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Oily Bilge Wastes Harbor a Set of Persistent Hydrocarbonoclastic Bacteria Accompanied by a Variable *alkB* Gene Composition in Marine Vessel Samples from Southwestern Atlantic Port of Mar del Plata, Argentina

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Abstract The present work provides the first knowledge about the diversity and functionality of the microorganisms occurring in oily bilge wastes, from the port of Mar del Plata, Argentina. We used a culture independent approach, based on massive sequencing of the 16S rRNA gene (Illumina), to analyze the microbiome structure from different oily bilge wastes. To gain insight into functional biomarkers for hydrocarbon (HC) degradation in these wastes, the occurrence of *alkB* genes in the degrading bacteria communities was also investigated trough PCR-DGGE method. The microbial compositions were determined in bilge waste oily phase (BWOP) samples from a deep-sea, a fishing ship, a dredge ship, a small coastal vessel, and an open pool, where bilge wastes from Mar del Plata's vessels are deposited. A core microbiome with OTUs classified as bacterial types associated with HC biodegradation was found across all samples. Seven highly predominant and stable bacterial types accounted for 82% of the relative composition determined in all the BWOP samples. These results indicate that oily bilge is a strong selector for bacteria having common taxonomical and functional features, independent of the vessel of collection. Alkane degradation marker gene *alkB* PCR-DGGE fingerprinting showed distinct diversity patterns among the samples. Our analysis showed that there is a well-defined bacterial assemblage able to withstand and thrive in the harsh conditions of the oily bilge wastes. Also, a well-established aliphatic biodegradation potential marker was detected, indicating that it could promptly be used for the generation of new bioremediation technologies.

Keywords Hydrocarbon · Bilge waste · Microbiome · Alkane monooxygenase gene

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1 Introduction

Microorganisms are the main source of biodiversity on our planet and play an essential role in maintaining biogeochemical cycles and in the purification of contaminated environments. In recent years, metagenomic techniques have provided a new perspective on microbial diversity and functionality in these environments. Standard culture methods permit the characterization of less than 0.1–1% of microbial diversity in the majority of natural ecosystems (Amann et al., 1995). Metagenomics has helped us in closing the gap left by culturing techniques and has provided insights into in situ microbial structures, dynamics, and functioning, thereby enhancing bioremediation processes (Malla et al., 2018).

The HC input into the oceans has a great impact on the pollution of the marine ecosystems and is of major environmental and health concern worldwide. One of the main anthropogenic sources of HC in the sea is the discharge of bilge wastes, which is estimated to be even higher than accidental oil spills (Cazoir et al., 2012; Etkin, 2010; GESAMP, 2007; National Research Council, 2003; Pavlakis et al., 2001).

Bilge wastes are liquid residues generated in normal ship operations that are collect in the bottom of the hull. They are mainly composed of a seawater phase and an oily phase (bilge waste oily phase, BWOP), which usually contains diesel and fuel oil, and other HC residues as hydraulic oils and lubricant oils (EPA, 2008). International maritime legislation limits the discharge of bilge wastes in the open ocean to less than 15 ppm of HC for vessels bigger than 400 t, while in certain sensitive areas the discharge is totally prohibited and all bilge wastes must be treated on land (IMO, 1988). Different technologies have been proposed and used to treat bilge wastes, among them, ultrafiltration (Tomaszewska et al., 2005), oxidation of humid air (López Bernal et al., 1999), electrocoagulation (Aswathy et al., 2016), and incineration and biodegradation (Emadian et al., 2015). However, the development and optimization of environmentally friendly and low-cost treatments are of great interest.

Microbial biodegradation is one of the main processes for removal of contaminants from the environment (Prince & Atlas, 2005). Advancing our knowledge on the ecology of HC degraders in wastewaters is important to improve reliance on natural attenuation and/or optimize bioremediation processes at contaminated wastes. Many studies have demonstrated the presence of degrading microorganisms at different sites and residues contaminated with HC, including bilge wastes (Olivera et al. 2003; Santisi et al. 2015ab; Nievas et al. 2006; Sivaraman et al. 2011; Cappello et al. 2012; Nisenbaum et al. 2020; Corti-Monzón et al. 2020). The utilization of indigenous microorganisms in the treatment of wastewater is advantageous over the use of non-native microorganisms, because they are better adapted to toxic residues and to the inherent fluctuation on its composition.

Saturated HC (n-alkanes, branched alkanes and cycloalkanes) constitute the main fraction of BWOP (Nievas et al., 2005, 2006). Several microorganisms are known to utilize alkanes as carbon sources, using different uptake strategies followed by their chemical activation via highly specific enzymatic complexes (Guibert et al., 2016; Nie et al., 2014; Rojo, 2009). Enzymatic activation of alkanes involves enzymes from different families, being integral membrane nonheme iron monooxygenases (AlkB) the most widely found among HC-degrading bacteria (Van Beilen & Funhoff, 2007). More than 250 AlkB homologs have been discovered in at least 45 bacterial species of multiple genera from a wide range of environments (Wang et al., 2010). These enzymes catalyze the terminal or subterminal oxidation of n-alkanes to primary or secondary alcohols, being the initial step in the aerobic degradation of n-alkanes (Van Beilen et al., 2003). Various oligonucleotide primers and DNA probes targeting *alkB* genes have been developed over the last years (Smits et al., 2002; Kloos et al., 2006; Hamamura et al., 2008; Olivera et al., 2009; Viggor et al., 2013), which can be applied to the study of specific bacterial groups or general HC-degrading bacteria in the environment. As a result, the *alkB* gene has been used as a molecular marker to evaluate the bioremediation capacities of oil-polluted environments (Kuhn et al., 2009; Wang et al., 2010). Therefore, knowledge about the diversity of the *alkB* gene, as well as information about their host bacteria in oily wastewater, can help to assess the potential of indigenous microorganisms for the design of bioremediation strategies. Although alkanes are

major bilge wastes components, data about *alkB* gene in these highly toxic wastes have not been published, and also little research has been performed regarding microbial diversity inhabiting these residues.

The present work aims to provide the first knowledge about the diversity and functionality of the microorganisms present in BWOP, collected in the Port of Mar del Plata, Argentina. We used a culture independent approach, based on massive sequencing of the 16S rRNA gene (Illumina), to analyze the microbiome structure from different BWOP. To gain insight into functional biomarkers for HC degradation in these residues, the occurrence of *alkB* genes in the degrading bacteria communities was also investigated trough PCR-DGGE method.

2 Materials and Methods

2.1 Bilge Waste Oily Phase Sampling

Four different kinds of BWOP samples coming from Mar del Plata Port (38° 02′S, 57° 31′ 30″ W) were collected from three kinds of vessels: a deep-sea fishing ship (A), a dredge ship (D), a small coastal vessel (C), and a sample obtained from a pool containing bilge wastes taken in port wastewater reception facilities (M). BWOP samples were aliquoted and stored at 4 °C for further different analyses. Aliquoted fractions used for the detection of HC were acidified to pH 2 (American Society of Testing Materials, ASTM Standard D7678, 2011).

