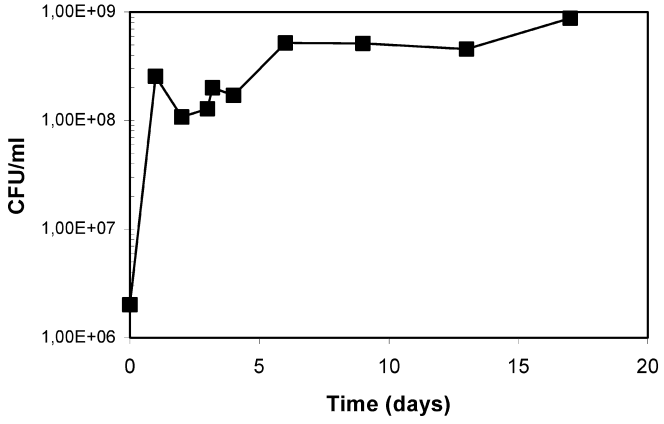

Fig. 4 Viable counts in Culture reactor. ■ Average values of duplicate samples

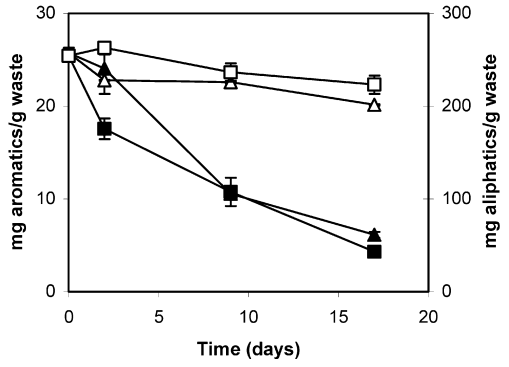

Fig. 5 Total aliphatic (*square symbols*) and total aromatic (*triangles*) hydrocarbon concentrations in Culture and Control bioreactors. *Solid symbols* Culture, *open symbols* Control. Values are the average of duplicate samples

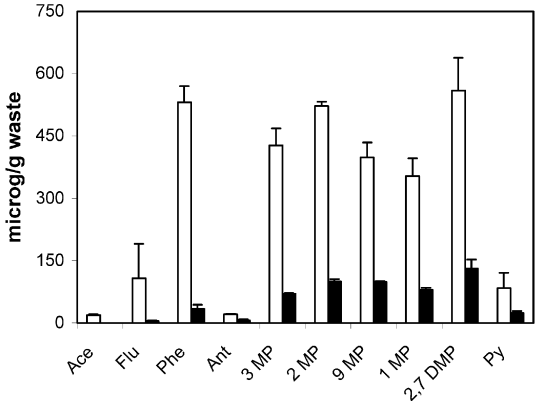

Fig. 6 Concentrations of individual polycyclic aromatic hydrocarbons (PAHs) at the beginning (*white bars*) and after 17 days of treatment (*black bars*) in the Culture reactor. *Ace* Acenaphtylene, *Flu* fluorene, *Phe* phenanthrene, *Ant* anthracene, *3MP* 3-methyl phenanthrene, *2MP* 2-methyl phenanthrene, *1MP* 1-methyl phenanthrene, *9MP* 9-methyl phenanthrene, *2,7DP* 2,7-dimethyl phenanthrene, *Py* pyrene. Values are the average of duplicate samples

growth of E + 03 cfu ml^{-1} at day 17 was observed. Even so, counts in the Culture were much higher than in the Control throughout the experiment, so it was assumed that the loss of hydrocarbons in the Control was due mainly to evaporation.

After a 17-day period, the amounts of aliphatic and aromatic hydrocarbons removed in the Culture reactor were 229.2 ± 7.51 and 19.6 ± 0.45 mg (g waste)$^{-1}$, respectively. These values corresponded to a decrease in their initial concentrations of 83.1 and 76.2%, respectively. In the Control, 12.2 and 21.7% of the initial concentrations of aliphatic and aromatic hydrocarbons, respectively, disappeared in the same period (Fig. 5).

