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ORIGINAL PAPER

# Enrichment and key features of a robust and consistent indigenous marine-cognate microbial consortium growing on oily bilge wastewaters

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**Abstract** Oily bilge wastewater (OBW) is a hazardous hydrocarbon-waste generated by ships worldwide. In this research, we enriched, characterized and study the hydrocarbon biodegradation potential of a microbial consortium from the bilges of maritime ships. The consortium cZ presented a biodegradation efficiency of 66.65% for total petroleum hydrocarbons, 72.33% for aromatics and 97.76% removal of n-alkanes. This consortium showed the ability to grow in OBWs of diverse origin and concentration. A 67-fold increase in biomass was achieved using a Sequential Batch Reactor with OBW as the only carbon and energy source. The bacterial community composition of the enriched OBW bacterial consortium at the final stable stage was characterized by 16S amplicon Illumina sequencing showing that 25 out of 915 of the emerged predominant bacterial types detected summed up for 84% of total composition. Out of the 140 taxa detected, 13 alone accumulated 94.9% of the reads and were classified as *Marinobacter*, *Alcanivorax*, *Parvibaculum,* Flavobacteriaceae, Gammaproteobacteria PYR10d3, *Novispirillum* and Xanthomonadaceae among the most predominant,

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followed by *Thalassospira*, *Shewanella*, Rhodospirillaceae, Gammaprotobacteria, Rhodobacteriaceae and *Achromobacter*. The microbial community from OBW bioreactor enrichments is intrinsically diverse with clear selection of predominant types and remarkably exhibiting consistent and efficient biodegradation achieved without any nutrient or surfactant addition. Due to there is very little information available in the OBW biodegradation field, this work contributes to the body of knowledge surrounding the treatment improvement of this toxic waste and its potential application in wastewater management.

**Keywords** Oily bilge wastewater · Consortium · Microbiome · Hydrocarbons · Biodegradation

## Introduction

The oily bilge wastewater (OBW) generated from ships worldwide is one of the main threats of pollution to marine coastal ecosystems. OBW is a complex mixture of compounds that accumulates in shipboard bilges and includes oily fluids and other pollutants such as metals, detergents and solvents, which come from a variety of sources (e.g., engines, piping and mechanical sources) (EPA-842-R-99-001 1999; EPA-842-R-07-005 2008; EPA-833-R-10-005 2010; EPA-833-F-11-001 2011). OBW can be purified on board through oil/water separators, leaving most of the hydrocarbons on board for collection in port for their treatment or final disposal. International maritime legislation limits the discharge of OBW in the open ocean to less than 15 ppm of hydrocarbons for vessels of more than 400 tons, while in certain sensitive areas the discharge is totally prohibited (IMO 1988). However, the direct discharge of these sewage into the sea, legally or illegally, negatively affects marine resources, which represents a risk to human health (Emadian et al. 2015).

Although physicochemical methods have been successfully applied to on board treatment (McLaughlin et al. 2014), they impose several issues with regards to chemical consumption, high energy requirements and secondary pollution. Conversely, the use of biological treatments is becoming increasingly popular in the field of saline wastewater characterized by high organic content and petroleum hydrocarbons. Considerable work in the microbiology of various hydrocarbon-rich environments has demonstrated the natural abundance of degrading microorganisms that use a wide range of hydrocarbons as nutritional resources, particularly bacteria and archaea (Rosenberg et al. 1992; Head et al. 2006; An et al. 2013; Fowler et al. 2016). The use of indigenous consortia in the treatment of OBW could be advantageous since these microorganisms are better adapted to these fluctuating wastewater characterized by high salinity, low temperature, presence of heavy metals and other organic pollutants. In addition, they act synergistically in the mineralization of the residue, which may result in greater degradation efficiency than using pure cultures (Gouveia et al. 2018). Likewise, microbial communities can vary according to the composition of the effluent and the environmental conditions presented. The biotreatment of wastewaters have great efficiency and low environmental impact (Nievas et al. 2005), and the optimal conditions for a biologically active environment can be obtained using bioreactors. Therefore, studies on OBW degradation by enriched autochthonous cultures have suitable application prospects and clear research value (Huang et al. 2019).

Accordingly, microbial communities characterization, in terms of their diversity, metabolic potential and response toward external agents, is essential for developing biodegradation technology (Sarkar et al. 2016). Culture-independent molecular techniques have identified diverse and complex assemblages of aerobic and anaerobic bacteria and archaea capable of hydrocarbon degradation, nitrate/sulfate/iron-reduction, fermentation, and methane metabolism in various hydrocarbon rich environments (Kostka et al. 2011; Hu et al. 2013; An et al. 2013; Sarkar et al. 2016; Techtmann and Hazen 2016). The few research studies available in OBW treatment have been focused mainly on understanding the rates of hydrocarbon biodegradation (Olivera et al. 2003; Nievas et al. 2005, 2006; 2008; Sun et al. 2009; Sivaraman et al. 2011; Mancini et al. 2012; Di Bella et al. 2015; Santisi et al. 2015; Vyrides et al. 2018). Although microbes are recognized as essential to the process, very little is known about the structure, composition, dynamics and metabolic capacity of OBW microbial communities. Therefore, the objective of this study was to determine the detailed composition of an enriched OBW autochthonous microbial community by a culture-

independent metagenomics approach, explore its hydrocarbon degradation ability in laboratory-scale bioreactors and study its growth performance under variable operation conditions with OBW as the only carbon and energy source. This information will contribute the scaling up success, since it reveals the essence and the abilities of the microbial community; helps to evaluate and select strains, optimize experimental and process variables, reveal the controlling or critical steps in the operations, and provide information to engineering calculations and reactor designs.