2.2 Physicochemical BWOP Characterization

BWOP settleable solids and relative density were measured according to methods 2540 and 2710F (American Public Health Association, APHA et al. 1998), respectively. Water content, density, and kinematic viscosity were analyzed according to ASTM D4928 (2012), ASTM Standard D854 (2014), and ASTM Standard D88-07 (1999) methods, respectively. Conductivity and pH were measured by portable meters HI933100 and HI98103 (Hanna Instruments, Inc.).

Total petroleum hydrocarbon (TPH) determination was carried out on HP 6890 gas chromatograph with FID detector, using the method 1005 (TNRCC, 1005, 2001) by liquid–liquid extraction with n-pentane (Merck) as extraction solvent. The characterization of aliphatic (AlH) and aromatic (ArH) fractions nC6 to nC35 was carried out from the fraction obtained with n-pentane, on a HP 7890 Gas Chromatograph with MSD detector, using the TNRCC 1006 (2000) standard.

To analyze the variation in the means of the different properties according to the origin of the samples, the Krustall-Wallis test or ANOVA test were applied, depending on the normal distribution of the variables. A significance value of 0.05 was used in all analyses.

2.3 Microbiological Characterization of the BWOP

2.3.1 DNA Extraction

For microbiome DNA extraction and purification, we followed standard procedures (Sambrook et al., 1989) with additional steps reported to preserve stable microbiome compositions in the collected samples (Neu et al., 2021) and to work in oil-contaminated samples metagenomes (Vilchez-Vargas et al., 2013). Fifty milliliters of each homogenized BWOP sample were taken for total DNA extraction. Cells were harvested by centrifugation (20 min at 8,000 rpm). Pellets of approximately 200 mg wet weight were obtained from each sample. Absolute ethanol was added to these pellets to a final volume of 2 ml, and microbiome composition in samples was preserved at 4 °C until further processing. Four hundred milligrams of silica beads and 400 µL of Extraction Buffer (10 mM Tris–HCl [pH 8.0], 20 mM EDTA [pH 8.0], 2% (w/v) SDS, 6 M Urea) were added to 1 mL of the ethanol diluted sample, and placed in a vortex mixer at a maximum speed for 5 min. Twenty microliters of 10 mg/mL proteinase K were added, followed by incubation during 2 h at 55 °C. Then, 0.1 volume of NaCl 5 M was added and mixed by inversion. An equal volume of phenol–chloroform-isoamyl alcohol (25:24:1, pH 8) was added, vortexed briefly, and the aqueous phase was separated by centrifuging for 10 min (13,000 rpm at 4 °C), mixed with an equal volume of chloroform-isoamyl alcohol (24:1), and followed by 10 min of centrifugation (13,000 rpm at 4 °C) for aqueous phase separation. Total DNA was precipitated from the extracted aqueous layer, adding an equivalent of 0.1X supernatant

volume of 3 M sodium acetate, and 0.6× supernatant volume of isopropanol. Samples were incubated overnight at − 20 °C, followed by 30 min of centrifugation (13,000 rpm at 4 °C). Resulting DNA pellets were washed in ice-cold 70% (v/v) ethanol, dried, and re-suspended in 30 µL of TE Buffer. Finally, DNA was quantified using Nanodrop ND-1000 (Thermo Scientific).

2.3.2 16S rRNA Gene Amplification and Sequencing

PCR amplicon libraries of the variable V4 region of the 16S rRNA genes were prepared to determine the bacterial and archaeal compositions in the samples. To obtain these amplicons from metagenomic DNA, the primers 515F [5′-GTGYCAGCMGCCGCG GTAA-3′] (Moyer et al., 1998) and 806R [5′-GGA CTACNVGGGTWTCTAAT-3′] (Kolmakova et al., 2014) were used according to the Earth Microbiome Standard Protocol https://earthmicrobiome.org/protocols-and-standards/16s/. The PCR mixture contained 1X buffer, 0.3 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.04 U μL^{-1} accuzyme polymerase (Bioline), and 1 ng of template DNA. The PCR cycle conditions used were initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 50 °C for 60 s, and extension at 72 °C for 90 s. A final extension was done at 72 °C for 10 min. Sequencing was made using the MiSeq Illumina platform (Baylor College of Medicine, USA).

2.3.3 Diversity Analysis

All sequence analyses were performed using QIIME 1.9.1 (Kuczynski et al., 2011). Initially, the set of Illumina reads was filtered and split according to the barcodes. Chimera sequences were identified, extracted, and excluded out of the datasets by Usearch 6.1. A multi-step open-reference OTU picking workflow was performed within the QIIME system. Using this methodology, OTUs were picked for assigning reads to species groups based on 97% sequence similarity. Naïve Bayesian Classifier (Wang et al., 2007) was used with SILVA 132 (Glöckner, 2019) as taxonomic reference. Alpha and beta diversity analysis were performed as previously described (McMurdie & Holmes, 2013). Tax4fun (Aßhauer et al., 2015) implemented in Microbiome Analyst (Chong et al., 2020) was used to infer functions that could be potentially enriched in these microbiomes. The raw sequences produced and analyzed in this manuscript are available in the NCBI BioProject https://www.ncbi.nlm.nih.gov/bioproject/PRJNA667237/ with SRA accessible under https://www.ncbi.nlm.nih.gov/sra/PRJNA667237/.

2.4 *Denaturing Gradient Gel Electrophoresis (DGGE) of alkB Genes*

2.4.1 DNA Extraction

A pre-extraction method was included and optimized in order to remove the organic pollutants present in the BWOP samples (Purohit et al., 2003). Four subsamples of 2 mL were centrifuged at 12,000× *g* for 15 min. The BWOP pellets were re-suspended in Buffer 1 (50 mM Tris HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.05% (v/v) Triton X-100) and harvested at 12,000× *g* for 15 min. When the samples had high oil content, this step was repeated. Then, the pellets were washed in Buffer 2 (50 mM Tris HC pH 8.0, 200 mM NaCl, 5 mM EDTA), centrifuged and re-suspended in Buffer 3 (10 mM Tris HCl pH 8.0, 0.1 mM EDTA). The sludge pellet was recovered by centrifugation at 2,500× *g* for 10 min and subjected to two more washes with double-distilled water. The pellet bilge sub-samples were put together in a 2-mL microcentrifuge tube, pelleted once more at 12,000× *g* for 15 min and used for DNA extraction.

DNA was extracted using cetyltrimethylammonium bromide (CTAB) method (Ellis et al., 2003). This technique was adapted to extract DNA from BWOP. Buffer CTAB (1 mL of 100 mM Tris–HCl, 100 mM EDTA [pH 8.0], 100 mM phosphate buffer [pH 8.0], 1.5 M NaCl–1% CTAB) plus 20 µL lysozyme (1 mg mL^{-1}) and 20 µL proteinase K (20 mg mL^{-1}) were added to the BWOP pellets obtained from the pre-extraction steps. Tubes were then agitated on a platform shaker at maximum speed for 20 min. Subsequently, 200 µL of 10% (w/v) SDS (sodium dodecyl sulfate) was added, and the samples were incubated at 65 °C for 30 min. The lysates were maintained on ice for 2 min and centrifuged at 12,000× *g* for 5 min. The supernatant was transferred to a fresh tube and the pellet was re-extracted with 300 µL of buffer CTAB and centrifuged again. The supernatants were extracted with an equal volume of

chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was precipitated with 0.6 volume of isopropanol at 20 °C for 30 min. The pellet was washed in 300 µL of 80% (v/v) ethanol and air dried before resuspending in 100 µL of MiliQ water. DNA was quantified using Nanodrop One/One (Thermo Fisher, Scientific) and stored at − 20 °C until subsequent PCR amplification.