After 17 days of Culture reactor operation, removal rates of acenaphtylene, fluorene, phenanthrene, methyl-substituted phenanthrenes, anthracene, and pyrene were evaluated (Fig. 6). The removal of non-substituted PAHs decreased as the molecular weight increased (acenaphtylene 100%, fluorene 95.2%, phenanthrene 93.6%, anthracene 70.3%, and pyrene 71.5%). The removal percentages of methyl phenanthrenes were lower than that of their parent compound (3-methyl phenanthrene 83.6, 2-methyl phenanthrene 80.8, 1-methyl phenanthrene 77.3, 9-methyl phenanthrene 75.1, and 2,7-dimethyl phenanthrene 76.6). In bilge waste, the presence of these types of hydrocarbons is in agreement with its pethrogenic origin.

Discussion

Development of an efficient biological treatment for ship bilge waste is largely motivated by the need for more effective remediation processes that avoid hydrocarbon pollution of terrestrial as well as marine environments. The results obtained demonstrate the feasibility of applying a microbial treatment to reduce hydrocarbon content in this waste. An important reduction in the concentrations of aliphatic and aromatic hydrocarbons

in the waste was reached after 17 days of treatment in 80-l batch stirred-tank reactors. In comparison with previous laboratory studies [16, 18], these systems allow the waste concentration in the medium to be increased by 10 times (5% v/v), while maintaining high biodegradation percentages.

Despite the complexity of the hydrocarbon biodegradation process, first-order kinetics proved to be suitable to describe biodegradation of aliphatic and aromatic hydrocarbon mixtures, diesel oil, and petroleum refinery sludges [11, 12, 22]. Aliphatic and aromatic hydrocarbon concentrations from the Culture reactor were corrected considering the hydrocarbon loss determined in the Control, and then plotted against time. For both fractions, a linear correlation was observed within 17 days (aliphatic $r^2 = 0.9656$, aromatic $r^2 = 0.9105$). The calculated rate constants were 0.0668 and 0.0513 day^{-1} with a corresponding half-life, defined as the time taken for half of the original substrate amount to be biodegraded, of 10.3 and 13.5 days for the aliphatic and aromatic hydrocarbon fractions, respectively.

At day 2, the aliphatic concentration in the Culture reactor was lower than in the Control; however, aromatic concentrations were similar in both treatments (Fig. 5). This indicates that abiotic losses were the main component of the aromatic decrease in the first hours of incubation. Therefore, hydrocarbons from the aliphatic fraction must have accounted for microbial growth observed in this period. Such differences in the evolution of aliphatic and aromatic degradation rendered a lower constant rate for the latter. Even so, the results demonstrated a high removal rate for the PAHs analyzed (Fig. 6). Higher phenanthrene degradation (93.6%) over anthracene (70.3%) was probably due to the higher solubility of the former. A similar difference in the relative magnitude of the removal rate of these compounds was reported by Gamati et al. [6]. They found 79.3% and 60.5% of phenanthrene and anthracene degradation, respectively, after a residence time of about 10 days in a pilot plug-flow slurry bioreactor.

The non-substituted PAHs identified belong to the group of priority pollutants defined by the Unites States Environmental Protection Agency (see [10]). Although the methyl substituted PAHs are not defined in the same way, the similarity in structure to their parent compounds together with their abundance in this kind of waste suggest the importance of studying their fate in biodegradation experiments. According to Irwin et al. [8], although there is less toxicity information available for most of the alkyl PAHs than for their parent compounds, most alkyl PAHs appear to be at least as toxic or hazardous as the parent compounds. Thus, for now, risk assessment experts suggest interpreting alkyl homologue values against known toxicological effect benchmarks and criteria for the corresponding parent compound.

Neither the pure cultures nor the microbial community from bilge wastes showed extracellular biosurfactant production, suggesting that the addition of an exogenously produced biosurfactant may be important, as shown by the results of previous hydrocarbon biodegradation experiments [16, 18].

Our results revealed a ubiquitous distribution of the *nahAc* genotype in bilge waste dump pools. On the other hand, although almost all of the isolates grew on naphthalene as sole carbon source, only four of them yielded *nahAc* amplification. Bilge wastes contain a mixture of many different PAHs and this variety of compounds could support the development of bacteria with multiple degradation pathways and a diversity of catabolic genes divergent from the classical *nah*-like type. Laurie and Lloyd-Jones [13] described a divergent set of PAH catabolic genes, the *phn* genes, from *Burkholderia* sp. strain RP007 which, although isofunctional to the *nah*-like genes, showed very low homology to them. Considering these results, new studies are being carried out to amplify hydrocarbon catabolic genes from the isolates that have not produced positive results for *nah* detection, which include the design of new primers and Southern-blot analysis.