## Materials and methods

### Oily bilge wastewater sampling

Oily bilge wastewater (OBW) samples were taken from an open pool where OBW from ships that arrive to Mar del Plata's port are deposited (Mar del Plata, Buenos Aires, Argentina) (OBW M). The samples were fractionated for characterization and stored at 4 °C for further analysis. Fractions to be used in the detection of hydrocarbons were acidified to pH 2 (ASTM Standard D7678 2011).

Another OBW samples from three different kind of ships (A: deep sea fishing ship; D: dredge ship; and C: coastal fishing boat) were also taken to test the ability of the consortia to grow in residues of different compositions.

### OBW characterization

The OBW settleable solids and relative density were measured according to 2540 and 2710F methods (APHA et al. 1998), respectively. Water content, density and kinematic viscosity were measured according to ASTM D4928 (2012), ASTM D854 (2014) and ASTM D88 (1999), respectively. Conductivity and pH were measured by a portable meters HI933100 and HI98103 (Hanna Instruments, Inc.). The Total Petroleum Hydrocarbon (TPH) determination was carried out in an IR Oil Content Analyzer OCMA-350 (HORIBA), using the 418.1 EPA method (EPA-821-R-98-002 1999) with perchlorethylene (Sintorgan) as extracting solvent. Aliphatic Hydrocarbons (AlH) and Aromatic Hydrocarbons (ArH) were extracted with n-hexane (UVE, pesticide grade), according to 418.1 EPA method. AlH were analyzed by High-Performance Gas Chromatography on a Hewlett-Packard 5890-A chromatograph, equipped with a Varian VF-1 ms capillary column (20 m × 0.39 mm × 0.1 µm), Split injection port (Split ratio: 25:1) and FID detector. Samples were analyzed using hydrogen (25 ml $min^{-1}$) as the carrier gas. The volume injected was 3 µl. Injector and detector temperatures were 320 °C and 350 °C respectively. The column temperature was programmed as follows: 35 °C, 1 min; 25 °C $min^{-1}$ until 100 °C, 12 °C $min^{-1}$ until 320 °C, and 10 min at 320 °C. Detection was performed using an Agilent 8453E UV–Vis absorption spectrophotometer. Hydrocarbon identification was performed by comparing retention times with corresponding standards, consisting of a high boiling mixture of n-$C_6$ to n-$C_{40}$ (Hewlett-Packard). ArH were determined by Synchronous Fluorescence Spectroscopy (SFE) on a Jasco FP6200 Spectrofluorometer (Wakeham 1977; Kerkhoff et al. 1985). They were first analyzed in an Agilent 8453E UV–Vis absorption spectrophotometer to select the excitation wavelength suitable for use in the SFE. Synchronous spectra were collected in the range of 300 to 550 nm, with wavelength ($\Delta\lambda$) intervals of 10 and 50 nm at 250 nm $min^{-1}$.

The abundance of cultivable microorganisms was determined by Colony Forming Units (CFU/mL) as follows: from 1 mL of each homogenized sample a 1:10 dilution was made in sterile seawater and vortexed for 2 min. One mL of the aqueous phase was extracted to carry out serial dilutions (Finney 1951) and plated by surface dissemination on Petri dishes with nutritive agar (Merck) 1.5% (w/v) in sterile marine salts solution [MS: $NH_4NO_3$ 1 g $l^{-1}$ and phosphate solution 4 ml $l^{-1}$ ($Na_2HPO_4{\cdot}12H_2O$ 20 g $l^{-1}$ and $NaH_2PO_4{\cdot}H_2O$ 4 g $l^{-1}$ in seawater), pH 7] with 0.5% (v/v) gas oil (Nievas et al. 2006). The seawater used was previously filtered with Whatman ash less 1440-25 filter paper and sterilized in an autoclave. The gas oil used was sterilized by filtration with sterile nylon filter (0.22 µm). Colonies were counted daily during 20 days, after being in the dark at 25 ± 2 °C. The tests were carried out in triplicate.

### Consortium isolation and enrichment

Discontinuous liquid culture was carried out in 250 ml Erlenmeyer flasks with 0.5% (v/v) of non-sterile OBW in 100 ml of SM. Culture was kept in an orbital shaker (Shaker Pro, Vicking SRL) at 150 rpm and 25 ± 2 °C. The cell growth of the culture was followed by measuring turbidity at $OD_{600nm}$ (Gauthier et al. 1992a, b), in a UV–Visible spectrophotometer SHIMADZU UV1601PC. This consortium (cZ consortium) was maintained with medium renewals every 20 days for its enrichment and stabilization before the degradation assays. A correlation between $OD_{600nm}$ and number of cells per milliliter was done using a Neubauer Improved Chamber (Reichert, Bright-Line) in a Nikon E200 microscope with contrast phase and 40X/0.65 NA objective. A regression line with the following form was obtained: $y = 2 \times 10^9 x$ ($R^2$ = 0.9051); where $y$ = number of cells $ml^{-1}$ and $x = OD_{600nm}$.

### Hydrocarbon degradation potential

Biological degradation was tested in batch mode. A laboratory scale reactor composed of a cylindrical glass vessel (Omi-Cultura, Virtis Co) with a working volume of 2.5 l was set up. The reactor was made of stainless steel with a hole for sample extraction, an air inlet and support for the baffles that help the mixing of the components and avoid vortex formation. It was operated in a controlled temperature room of 25 ± 2 °C, in darkness. Aeration was supplied at the bottom of the reactor with a Balston DFU filter (grade AQ) at a flow rate of 1 l $min^{-1}$ and constant stirring of 150 rpm with two Rushton type turbines. The reactor was fed with 0.5% (v/v) of sterilized OBW (∼ 5000 ppm), as the sole source of carbon and energy. The supplementation with 0.2 g $l^{-1}$ of yeast extract according to Nievas et al. (2008) was tested, but no changes in growth were observed, therefore the use was discontinued (data not shown). Assays were performed in triplicate. A control assay was carried out without microorganism and another one with bacteria and without OBW.