2.4.2 PCR Amplification of alkB Gene Fragments

The *alkB* gene fragments were amplified using the *alkB*484F (5′-GGKCAYTTCTWCRTYGARCA-3′) *alkB*824R (5′-CCGTAGTGYTCRABRTARTT-3′) primer set (Olivera et al., 2009). PCR reactions were performed in 25 µL volume containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1 U of T-PLUS DNA polymerase (Inbio Highway, Tandil, Argentina). Template DNA concentration was optimized for each sample (1–5 ng/reaction). Cycling conditions involved an initial 5-min denaturing step at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 45 °C, and 30 s at 72 °C, and a final 15 min elongation step at 72 °C. Four separate reactions were run for each sample, pooled and purified from 1.5% (w/v) agarose gels using Wizard SV Gel and PCR clean-up System kit (Promega, Madison, WI, USA).

2.4.3 DGGE Fingerprinting of alkB Genes

For DGGE analysis, a 39-bp GC-clamp (Baek et al., 2007) was added to the 5′ end of the *alkB*F primer using the same PCR conditions. DGGE was carried out following the method of Muyzer et al. (1993). Approximately 500 ng of the amplified samples were loaded on a 6% (w/v) polyacrylamide gel (acrylamide and *N*,*N*′-methylene bisacrylamide at a ratio of 37:1) with a linear DNA denaturing gradient that ranged from 40 to 70% (where 100% is defined as 7 M urea and 40% deionized formamide). Gels were electrophoresed in a 1 × TAE buffer (40 mM Tris pH 7.4, 20 mM sodium acetate, 1 mM EDTA) for 16 h at a constant temperature (60 °C) and voltage (100 V), using a DGGE-2000 system (CBS Scientific Company). Gels were then stained with the nucleic acid stain SYBR Gold (Molecular Probes) for 30 min and visualized with UV in GelDoc EQ (Bio-Rad, Hercules, CA, USA). In addition, the DGGE bands of stronger intensities and differences in electrophoretic mobility were excised using a razor blade and soaked overnight at 4 °C in 20 µL of MilliQ water, followed by a freeze–thaw cycle. Three microliters of the dissolved DNA was used as a template in a further PCR reaction with the primers and conditions above described. PCR products were purified with Wizard® Genomic DNA Purification Kit (Promega) and sequenced by Sanger method (INTA Castelar, Argentina).

2.4.4 Phylogenetic Analyses

Retrieved nucleotide sequences without chimeras were edited using the FinchTV 1.4.0 program (Geopiza Inc.) and overlapped with the BioEdit Sequence Alignment Editor 7.0.9. Software (Hall, 1999). Each obtained consensus sequence was compared with sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov), using BLAST to identify them (Altschul et al., 1997). Then, sequences were aligned to their nearest neighbor using CLUSTAL X (Thompson et al., 1997). Phylogenetic trees were constructed based on the Kimura 2-parameter (K2P) model (Kimura, 1980) and the neighbor-joining algorithm (Saitou & Nei, 1987) using the MEGA 4.0 software (Tamura et al., 2007). Bootstrap analysis with 1000 replicates was applied to assign confidence values to the nodes of the consensus trees.

Sequences reported in this study were deposited at the GenBank database under accession numbers MT758583 to MT758601.

3 Results and Discussion

3.1 Bilge Waste Oily Phase Properties

Bilge wastes have particular characteristics depending on their origin (type and age of ship, fuel used, lubricants and chemicals used on board, age of the waste, among others) (Church et al., 2019). In this work, BWOP from different ships (a deep-sea fishing ship (A), a dredge ship (D), a small coastal vessel (C), and a mixed parked sample (M)) were analyzed, and their physical and chemical properties are shown in Table 1. The density and specific gravity values denote the fluidity of these samples. The observed density values for BWOP A, C, and D agreed with

Table 1 Physical–chemical characteristics of the BWOP samples analyzed

	BWOP A	**BWOP C**	**BWOP D**	BWOP M
Settleable solids ($mL \cdot l^{-1}$)	0.6 ± 0.02	0.1 ± 0.01	≤ 0.10	≤ 0.10
Specific gravity	0.96 ± 0.01	0.85 ± 0.03	0.99 ± 0.01	0.79 ± 0.01
Conductivity ($mS \cdot cm^{-1}$)	29 ± 0.30	≤ 1.50	≤ 1.50	≤ 1.50
Density ($g \cdot mL^{-1}$)	0.93 ± 0.01	0.92 ± 0.02	0.95 ± 0.00	0.86 ± 0.01
Kinematic viscosity (cSt, 40 °C)	19.08 ± 0.00	59.36 ± 0.29	44.52 ± 0.58	55.4 ± 0.06
Water content (%, v/v)	30.00 ± 0.20	1.59 ± 0.04	2.15 ± 0.21	0.78 ± 0.03
pH	4.99 ± 0.18	4.85 ± 0.07	6.75 ± 0.07	6.50 ± 0.13
COD ($mgO_2\ L^{-1}$)	665.66 ± 14.01	1344.66 ± 30.09	1238.33 ± 43.68	1530.00 ± 43.59
BOD ($mgO_2\ L^{-1}$)	2932 ± 0.01	1113.33 ± 32.15	948.00 ± 50.71	1056 ± 37.59
Total petroleum hydrocarbons (TPH) ($g \cdot L^{-1}$)	554	664	1540	910
Gasoline range organics (GRO) ($g \cdot L^{-1}$)	62	54	100	86
Diesel range organics (DRO) ($g \cdot L^{-1}$)	430	570	1380	796
Aliphatic hydrocarbons (AlH)				
nC8-nC10	29.4	70	142	96
nC10-nC12	50	138	440	190
nC12-nC16	190	378	740	488
Aromatics hydrocarbons (ArH)				
nC8	15.6	33	48	32.4
nC8-nC10	176	34	106	41

the estimated for the fuel oil No. 4 (ASTM 396-15a), with no significant differences among them. BWOP M had a lower value than fuel oil No. 2 (ASTM-D396-15a), but closed to that reported by Nievas et al. (2006) for the BWOP from Puerto Madryn (0.888 g mL^{-1}), being both parked residues collected from ships of the port. The fuel used by the vessels is mostly diesel whose density ranged from 0.840 (YPF, 2014) to 0.970 g mL^{-1} (YPF, 2012). In addition, an important diesel range organic (DRO) HC fraction was found, with the highest concentration in BWOP D. The values observed in this work are coherent with the density reported for diesel, and the fluctuation was expected due to the presence of other lubricant compounds, water from filtrations or washing and emulsifiers in the mixture. The specific gravity and the kinematic viscosity values presented significant differences among the samples ($p = 0.00013$ and $p = 0.08326$, respectively).