Acknowledgements This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT 07-04069. BID 1201/OC-AR), (PICT-PIP 4271/96), Argentina.

References

1. Bosh R, Garcia-Valdes E, Moore ERB (1999) Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. Gene 236:149–157
2. Commendatore MG, Esteves JL, Colombo JC (2000) Hydrocarbons in coastal sediments of Patagonia, Argentina: levels and probable sources. Mar Pollut Bull 40:989–998
3. Cooper DG (1986) Biosurfactants. Microbiol Sci 3145–3150
4. Cooper DG, Goldenberg BG (1987) Surface active agents from two *Bacillus* species. Appl Environ Microbiol 53:224–229
5. Ferrero M, Llobet-Brossa E, Lalucat J, García-Valdés E, Rosselló-Mora R, Bosch R (2002) Coexistence of two distinct copies of naphthalene degradation genes in *Pseudomonas* strains isolated from the western Mediterranean region. Appl Environ Microbiol 68:957–962
6. Gamati S, Gosselin C, Bergeron E, Chenier M, Truong TV, Bisaillon JG (1999) New plug flow slurry bioreactor for polycyclic aromatic hydrocarbon degradation. In: Alleman B, Leeson A (eds) Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. Battelle Press, Columbus, Ohio, pp 1–6
7. Haigler BE, Gibson DT (1990) Purification and properties of NADH-ferredoxin NAP reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J Bacteriol 172:457–464
8. Irwin RJ, VanMouwerik M, Stevens L, Seese MD, Basham W (1997) Environmental contaminants encyclopedia. National Park Service, Water Resources Division, Fort Collins, Co.
9. Jeffrey AM, Yeh HJ, Jerina DM, Patel TR, Davey JF, Gibson DT (1975) Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry 14:575–584
10. Kanaly RA, Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067
11. Karin A, Berry T, Burton DL (1997) Natural attenuation of diesel fuel in heavy clay soil. Can J Soil Sci 77:469–477
12. Lagrega MD, Buckingham PL, Evans JC (1994) Hazardous waste management. McGraw-Hill, New York

13. Laurie AD, Lloyd-Jones G (2000) Quantification of *phnAc* and *nahAc* in contaminated New Zealand soils by competitive PCR. Appl Environ Microbiol 66:1814–1817
14. Mills AL, Breuil C, Colwell RR (1978) Enumeration of petroleum-degrading marine and estuarine microorganisms by the most probable number method. Can J Microbiol 24:552–557
15. Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:3518–3525
16. Morán AC, Olivera N, Commendatore M, Esteves JL, Siñeriz F (2000) Enhancement of hydrocarbon waste biodegradation by the addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11:65–71
17. Olivera N (2001) Biorremediación de sistemas contaminados con hidrocarburos. Universidad Nacional del Sur. Bahía Blanca, Argentina. PhD thesis
18. Olivera N, Commendatore MG, Morán AC, Esteves JL (2000) Biosurfactant-enhanced degradation of residual hydrocarbons from ship bilge wastes. J Ind Microbiol Biotechnol 25:70–73
19. Rosenberg E, Ron EZ (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162
20. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. Sepic E, Trier C, Leskovsek H (1996) Biodegradation studies of selected hydrocarbons from diesel oil. Analyst 121:1451–1456
22. Sepic E, Bricelj M, Leskovsek H (1997) Biodegradation studies of polyaromatic hydrocarbons in aqueous media. J Appl Microbiol 83:561–568
23. Stapleton RD, Sayler GS (1998) Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. Microb Ecol 36:349–361
24. Stapleton RD, Bright NG, Sayler GS (2000) Catabolic and genetic diversity of degradative bacteria from fuel-hydrocarbon contaminated aquifers. Microb Ecol 39:211–221
25. United Nations Environmental Programme (1992) Determinations of petroleum hydrocarbons in sediments. Reference methods for marine pollution studies, no. 20. UNEP, Nairobi, Kenya