Periodically, pH and cell growth were monitored. Biomass growth was evaluated spectrophotometrically at 600 nm at different times, in triplicate. The $OD_{600nm}$ value was then transformed on number of cells $ml^{-1}$ using the regression line described above. At the beginning (day 0) and the end (day 32) of the microbial exponential phase of growth, TPH, AlH and ArH were determined as was described in "OBW characterization". A negative control of MS with bacteria was used for the SFE determinations, and its value was discounted from the samples. The hydrocarbons were extracted from 1 l from the reactor volume. The biodegradation efficiency (%, BE) was calculated as (Nievas et al. 2006) based on the hydrocarbon residual mass found at the end of the inoculated and the respective control assay.

### Determination of consortium microbial composition by culture independent means

Ten ml composite sample (CS) was used for DNA extraction and purification. This sample consisted of homogenized aliquots of the enriched cZ consortium taken at the end of the exponential growth phase (day 14). Cells were harvested by centrifugation (10 min at 10,000×$g$) for 10 min, and the cell pellet was resuspended in 70% (v/v) ethanol and stored at 4 °C until the DNA extraction. DNA extraction was done by our reported customized protocol (García-Bonilla et al. 2019). Total DNA was extracted from a 0.5 ml aliquot of the CS.

The primers used were 515F [5′-GTGY-CAGCMGCCGCGGTAA-3′] and 806R [5′-GGAC-TACNVGGGTWTCTAAT-3′] (Walters et al. 2016) amplifying the hypervariable region V4 of bacterial and archaeal 16SrRNA gene sequenced on an Illumina-MiSeq platform, using 250X paired-end sequencing chemistry.

The analysis was performed according to the workflow proposed in the QIIME package version 1.9.1 (Caporaso et al. 2010). The raw data were filtered to maintain a quality score of 28. Chimeras were identified and extracted from the data using Usearch 6.1 (Edgar et al. 2011). The taxonomic assignment was made through the "Open-reference OTU picking" option using the UCLUST algorithm (Edgar 2010). Sequences with more than 97% similarity were grouped into operational taxonomic units (OTUs). The centroid sequences representative of each OTU were used to make the alignment against the SILVA 132 database (version 132, released April, 2018) using the Naïve Bayesian Classifier (Wang et al. 2007). Complete description of the amplicon sequencing

protocol and the bioinformatics pipeline is available in the Online Resource 1. The sequence reads produced in this study had been deposited in European Nucleotide Archive (ENA) EMBL under accession number PRJEB36985 (https://www.ebi.ac.uk/ena/data/view/PRJEB36985).

### Consortium flexibility: growth under different conditions and biomass increase

To study the consortium growth under different conditions, 500 ml flasks with a final volume of 400 ml were used in a HACH BOD2173 agitator (Hach Chemical Company, USA), operated at 150 rpm in darkness at 25 ± 2 °C. The cZ consortium was grown with 0.5% (v/v) OBW from different kind of ships (OBW A, OBW C, OBW D) in order to analyze its adaptation to wastewaters of different composition and check its operational flexibility. Different OBW initial concentrations, 0.25%, 0.5% and 1% (v/v), were also tested. Biomass growth was evaluated spectrophotometrically at 600 nm at different times, in triplicate. To evaluate the differences on the microbial consortium growth the Kruskal–Wallis nonparametric test was applied. A significance value of 0.05 was used in all analysis.

We plotted the measured OBW hydrocarbon concentration versus time on a semi-log plot and the reaction followed first order kinetics (data not shown). Then, Monod kinetic parameters were estimated for the different conditions tested (Clark and Blanch 1997; Nisenbaum et al. 2013; Corti Monzón et al. 2018). Monod's model adequately describes cell growth kinetics and can be used to describe complex degrading systems of different strains. Thus, we calculated the generation time of the microorganisms ($t_g$, d), time period in which cell population is doubled); the maximum specific cell growth rate ($\mu_{max}$, $d^{-1}$); and the productivity ($p_x$, cell $ml^{-1}$ $day^{-1}$) of the system.

In order to increase the cZ microbial consortium biomass, a Sequential Batch Reactor (SBR) was proposed in the same glass vessel described in "Hydrocarbon degradation potential" and operated in the same conditions. The reactor was fed with 0.5% (v/v) of sterile OBW in MS, pH 7. SBR operation was monitored by absorbance at 600 nm during each cycle of approximately 20 days (end of the exponential phase). Sedimentation and decanting periods were 1 h for all runs and the volume exchange ratio per cycle was ca. 60% of the reactor. The number of cycles carried out was determined according to the growth curves obtained, since the objective was to obtain three curves with the same stationary phase that indicated the stabilization of the culture. Three independent replicates were done. The variable Absorbance measured in all the reactors was described in univariate form for the SBR and batch tests. The data were then analyzed in their adjustment to a normal distribution using normal fit tests from Shapiro Wilks. To analyze the variation in the means of the different reactors of the SBR, a test of means analysis (nested ANOVA) was carried out, followed by a test of contrast analysis (Tukey test). A significance value of 0.05 was used in all analysis.

## Results and discussion

### OBW properties

Physical and chemical properties of OBW are shown in Table 1. The density, specific gravity and API gravity values denote the fluidity of this hydrocarbon mixture. The observed density value is within the density parameters for diesel, 0.840 g $ml^{-1}$ (YPF 2014) to 0.970 g $ml^{-1}$ (YPF 2012), which is fuel generally used by the vessels, and can fluctuate for the presence of other lubricant compounds, water from filtrations or washing and emulsifiers in the mixture. It is close to that presented by Nievas et al. (2006) for the OBW of Puerto Madryn (0.888 g $ml^{-1}$). The hydrocarbon chromatographic analysis showed the *n*-alkanes series from C10 to C38 and an unresolved complex mixture (UCM). Synchronous fluorescence spectroscopy (EFS) showed the presence of Aromatic Hydrocarbons (ArH), considered by the United States Environmental Protection Agency (Smith et al. 1989) as priority pollutants. An important diesel range organic (DRO) hydrocarbon fraction was found as expected, since fuel and diesel oil are the main fuels used in ships.