BWOP from fishing ships C and A showed lower pH values, in comparison with the other samples. This could suggest the presence of decomposing fish remains that rapidly acidify the environment (Manning, 2001). Likewise, the pH values of the samples were slightly acidic, lower than other reports which ranged from 6.8 to 9 (Church et al., 2019; Dastgheib et al., 2012). The chemical oxygen demand (COD) and biological oxygen demand (BOD) values showed high organic loads, doubling those of concentrated crude oils (Metcalf & Eddy, 1996). The BOD of sample A far exceeded the rest of the values with an average of 2392.66 mg O2 L^{-1} ($p = 0.00706$). COD values showed significant differences among all the samples and with other reported values (Emadian et al., 2015). Only BWOP A had significant water content (30% (v/v)), while the rest of the studied residues exhibited less than 2% (v/v). Settling solids ($p = 0.2366$) were found only in samples A and C, with values of 0.01 and 0.06 mL L^{-1}, similar to those obtained for fuel oils up to grade 4 inclusive (ASTM D396-15a). The conductivity values presented by samples D, C, and M were less than 1.5 ms cm^{-1}, which was consistent with the low water content of the samples. Sample A, had a high conductivity value of 29 mS cm^{-1}, like the bilge waste reported by Nievas et al. (2008), which indicates a content of sea water (approximately 63%) mixed with fresh water (Nievas et al., 2008). Values differed greatly between these samples ($p = 0.02929$), as reviewed by Church et al. (2019) for previous research. Oil content ranges from 554

to 1540 ppm displaying large variation among samples, and it is consistent with the findings from previous reports (Church et al., 2019). The HC chromatographic analysis showed the n-alkanes series from C8 to C16, and the aromatic HC fraction from C8 to C10. The absence of higher HC chains may be due to previous degradation of the sample, due that long-chain HC may have been shortened during navigation or parking time at port as a result of biocatalytic action. According to the results, it can be observed that the physicochemical characteristics of BWOP varies among vessels, as was also described in Church et al. (2019) and Tiselius and Magnusson (2017). Physicochemical values previously published in other reports (Nievas et al., 2006, 2008; Sivaraman et al., 2011; Mancini et al., 2012; McLaughlin et al., 2014; Ulucan et al., 2014; Emadian et al., 2015; Vyrides et al., 2018; Church et al., 2019) also differ among them and with this report.

3.2 Microbial Composition of BWOP

The microbiome composition of the BWOP was determined with an average of 102.225 high-quality reads per sample, achieving a near complete representation of the amplicon complexity and saturated description of the microbial composition, which can be inferred using this approach. Rarefaction curves and values of Good's coverage were in all cases above 99.5% in all the samples, indicating that the sequence depth was sufficient (see Online Resource 1). The four samples had a similar saturation trend, indicating a relatively low diversity, possibly of similar composition across all the samples, and the presence of a few bacterial groups of high predominance common to all them. We observed that it was indeed the case, when looking at the taxonomic composition and relative abundances of OTUs clustered at 97% similarity and maximum classification, which can be assigned with a confidence above 80% using the RDP Naïve Bayesian Classifier with SILVA 132 taxonomy nomenclature.

A core microbiome was observed (Fig. 1), with OTUs of identical classification being constantly found through the BWOP wastes analyzed (221 OTUs common to all the samples out of 1,641 detected in all them, see Online Resource 1). While the samples have in common to be oily bilge wastes, all are coming from a quite distinct source. The stability of the core microbiome members and its remarkable predominance on the microbiome composition across the set of samples analyzed indicate that the oily bilge waste is a strong selector for bacteria, having common taxonomical and functional features, independent of the vessel of collection. The bacteria found in higher densities in these samples are able to resist and thrive in oily bilge wastes of complex composition and high ecotoxicity. Some of them might be able to detoxify and grow from such HC sources, while others may be sustained from the primary consumers metabolites. The bacterial composition that collectively composed 82% of the bacterial community common to all the samples are OTUs classified as *Marinobacter* spp., a betaproteobacteria of the environmental order known as MOB121, bacterial members of undescribed genus/species belonging to the *Flavobacteriaceae* family, bacteria belonging to the genera *Thalassospira*, *Parvibaculum*, and *Alcanivorax*, as well as two OTU variants of *Pseudomonas* genus. Finally, a member of an undescribed species inside the *Rhodospirillaceae* family and an undescribed species of the gammaproteobacterial order known as PYR10d3 are important members of the core microbiome consistently detected in BWOP.

The core microbiome sequences detected are representing novel divergent branches in all the genera detected, with distances of 3% or higher, versus closest reference type strains 16S rRNA gene sequences in databases, suggesting they are possibly coming from bacterial species not yet described.

Several OTUs of the core community detected have been associated with HC degradation or HC contaminated places, which is consistent with the composition of oily bilge wastes. Bacteria of the genus *Marinobacter* are ubiquitous in the marine environment and the degradation of HC is a common feature in many strains of this genus. Many are alkane degraders, such as *Marinobacter hydrocarbonoclasticus* SP17, *M. maritimus*, and *M. algicola* DG893 (Christen et al., 1992; Shivaji et al., 2005; Green et al., 2006). Others are able to degrade polyaromatic HC such as fluoranthene (Brito et al., 2006), naphthalene (Cui et al., 2016; Hedlund et al., 2001), phenanthrene (Dastgheib et al., 2012; Cui et al., 2016), and anthracene (Cui et al., 2016). Betaproteobacteria type of the order MOB121 was identified in a bacterial screening of a Malaysian crude oil (Liew & Jong, 2008) and in a microbial community in refinery