The composition of OBW varies between vessels and also from day to day within a vessel (Tiselius and Magnusson 2017), generating differences in the physicochemical parameters reported. Unlike the OBW used in this work, Vyrides et al. (2018) reported pre-settled OBW from Zygi, Cyprus, with a pH of

**Table 1** OBW properties

| | |
|---|---|
| Settleable solids (ml $l^{-1}$) | $\leq 0.1$ |
| Specific gravity | 0.785 |
| Conductivity (mS $cm^{-1}$) | $\leq 0.2$ |
| Density (g $ml^{-1}$) | 0.863 |
| Kinematic viscosity (cSt, 40 °C) | 55.4 |
| Water content (%, v/v) | 0.783 |
| pH | 6.5 |
| Total petroleum hydrocarbons (TPH) (g $l^{-1}$) | 408 |
| Gasoline range organics (GRO) (g $l^{-1}$) | 0.43 |
| Diesel range organics (DRO) (g $l^{-1}$) | 3.98 |
| Aliphatic hydrocarbons (AlH) | c10–c38 |
| Aromatics hydrocarbons (ArH) | +[a] |
| Unresolved complex mixture (UCM) | +[a] |
| Colony form units (CFU $ml^{-1}$) | $2.7 \times 10^5 \pm 3 \times 10^{-3}$ |

[a](+) Presence

7.5–8.5 and conductivity of 38.01 mS $cm^{-1}$. In relation to the hydrocarbons present in the other OBW previously reported, Sivaraman et al. (2011) found 43.16% of AlH and 2.79% of ArH and Nievas et al. (2006) reported 542 g $kg^{-1}$ of TPH in the OBW, which includes total HPLC-resolved hydrocarbons, as well as the complex unresolved mixture (UCM), 89% were AlH and 11% ArH. The results obtained for OBW M agree with the amount of TPH found by Nievas et al. (2008, 2006) for OBW waste of Puerto Madryn, Argentina.

Cultivable heterotrophic microorganisms count were $2.7 \times 10^5$ CFU $ml^{-1}$. This value was greater to the CFU reported in the intertidal water contaminated with hydrocarbons (Pucci et al. 2009), OBW from an oil company (Roy et al. 2014) and hydrocarbon contaminated water (Youssef 2010), which were of the order of $10^2$ to $10^4$ CFU $ml^{-1}$. Youssef et al. (2010) assigned the observed variations in the counts to the chemical nature of the samples.

## Biodegradation potential

Hydrocarbon biodegradation potential of the enriched cZ consortium was tested in a 2.5 l reactor in batch mode, with 0.5% (v/v) OBW as the sole carbon and energy source. Cell growth was determined by turbidity (Fig. 1a) and a correlation between $OD_{600nm}$ and number of cells $ml^{-1}$ was done ($y = 2 \times 10^9 x$, $R^2 = 0.9051$). During the first 4 days, the microbial population increased from $3.00 \times 10^8$ to $3.40 \times 10^9$ cells $ml^{-1}$, but continued growing gradually up to $1.10 \times 10^{10}$ cell $ml^{-1}$ after 32 days, 33 times higher than its initial concentration. During this time, the pH value remained between 7 and 7.5 and no microbial growth or pH change was observed in the negative control assay (data not shown). The hydrocarbons disappearance by day 32 is shown in Fig. 1. The hydrocarbon biodegradation efficiency (BE, %) was calculated, as mention in "Hydrocarbon degradation potential". The BE of the cZ consortium was 66.66% of TPH, and the removal of AlH and ArH were 97.76% and 87.2% respectively (Fig. 1c). The TPH BE was similar to that obtained by Nievas et al. (2006, 2008) working with autochthonous OBW consortia from Puerto Madryn. Regarding to ArH, Olivera et al. (2003) and Nievas et al. (2006) reported a BE of $\sim$ 80% and $\sim$ 89.4%, respectively and Nievas et al. (2005) of 7.1% and 18.3%.

The hydrocarbon chromatographic profiles (Fig. 2a) show a marked decrease in the resolved aliphatic fraction and in the unresolved complex mixture (UCM), which is shown as a "hump" below the resolved compounds (Olivera et al. 2003). The UCM is normally present in petrogenic hydrocarbon chromatographic profiles and could be composed of branched aliphatic hydrocarbons, cycloalkanes and aromatic hydrocarbons, that cannot be resolved (Frysinger et al. 2003), which usually show the greatest resistance to biodegradation (Atlas 1981; Frysinger et al. 2003). UCM biodegradation has been reported in other studies of different hydrocarbon mixtures (Geerdink et al. 1996; Marchal et al. 2003; Penet et al. 2004; Nievas et al. 2008). UCM has generally

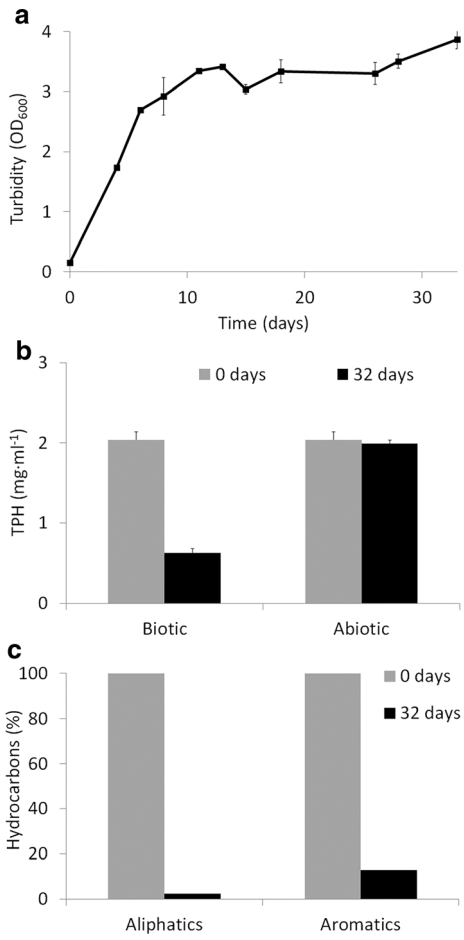


**Fig. 1** Cellular growth of the cZ consortium and hydrocarbon degradation of OBW as the sole carbon and energy source. cZ consortium was cultivated at an initial concentration of 0.1 ($OD_{600nm}$) in MS medium with OBW (0.5% v/v) as the only carbon and energy source; at 25 ± 2 °C and150 rpm in a 2.5 l batch reactor. The turbidity was monitored spectrophotometrically over time at 600 nm. The shown values are averages of triplicate samples. Hydrocarbons present in the reactor were measure at day 0 and day 32 by IR, GC and SFE respectively. Abiotic assays were done without cells. **a** Cellular growth curve, **b** Initial and residual TPH; **c** Initial and residual hydrocarbons (aliphatics and aromatics)

less microbial preference with respect to n-alkanes, which is usually attributed to the intrinsic difficulty of microorganisms to biodegrade these recalcitrant compounds.

Figure 2b shows the almost total disappearance of lighter aliphatic hydrocarbons up to n-C13. The range n-C8 to n-C11 consists of volatile hydrocarbons (Rahman et al. 2003), so the disappearance of the fraction C10–C13 could not be completely related to microbial degradation. The low percentage of volatile hydrocarbons present in the sample is due to the fact that it spends time on the ship and in the storage tanks before its treatment, which allows their loss. The cZ consortium achieved a significant reduction of the n-alkanes belonging to the n-C14 to n-C38, observing greater disappearance when the length of the carbon chain is shorter. Calculating the relative quantification of n-alkanes areas from the chromatographic profiles, the disappearance percentages were approximately: 99.88% (n-C10 to n-C13), 98.57% (n-C14 to n-C21), 97.13% (n-C22 to n-C33), 92.73% (n- C34 to n-C37), 71.43% (n-C38). In agreement with our results, Atlas (1981) reported that alkanes up to a chain length of C18 are the most easily biodegraded, while longer chains can be resistant to degradation (Singer and Finnerty 1984; Vestal et al. 1984; Atlas and Uterman 2002). In contrast, Nievas et al. (2006) obtained increasing biodegradation percentages of *n*-alkanes as the length of the carbon chains increases, which could be due to the differences in the microbial composition of the consortia.

In this work, the pH value remained between 7–7.5 throughout the time. Nievas et al. (2006) reported a pH change from 7 to 5.5 in the same MS medium with OBW. Rosenberg et al. (1992), Cerqueira et al. (2011), Janbandhu and Fulekar (2011) and Marín et al. (1995) also observed a decrease in pH during biodegradation with microorganisms growing at the expense of hydrocarbons. It has been reported that a pH of 7 is optimal for hydrocarbon biodegradation (Atlas 1988; Salmon et al. 1998) and extreme pH can have a negative influence on the ability of microbial populations to degrade hydrocarbons (Rahman et al. 1999; Meredith et al. 2000). Due to this background, the microbial cZ consortium grown in MS medium could have a great advantage in the biodegradation of OBW over other minimal mediums.

## Determination of consortium microbial composition

Bacteria are the most active microorganisms in the degradation of petroleum and they function as the primary degraders in spills of hydrocarbons in the environment (Gunkel 1968; Atlas 1981). In this work, total DNA was isolated at day 14 from the beginning of the batch culture and a molecular identification of bacterial amplicons of the 16S rRNA gene was done.