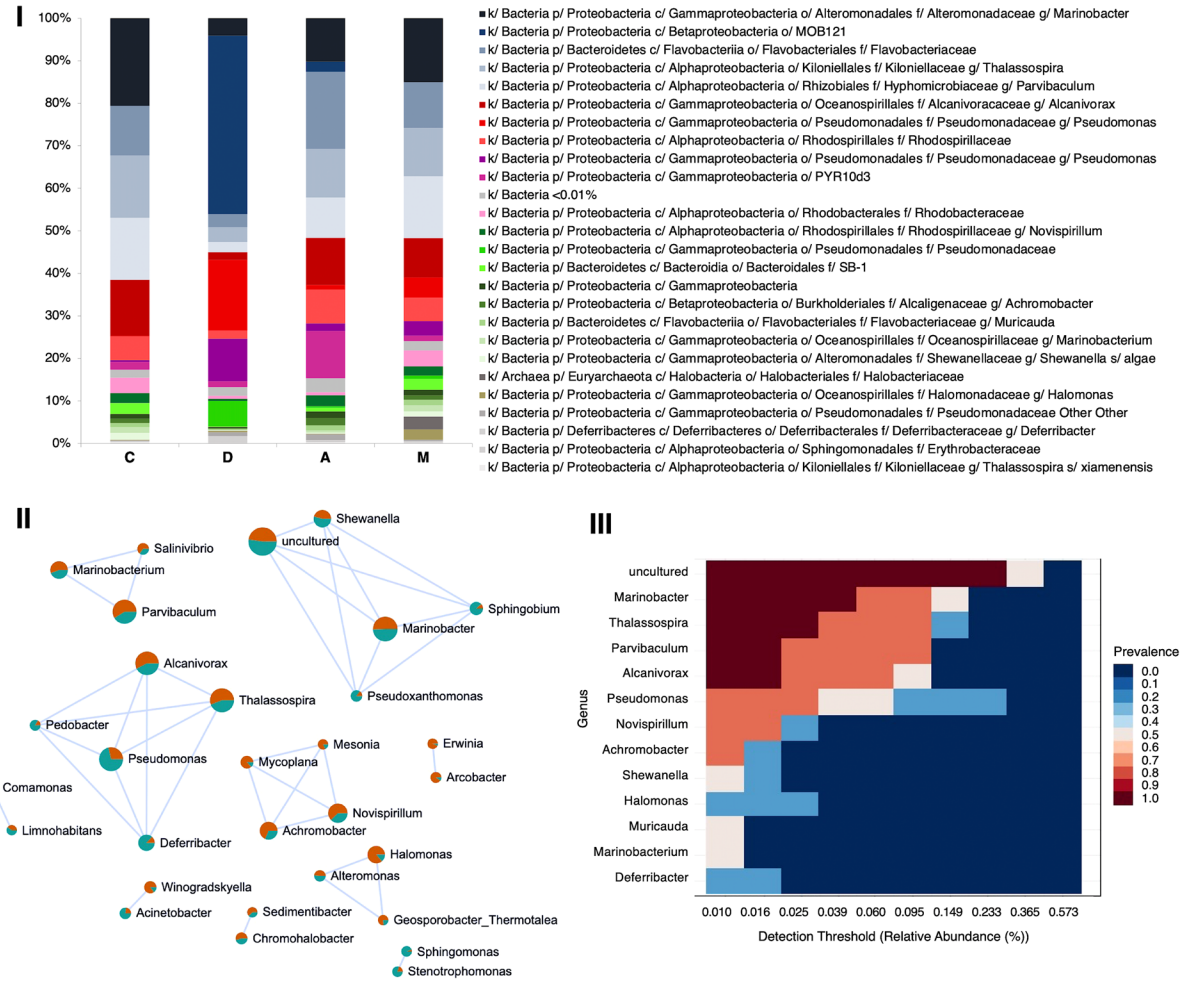

Fig. 1 The microbiome composition of the BWOP from 4 distinct vessel samples in Mar del Plata (Argentina), as determined by analyses of the hypervariable region V4 of the 16S rRNA Illumina sequencing. Pair-end amplicon sequences were obtained from each sample and processed as described in materials and methods. **I** OTUs having identical classification across the samples and having a cumulative averaged frequency lower than 0.01% are grouped in the category k/ Bacteria, 0.01%. The maximum resolution obtained for OTUs and frequencies grouped in the same taxa is shown, using as a minimum threshold a confidence value of 80% of the RDP Naïve Bayesian Classifier with SILVA 132 as reference taxonomy dataset. The samples are BWOP from a small coastal vessel (C), 115,308 reads; BWOP sample from a large dredger ship (D) 109,190 reads; BWOP from deep-sea fishing ship (A) 83,460 reads; and pooled whole port mixture of oily bilge wastes collected from multiple ships (M) 95,260 reads. The average read length size is 300 bp. **II** Network graphical display of co-occurring genera across the samples. Correlation analysis by Spearman rank correlation, permutation (SparCC) 100, P-value threshold 0.05; correlation threshold 0.3. **III** Core microbiome at genus taxonomic level. Thresholds of sample prevalence 20% and relative abundance 0.01%, represented as heatmap clustering, distance measure Euclidian, clustering algorithm average

waste with HC degradation ability (Sarkar et al., 2016). Different members of the genus *Alcanivorax* are alkane degraders. For example, *Alcanivorax borkumensis* is a marine bacterium that exclusively uses n-alkanes as carbon and energy sources (Yakimov et al., 1998; Warr et al., 2018). Studies indicate that *Alcanivorax* plays a critical role in the biodegradation of marine waters contaminated with HC, since the bacteria of this genus are found in low quantities in uncontaminated marine waters but their quantity increases significantly in response to oil spills (Kasai et al., 2002; Harayama et al., 2004; Kostka

et al., 2011). On the other hand, members of *Flavobacteriaceae* family have been described as aromatic HC degraders, including monoaromatic and polyaromatic HC and HC mixtures as diesel fuel (Hemalatha & VeeraManikandan, 2011; Chaudhary et al., 2019). Also, one *Parvibaculum* species (*Parvibaculum hydrocarbonoclasticum* sp. nov.) was reported to be an alkane-degrader (Rosario-Passapera et al., 2012). Some bacterial strains belonging to *Pseudomonas* genus are recognized as PAH-degraders (Prabhu & Phale, 2003; Qi et al., 2015; Chebbi et al., 2017) and alkane and alkene degraders (Smits et al., 2003; Nisenbaum et al., 2013; Muriel-Millán et al., 2019). Uncultured clone PYR10d3 has been found in a bacterial community originated from an oil-contaminated coastal sediment (Païssé et al., 2010) and was associated with the degradation of pyrene in a bioreactor for treating soil contaminated with PAH (Singleton et al., 2006). The family *Rhodospirillaceae*, which includes the genus *Thalassospira*, has also been reported in oil-degrading enrichments from soil and oil-polluted seawater, and members of this family can degrade PHAs (Kodama et al., 2008; Zhao et al., 2010).

In addition, some *Marinobacter* (Zenati et al., 2018), *Pseudomonas* (Bonilla et al., 2005), *Alcanivorax* (Qiao & Shao, 2010), and *Flavobacterium* (Bodour et al., 2004) members produce bioemulsifiers which increase the bioavailability of HC for biodegradation. It is known that bilge wastewater presents commercial surfactants and different metals in its composition. Members of *Marinobacter*, *Parvibaculum,* and *Thalassospira* genera have been reported as surfactants degraders (Dong et al. 2004; Bassey & Grigson, 2011), and *Marinobacter manganoxydans* MnI7-9 oxidizates manganese and tolerates high levels of metals/metalloids (Wang et al., 2012).

Other works that studied cultivable oil-degrading bacteria from bilge wastewater samples have found mainly members of the *Pseudomonas* genus (Olivera et al., 2003; Nievas et al., 2006; Sivaraman et al., 2011; Santisi et al., 2015a, 2015b), but *Bacillus*, *Halomonas* (Santisi et al., 2015b), *Alcanivorax* (Santisi et al., 2015a), *Marinobacter* (Sivaraman et al., 2011), *Acinetobacter*, and *Rhodococcus* (Cappello et al., 2012) genus have also been detected.

Articles exploring the diversity of non-cultivable microorganism by 16S RNA Illumina sequencing of bilge wastewaters, or enriched microbial cultures derived from it, have been recently published (Schaerer et al., 2019; Corti-Monzón et al., 2020; Nisenbaum et al., 2020). Schaerer et al. (2019) performed a global comparison of the bacterial communities of bilge water, boat surfaces, and external port water from different places. They compared the microbial communities of the hull and bilge compartments of 20 boats to those of the port water in 20 different ports in five regions around the world. Such work did not focus on oily bilge wastewaters; thus, the method of sampling the bilge liquid was probably favoring the aqueous phase collection and not the BWOP, where hydrocarbonoclastic bacteria are growing. Therefore, the communities reported were completely different in predominant types from those described in this work. We observed that many of the OTUs detected were associated with ribotypes found in marine microorganisms or retrieved from marine environments, which shows the expected influence of seawater microbiome on the bilge composition.