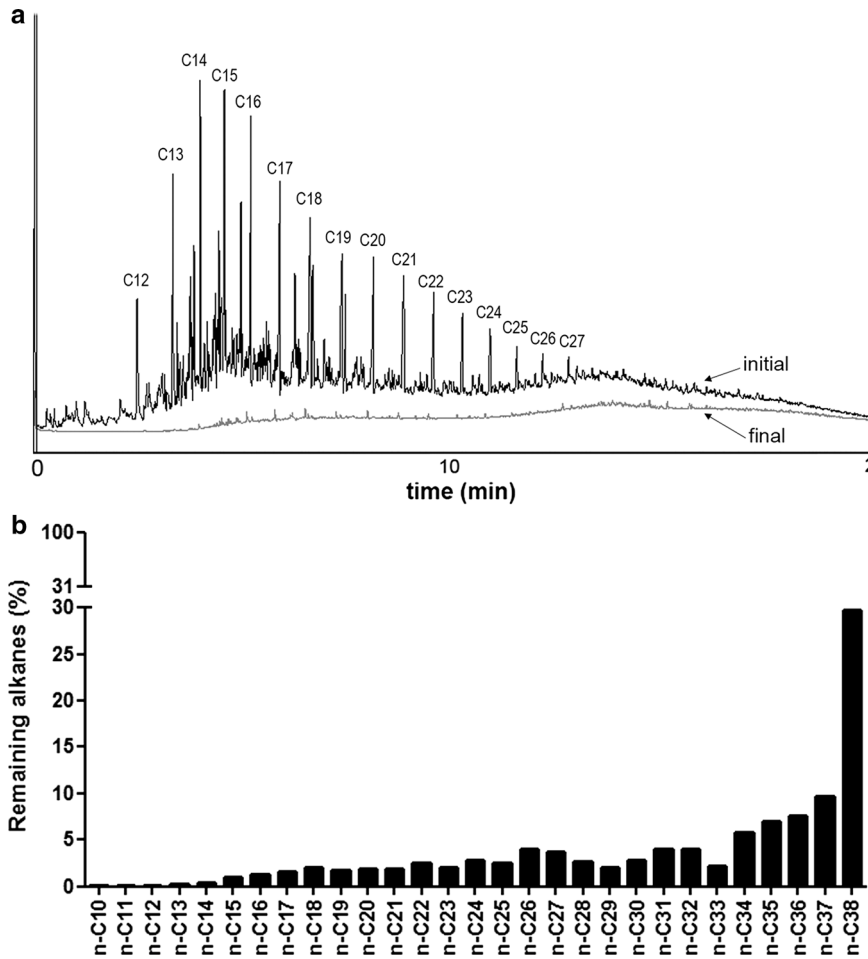


**Fig. 2** Determination of n-alkane series and aromatic compounds from OBW before and after the biodegradation assay. CZ consortium was cultivated at an initial concentration of 0.1 ($OD_{600nm}$) in MS medium with OBW (0.5% v/v) as the only carbon and energy source; at 25 ± 2 °C in agitation (150 rpm), in a 2.5 l batch reactor. **a** Hydrocarbon chromatographic profile analyzed by GC-FID at the beginning of the biodegradation assay (initial) and at the end of the assay (after 32 days) (final) **b** Relative quantification of *n*-alkanes areas from the chromatographic profiles, expressed as remaining percentage of hydrocarbons (after 32 days from inoculation) respect to initial time

A total of 73,702 amplicon reads of 16S rRNA gene hypervariable region V4 were obtained from the total DNA extracted from this consortium. By grouping them in Operational Taxonomic Groups (OTUs) at a 3% distance, 915 OTUs could be detected (Online Resource 1). Regarding the alpha diversity description of the amplicon complexity, it was reaching saturation, having a calculated Good's C coverage of 99.6%. A Simpson index of 0.936 indicates the presence of some predominant OTUs that accumulate a high relative frequency of reads, while a Shannon index of 5.196 indicates that evenness and richness of bacterial community OTUs are remarkably high for a sample coming from conditions that could be considered as strong selectors (contaminants and bioreactor culturing). Chao1 richness estimator of 1145.33 indicates that the number of OTUs that are possibly present but no detected is still significant, but given the high coverage obtained, they are very likely those coming from bacterial types at extremely low cell numbers in the original sample. Considering this information, we observed that, in fact, 2.7% of all the OTUs (25 out of 915) accumulated 83% of all the total reads, thus they are the predominant assemblage of the cZ consortium (at least on day 14, when the sample was taken). In contrast, OTUs that are having very low relative

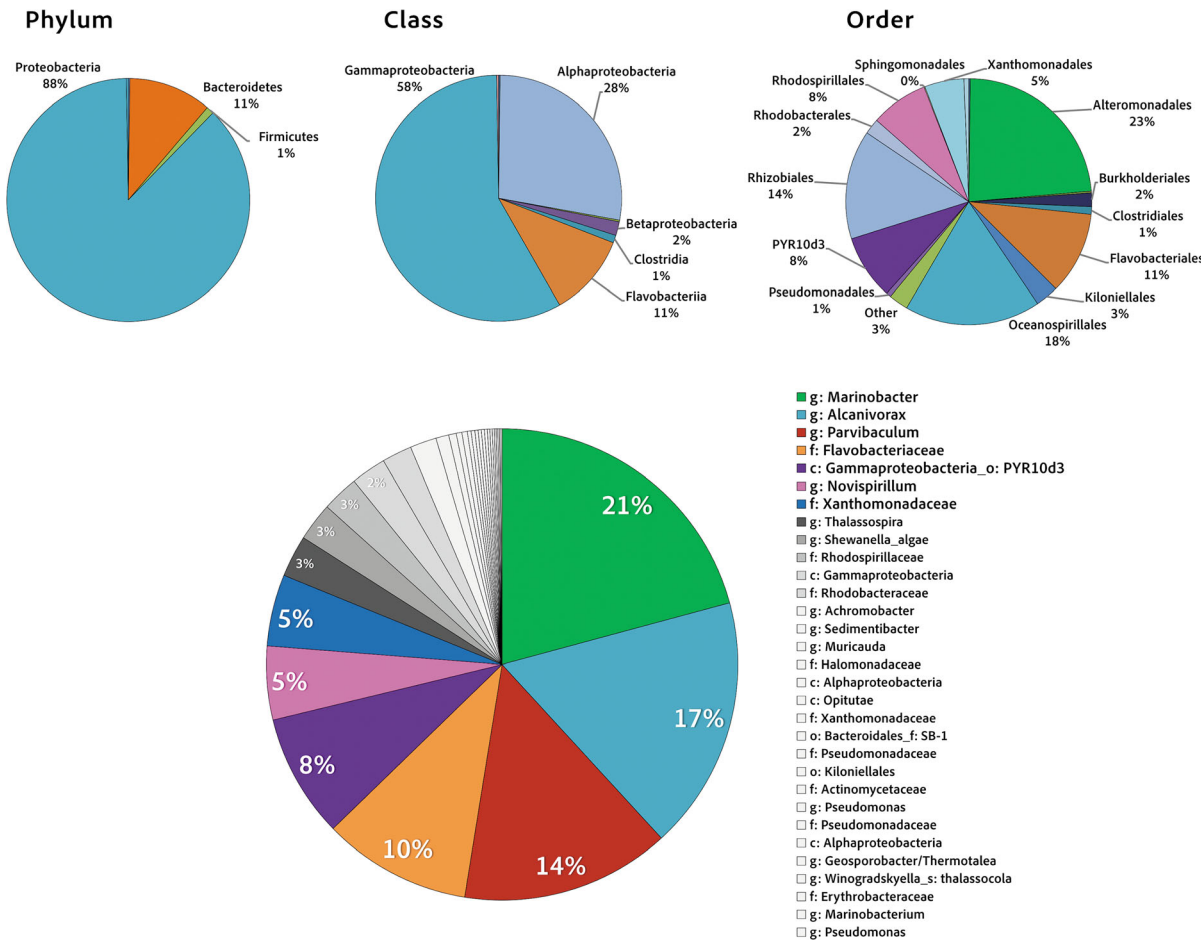


**Fig. 3** Bacterial members and relative abundance of the enriched microbial consortium cZ from OBW. The bacterial taxonomic composition at phylum, class and order level are shown. The distribution at the bottom shows the relative abundance of all the sequences obtained when classified in a given taxa with the maximum resolution attained when using RDP Naïve Bayesian Classifier (confidence value > 80%) and SILVA 132 as reference taxonomy. Seven predominant bacterial types, having a relative frequency of > 5%, account for 80% of the consortium cZ bacterial composition. The complete OTU identifiers and sequence dataset can be found as Online Resource 1