Regarding the functional potential inferred from the frequencies and classification of the 16S amplicon sequences detected in all samples with Tax4Fun, reconstructed metabolism of the mapped KEGG function IDs are very similar between the four samples (Bray–Curtis dissimilarity $< 0.2\%$ in frequencies of 6349 KO). It followed the expected patterns of complete pathways for central metabolism functions, or the module for beta oxidation, essential for energy production after aliphatic hydroxylation. Worth to mention is that the single KOs (KEGG Orthology IDs) with the highest predicted frequency (top 6 having $> 3\%$ relative abundance) are not from functions of energy production metabolic pathway, but from protein families of environmental signaling, sensing, and transporting as important adaptations in the bilge environment. For example, K02014 iron complex outer membrane receptor protein, K03406 methyl-accepting chemotaxis protein, K03296 surfactin self-resistance transporter, K03296 hydrophobic/amphiphilic exporter-1 sensory transduction histidine kinase, K00936 two-component system, sensor histidine kinase, K06147 multidrug ABC transporter permease, and K00059 fatty acid biosynthesis. The results are included as Online Resource 2.

We recently reported two different microbial consortia isolated from bilge wastewater from Mar del Plata port. The two consortiums were enriched from bilge samples using HC as the sole source of carbon and energy, making them possible HC degraders. One

was shown to possess the ability to degrade aromatic and aliphatic HC (Nisenbaum et al., 2020), while the other had the ability to demulsify HC emulsions in water (Corti-Monzón et al., 2020), two interesting characteristics for the biological treatment of bilge residues. The microbial identification of said consortia by 16S RNA Illumina sequencing showed highly consistent microbial groups with great similarities between them and with the bilge analyzed samples in this work. This indicates a strong selective pressure of the HC on the microbial communities present in the bilges analyzed that could be used as a particular bioresource to select and isolate emerging HC-degrading microbial consortia or pure microorganisms.

3.3 *alkB DGGE Profiling in BWOP Samples*

During the last years there has been an increasing worldwide concern about marine HC contamination into the sea, resulting from vessels bilge wastes disposal. Nevertheless, up to date no functional surveys regarding catabolic genes associated with alkane degradation, either in bacterial isolates or in metagenomic DNA, have been performed in these kinds of samples. Considering that alkanes represent the main component of these residues, we carried out a preliminary study about the occurrence and diversity of the *alkB* gene, which is widely used as a marker gene for the detection of alkane biodegradation potential in contaminated sites.

In order to characterize the *alkB* gene diversity in the BWOP assayed in this work, PCR-DGGE fingerprinting analyses were performed using the *alkB*484F/GC-clamped and *alkB*824R primer set. Our profiling results indicate that the *alkB*-DGGE band patterns were different for the four samples evaluated with bilges of similar microbiomes (Fig. 1) and different physicochemical characteristics (Table 1). Sample C was run in duplicate to verify the reproducibility of the method. About 3–8 discernible bands were observed for each sample with variable intensities. Most of them were specific to a given vessel, whereas a few showed to be common to more than one sample. BWOP A and C (Fig. 2) showed the highest number of bands, indicating higher *alkB* gene diversity. On the other hand, the community pattern of sample M revealed the least diversity, with only three bands, being one very predominant.

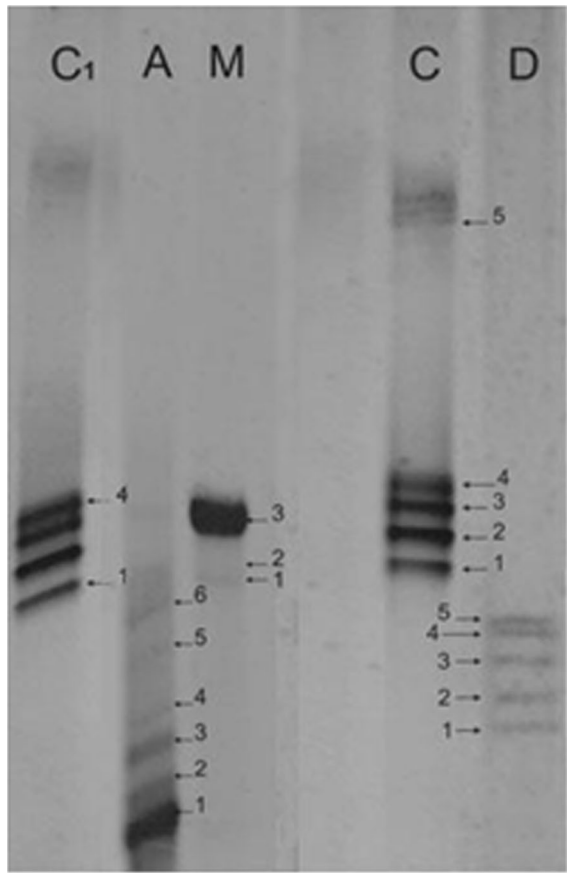

Fig. 2 PCR-DGGE *alkB* gene fingerprinting profiles detected in total DNA extracted from BWOP samples in vessels at the Southwestern Atlantic Mar del Plata port. Detection of *alkB* gene in bilge residues (A, C, D, and M). Numbers in gels indicate excised and sequenced bands. C1 is a replica of C

Assuming that one band represents one variant of the *alkB* gene, BWOP samples showed certain variety of genotypes. In accordance with previous works (Catania et al., 2018), the diversity of *alkB* variants observed suggests that the use of alkanes as substrates and the potential for alkane biodegradation are widespread attributes of microorganisms in HC polluted wastes, which enables a rapid adaptation of alkane-degrading bacterial communities. On the other hand, it shows how microbiomes of similar taxonomic composition could have different predominant content of a given catabolic gene encoding an advantageous function for growth.

A total of 21 dominant bands were excised from the DGGE gels and successfully sequenced. Phylogenetic affiliation analyses of the sequenced bands showed high similarities (95.3–100%) and low e-values to *alkB* genes sequences retrieved from the NCBI GenBank (Fig. 3, Table 2), demonstrating that the PCR primer set used in this study was efficient for detecting known *alkB* sequences. Two pairs of bands occupying the same position in the gel and sequenced from duplicate samples showed similar phylogenetic affiliations. Thus, C1-C1.1 and C4-C1.4 sequences were affiliated to alkane hydroxylases genes from an uncultured bacterium (KC466033) and from *Marinobacter* sp. P1-14D (GQ184210), respectively.

From the phylogenetic standpoint, 18 *alkB* gene variants were obtained, evidencing a considerable diversity among the assessed samples. In this sense, it was reported (Li et al., 2013) that, as these genes are quite divergent in diverse environments, it should be feasible to explore distinct alkane monooxygenase genotypes by means of molecular methods.