abundance (0.1%, frequency of 29 reads or below) are in turn accounting for 9% of the total reads, but are distributed in 842 OTUs. In Fig. 3 is shown the overall taxonomic composition of the microbial assemblage developed from the cZ consortium, determined for the 134 OTUs with a relative frequency above 0.1% (comprising 99.38% and classified in 31 taxa). This threshold was applied to simplify the pattern devoid of rare biosphere taxa (0.62% classified in 108 independent taxa). The most abundant bacterial species detected by this approach, by their relative abundance were: *Marinobacter* spp. 20.7%, *Alcanivorax* spp. 17.3%, *Parvibaculum* spp. 13.3%, Flavobacteriaceae 10.1%, Gammaproteobacteria PYR10d3 8.4%, *Novispirillum* spp. 5.0% and Xanthomonadaceae 4.9%; followed by *Thalassospira* spp., *Shewanella algae* and Rhodospirillaceae 3%, and Gammaproteobacteria, Rhodobacteriaceae and *Achromobacter* spp. 2%.

Two dominant phylogenetic classes constituted the OBW enriched microbial consortia, Proteobacteria (87.35%) and Bacteroidetes (11.03%) that together covered 98.4%. Firmicutes (1%), Verrucomicrobia (0.3%) and Actinobacteria (0.2%) represented the remaining. Archaeal sequences were detected but at very low abundances (0.03%). The presence of highly similar or identical sequences clustered in OTUs that can only be classified with certainty in a given family,

such as the case of the most abundant OTU classified as Flavobacteriaceae, Gammaproteobacteria PYR10d3 or Xanthomonadaceae, suggest the joint enrichment of bacterial types of not yet described genus or species representatives isolated or described so far. Thus, the consortium constitutes a resource where they can be maintained for further characterization or trials on pure culture isolation.

Phylum Proteobacteria was composed mainly of γ—(57.86%), α—(27.65%) and β—(1.84%) subdivisions. This phylum of Gram-negative organisms comprises the majority of the formally described genera of hydrocarbon-degrading bacteria (Timmis 2010). The observed predominance of Gammaproteobacteria in the cZ consortium is in agreement with several other hydrocarbon-rich environments including natural oil deposits, asphalt, crude oil, oil sand, oil contaminated water, soil and sludge (Batista et al. 2006; Militon et al. 2010; Yergeau et al. 2012; Mahjoubi et al. 2013; Head et al. 2014; Lamendella et al. 2014; Yang et al. 2014; Das and Kazy 2014). The most abundant genus found in cZ consortia, *Marinobacter* and *Alcanivorax*, have evolutionate as hydrocarbonoclastic bacteria and dominate in oil polluted sites (Abraham et al. 1998; Varjani 2017; Warr et al. 2018). Thirty four type strains of *Marinobacter* have been designated and the metabolic range of many of them remains largely unexplored. *Marinobacter hydrocarbonoclasticus* SP17, *M. maritimus* and *M. algicola* DG893 have been reported as alkane degraders (Gauthier et al. 1992; Shivaji et al. 2005; Green et al. 2006) and others were reported to degrade aromatic and polyaromatic hydrocarbons (Hedlund et al. 2001; Margareth et al. 2006; Dastgheib et al. 2012; Cui et al. 2016). Some *Marinobacter* species produce large quantities of bioemulsifiers that are attracting for various industrial applications and increase the bioavailability of hydrocarbons (Handley and Lloyd 2013). Others have been reported as Tween degraders and adsorb and tolerate high levels of metals/metalloids (Takai et al. 2005; Green et al. 2006). Therefore, this is a genus to be investigated from the cZ microbial consortium, since OBW presents commercial surfactants and different metals in its composition. The *Alcanivorax* group have been reported to produce glucolipids as biosurfactants when grow in *n*-alkanes (Abraham et al. 1998). Since *Alcanivorax* species can use alkanes more effectively than other hydrocarbon-degrading bacteria (Hara et al. 2003) and degrade branched alkanes, are commonly detected as predominant bacterial members in oil-containing seawater (Hara et al. 2003) and plays critical roles in the biodegradation of hydrocarbons contaminated sites. Uncultured clone PYR10d3, represents a group of three closely related γ-proteobacterial sequences (Singleton et al. 2006) and was previously found in a bacterial community originated from an oil contaminated coastal sediment (Païssé et al. 2010). This clone was associated with the degradation of pyrene in a bioreactor for treating soil contaminated with PAH (Singleton et al. 2006). Another family representing this subdivision in the cZ consortia was Xanthomonadaceae, also identified in microbial communities of oil sands tailings ponds (Rochman 2016), hydrocarbon-contaminated soil, seawater (Palleroni et al. 2010; Fan et al. 2019) and wastewater of an oil-polluted site (Timmis 2010) and able to utilize a wide spectrum of aromatic substrate as sole carbon source (Wojcieszyńska et al. 2011). *Shewanella* spp have been studied because they have an important role in co-metabolism during the bioremediation of halogenated compounds and petroleum derivatives, reducing magnesium, iron oxide and calcium (Kumar and Gopal 2015).