The closest matches in GenBank of the majority of *alkB* sequences obtained were related to uncultured or unidentified microorganisms, in agreement with previous studies from other underexplored ecosystems, like coastal and deep marine sediments (Kuhn et al., 2009; Wasmund et al., 2009; Guibert et al., 2012). This finding confirms the importance of independent culture techniques to detect microorganisms with biotechnological potential. Furthermore, those sequences closely related to cultured microorganisms showed high identity level (96.8–100%) with alkane monooxygenase gene variants reported in strains of *Marinobacter* sp., *Alcanivorax* sp., *Pseudomonas* sp., *Rhodococcus* sp., and *Nocardia* sp. The majority of these genera were indeed detected in the microbiome composition of the BWOP analyzed in this work as key members, even though the DNA extraction methods for rARN 16S sequencing and *alkB* gene-DGGE analysis were different. They have also been reported as HC degraders in different sites and conditions (Abbasian et al., 2015), including in bilge wastes as mentioned above, with the exception of *Nocardia* sp. In addition, all matches were *alkB* genes found in polluted zones (wastewaters, soils and coastal or oceanic environments). It is worth noting that *alkB* diversity in contaminated Subantarctic marine sediments in Argentina was analyzed by Guibert et al. (2012), and they also found that most of *alkB* sequences belonged to *Marinobacter* sp., *Alcanivorax* sp., *Pseudomonas* sp., and *Rhodococcus* sp.

Cluster analysis based on the 21 *alkB* nucleotide sequences, and those retrieved from GenBank, separates in 3 clades, and related to the different bilge samples, are shown in Fig. 3. K2P distances were calculated, showing higher values among the clades (61 to 163) than within them (2.6 to 13), confirming the different *alkB* variants among the bilge wastes.

Clade 1 comprised 12 sequences from BWOP C and D, and one from BWOP M, with 93–99.6% identity to *alkB* genes of *Alcanivorax marinus*, *Marinobacter* sp., and an uncultured bacterium. As mentioned previously, bacteria closely related to both genera are widely spread in marine areas and became predominant in HC contaminated seawaters. Thus, they are considered major actors in the bioremediation of oil-polluted marine environments (Catania et al., 2015).

Two sequences obtained from BWOP M, grouped in Clade 2, were included and associated with *Pseudomonas* sp., *P. putida*, and an unclassified bacterium with 97.7 to 98.7% identity. As one of the most widely studied bacterial genera, several *Pseudomonas* species are regarded as specialized hydrocarbonoclastic in chronically polluted sites (Pedetta et al., 2013; Catania et al., 2018), and strains of this genus has been detected in bilge wastes (Sivaraman et al., 2011; Santisi et al., 2015b). Moreover, *alkB* genes have been found in many sequenced genomes belonging to *Alcanivorax*, *Marinobacter*, and *Pseudomonas*, suggesting that they could be the core genes shared by these genera (Van Beilen & Funhoff, 2007; Nie et al., 2014).

Clade 3 was composed of six sequences from BWOP A, which clustered with alkane monooxygenase genes from *Rhodococcus* sp, *R. rhodochrous*, and *R. gingshengii*, as well as *Nocardia* sp. and an unclassified bacterium. *Actinomycetes* members have been characterized by their wide metabolic versatility and by the co-occurrence of genes responsible for concomitant metabolic bioconversion reactions of structurally-diverse HC in soils and marine systems (Brzeszcz & Kaszycki, 2018). All sequences in our study showed considerable evolutionary divergence from the sequence of *Acinetobacter* sp. included in the tree.

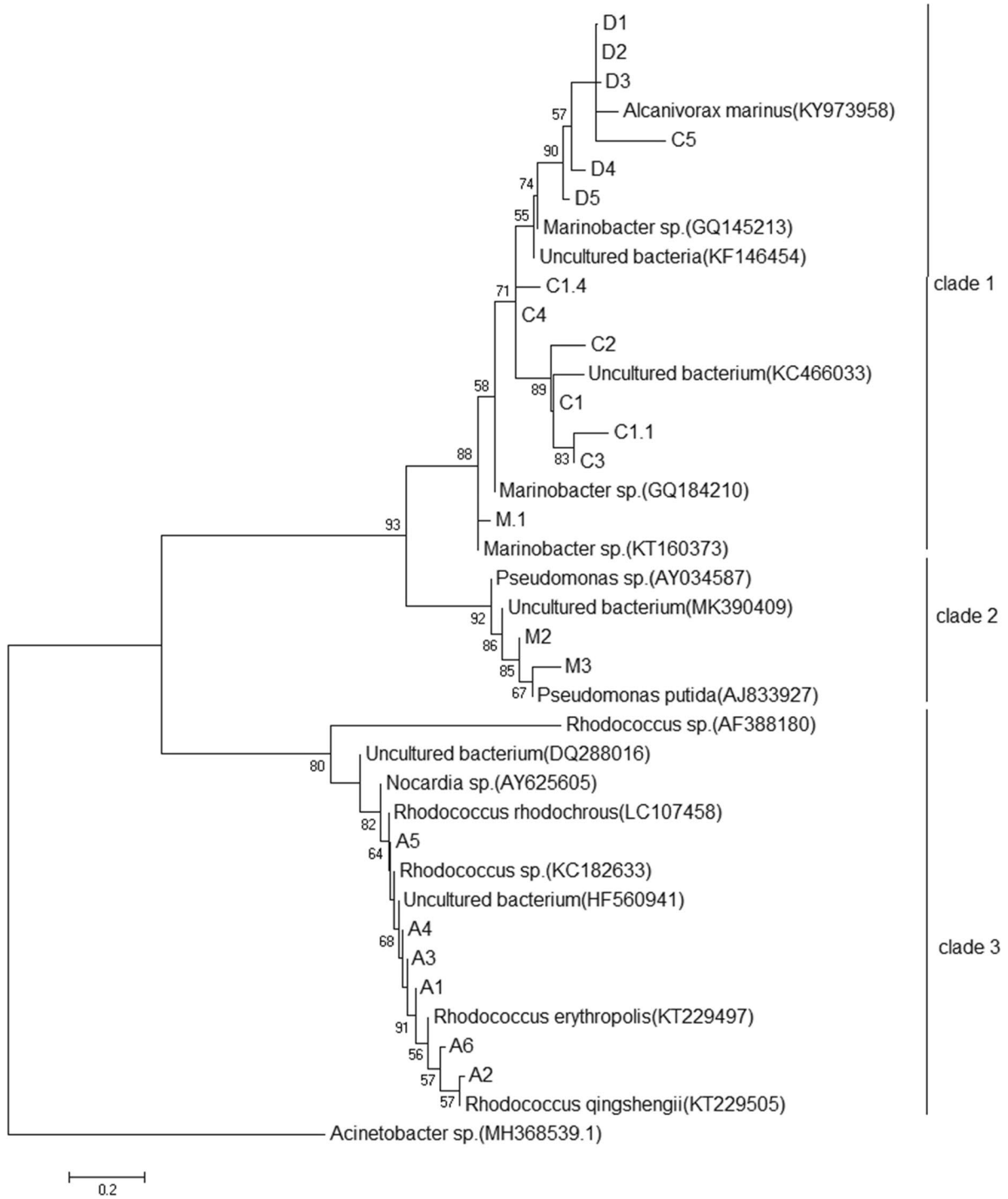

Fig. 3 Phylogenetic tree of the *alkB* sequences from bands of DGGE fingerprinting profiles. Reference *alkB* sequences of closer relatives are included. For details of tree construction, see "Materials and Methods" section

It is worth mentioning that despite the variation observed in *alkB* gene fingerprinting profiles, the

Table 2 Results of partial sequence and sequence similarity of *alkB*-DGGE bands obtained from total DNA extracted from BWOP collected in Mar del Plata port vessels

Sequence number	Closest relative	GenBank number	Similarity %	Mx Score
C1	Uncultured bacterium clone a4 alkane hydroxylase (*alkB*) gene, partial cds	KC466033	98.15	472
C2	Uncultured bacterium partial *alkB* gene for alkane monooxygenase, clone cl35, genomic DNA	FR828526	96.74	562
C3	Uncultured bacterium clone a4 alkane hydroxylase (*alkB*) gene, partial cds	KC466033	98.11	553
C4	*Marinobacter* sp. P1-14D alkane hydroxylase (*alkB*1) gene, partial cds	GQ184210	97.93	530
C5	*Alcanivorax marinus* strain RMR23 alkane monooxygenase gene, partial cds	KY973958	99.21	455
C1.1	Uncultured bacterium clone a4 alkane hydroxylase (*alkB*) gene, partial cds	KC466033	98.12	464
C1.4	*Marinobacter* sp. P1-14D alkane hydroxylase (*alkB*1) gene, partial cds	GQ184210	93.06	313
D1	Uncultured bacterium partial *alkB* gene for alkane monooxygenase, clone cl49, genomic DNA	FR828530	98.71	549
D2	Uncultured bacterium clonexj8-22 alkane monooxygenase (*alkB*) gene, partial cds	KJ653792	98.97	521
D3	Uncultured bacterium clone C9 alkane hydroxylase (*alkB*) gene, partial cds	KF154214	99.05	566
D4	*Marinobacter* sp. S17-4 putative alkane monooxygenase (*alkB*) gene, partial cds	GQ145213	99.63	488
D5	Uncultured bacterium clone *alkB*36 alkane hydroxylase (*alkB*) gene, partial cds	KF146454	98.23	494
A1	*Rhodococcus qingshengii* strain D1RHp1 alkane monooxygenase (*alkB*) gene, *alkB*-2 allele, partial cds	KT229505	99.61	466
A2	*Rhodococcus rhodochrous alkB* gene for alkane hydroxylase, partial cds, strain: NBRC 15,564	LC107458	99.66	540
A3	*Rhodococcus qingshengii* strain D1RHp1 alkane monooxygenase (*alkB*) gene, *alkB*-2 allele, partial cds	KT229505	100	477
A4	Uncultured bacterium partial *alkB* gene for alkane hydroxylase, clone P21-22_Cl.9_HRB2-7 m-2009	HF560941	99.64	514
A5	*Nocardia* sp. H17-1 alkane monooxygenase (*alkB*2) gene, partial cds	AY625605	99.33	544
A6	Uncultured bacterium clone alkG4-22 K putative alkane monooxygenase (*alkB*) gene, partial cds	DQ288016	98.28	610
M1	*Marinobacter* sp. SL014A-10A1-2 clone C11 alkane monooxygenase *AlkB* gene, partial cds	KT160373	98.61	510
M2	Uncultured bacterium clone AER-OPU-2_7 alkane monooxygenase (*alkB*) gene, partial cds	MK390409	98.38	544
M3	*Pseudomonas putida* partial *alkB* gene for putative alkane hydroxylase, strain SH41	AJ833927	98.77	433

nucleotide sequences of *alkB* revealed that the identities of the amplified fragments were, in general, coherent with the expected host in the microbiome composition assessed. *Alcanivorax*, *Marinobacter*, and *Pseudomonas* 16S rRNA sequences were relatively abundant in bilge samples, suggesting that *alkB* genes would be hosted by members of these genera. However, in BWOP A sample, *alkB* genes associated with *Rhodococcus* and *Nocardia* host were predominant and this result did not agree with 16S

data, where very low proportion of sequences were classified within *Rhodobacteraceae*, and sequences related to *Nocardia* sp. were not present. This lack of accordance could be due that bacteria other than *Rhodococcus* or *Nocardia* may contain this gene, acquired by horizontal gene transfer, which has been previously observed with *alkB* genes (Tourova et al., 2008; Yousaf et al., 2010; Guibert et al., 2012; Nie et al., 2014; Shahi et al., 2016). It is possible as well that the distinct DNA extraction methods used in this study for 16S and *alkB* genes analysis could contribute to this difference. Remarkably, the same divergence has been reported for *Rhodococcus alkB* genes in metagenomic studies carried out in oil-contaminated Arctic soils and Antarctic sediments (Tourova et al., 2008; Guibert et al., 2012). Guibert et al. found the presence of *alkB* genes associated to *Rhodococcus* in Subantarctic Coastal sediments, whereas only 0.25% of sequences from 16S gene pyrosecuencing data were classified as *Rhodococcus*. These authors attributed such lack of agreement to the *alkB* primer set used, which was designed with a majority of *Rhodococcus* sequences, or to the possible horizontal gene transfer. On the other hand, other microorganisms phylogenetically identified in the present study could also be functionally relevant, even when no information about *alkB* genes or biodegradation abilities have been reported.

4 Conclusions

An understanding of oily bilge waste components is required to improve bioremediation strategies. Here, we described the physicochemical and biological composition of BWOP coming from vessels with different shipping activities from Mar del Plata port. Variations in the physicochemical parameters assayed were found among the four bilge samples analyzed. The microbial identification of the distinct BWOP samples showed an interesting diversity but there was a core of bacteria that predominates the communities among the samples. Access to the composition of microbial communities present in the bilge wastes allows knowing the possible dispersion of microorganisms that would cause the ships transit. The stability of the core microbiome members and the remarkable predominance they have on the microbiome composition indicate that oily bilge waste is a strong selector for bacteria having common taxonomical and functional features, independent of the vessel of collection. The core microbiome was composed by bacterial types of the genus *Marinobacter*, a betaproteobacterial type of the environmental order known as MOB12; members of undescribed genus/species belonging to the *Flavobacteriaceae* family; bacteria of the genera *Thalassospira*, *Parvibaculum*, *Alcanivorax*, and *Pseudomonas*; a member of an undescribed species inside the *Rhodospirillaceae* family; and an undescribed species of the gammaproteobacterial order known as PYR10d3. All these OTUs possess microorganisms that have been associated with HC degradation or isolated from HC contaminated places, which is consistent with the composition of the bilge wastes.

The preliminary identification of *alkB* gene sequences performed in this study indicates its selection and role in adaptation in oily bilge wastes and could be used as functional biomarkers in both basic and applied contexts. The complementation of the phylogenetic approach with functional gene-based information in bilge wastes provides a first glance of the metabolic potential and physiological mechanisms that might drive HC biodegradation. On the other hand, in contrast to massive sequencing 16S identification, a lower number of *alkB* variants were described in this work; therefore, further studies would be necessary to get the whole picture of this gene diversity in bilge wastewaters.

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Declarations

Conflict of Interest The authors declare no conflicts of interest.

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