Alphaproteobacteria are a very diverse group characterized by their ability to degrade the polyaromatic hydrocarbons (PAH) of high molecular weight (Rehmann et al. 2001). In cZ consortium was represented mainly by *Parvibaculum* genus, which has been associated with diesel contaminated sites (Paixão et al. 2010) and heavy oil refinery wastewater (Wang et al. 2016). *Parvibaculum hydrocarboniclasticum* has been reported as an alkane-degrader (Rosario-Passapera et al. 2012) and *Parvibaculum lamentivorax* represents the first tier of a widespread microbial communities that degrade LAS, the commercial surfactant linear alkylbenzenesulfonate (Schleheck et al. 2004). The family Rhodospirillaceae, which includes de genus *Thalassospira*, both OTUs members of cZ consortia, have been also reported in oil-degrading enrichments from soil (Timmis 2010), oil polluted seawater and deep-sea sediment (Liu et al. 2007; Zhao et al. 2010). This family can grow in hydroxylated and aromatic compounds (Kodama et al. 2008; Giebel et al. 2016) and is capable of denitrification.

Betaproteobacteria was represented by *Achromobacter* spp. and *Novispirillim spp.* Some strains belonging to the genus *Achromobacter* reported

certain properties, e.g., biphenyl catabolism (Furukawa 2000), arsenite oxidation (Cai et al. 2009), (halo)aromatic acid degradation (Jencova et al. 2008), detoxification of chromium-containing slag (Chai et al. 2010), and hydrocarbon degradation (Deng et al. 2014), considered with high remediation potential. It has been reported to degrade phentachlorophenol (Murialdo et al. 2003; Gomila et al. 2011), petroleum hydrocarbons and produce biosurfactants, adapting to a wide range of salinity (Deng et al. 2010, 2014). The genus *Novispirillum* with an only known strain, *Novispirillum itersonii* previously known as *Aquaspirillum itersonii,* has been detected in aerobic hydrocarbon degrading microbial communities from oil sands (Rochman 2016), desert soil samples (Dashti et al. 2019) and fuel contaminated Antarctic soils (Eckford et al. 2002).

The phylum Bacteroidetes was represented mainly by the family Flavobacteriaceae. Members of this family have been isolated from a variety of sources (Jooste and Hugo 1999; McBride 2014) including and hydrocarbon-contaminated sites (Timmis 2010; Chaudhary and Kim 2018), and have been reported as fluorine and phenantrene (Viñas and Solanas 2005), diesel oil (Timmis 2010), crude oil (Rahman et al. 2002), and other aromatic and aliphatic compounds degraders (Krell 2018).

Our work contributes to the knowledge of microbiome composition of an enriched hydrocarbon-degrading consortia obtained from OBW samples of ships, that so far is very scarce. Olivera et al. (2003), Santisi et al. (2015), Nievas et al. (2006) and Sivaraman et al. (2011) have identified several cultivable oil-degrading bacteria from OBW samples, being found mainly members of the genus *Pseudomonas*. In our study, *Pseudomonas* represents less than 0.1% of the consortium.

Understanding the microbial community composition, structure and dynamics within contaminated OBW is critical for designing biodegradation strategies. The biochemical processes that prevail within this toxic residue largely depend on the microbial community and its metabolic potential, as well as the nature and amounts of available nutrients, highly fluctuating. In this study, we analyzed the microbial composition of the cZ consortium and evaluate the extent of biodegradation without chemical biostimulation. The bacterial diversity found indicates the presence of a metabolically rich community, with members that have previously been associated with the degradation of hydrocarbons, and capable of tolerating the adverse conditions of OBW. In this group, the presence of microorganisms capable of degrading both aromatic and aliphatic hydrocarbons (Fig. 1), and the potential capacity to reduce metals, produce biosurfactants, form biofilms, and degrade commercial surfactants (Online Resource 2), indicates a robust metabolic architecture for the OBW degrading community, retrieved and maintained in batch cultures. The cZ consortium is composed of aerobic and facultative anaerobic microorganisms, and some of them with the potential to function in an anaerobic environment (Online Resource 2). Within the bilge, forced aerobes prevail on the surface of the liquid. The scarce mixture presented by the waste only by the movement of the vessel, together with the low diffusion of $O_2$, generates anoxic and anaerobic zones inside and towards the bottom of the bilge tank where facultative anaerobic organisms are able to survive, constituting a functionally active part of the community (An et al. 2013; Sarkar et al. 2016).

## Consortium adaptability: growth under different conditions and biomass increasement

A comparative study of the cellular growth under different conditions was carried out to obtain preliminary information about the effect of wastewater quality and concentration, on the microbial consortium growth. Different initial OBW concentrations (0.25%, 0.5% and 1%, v/v) and OBW of diverse origin (OBW A, OBW C, OBW D and OBW M) were used for the assays (Table 2). Significant differences were found between the growth curves for different OBW initial concentration ($p = 3.677 \times 10^{-16}$). The cZ consortium showed an increasing capacity for growing with increasing concentration of OBW as well as maximum cell concentration ($X_{max}$) and cell productivity (average rate of production: $p_x$) were reached with 1% (v/v) of OBW. This observation coincides with that of Nievas et al. (2008) for OBW concentrations of 0.18% (v/v) and 0.53% (v/v). Between 0.5 and 1% of OBW M, there is a diminished effect in $\mu_{max}$ may be due to accumulation of toxic products or reduced availability of some other substrate (Stanbury et al. 2016). The threshold substrate concentration for OBW degradation inhibition will be determined in further studies, but this finding remarks the robustness of the microbial consortium obtained.

**Table 2** Growth parameters of cZ growing with different OBW and at different OBW initial concentration

| Assay | $X_0$ (cells ml$^{-1}$) | $X_{max}$ (cells ml$^{-1}$) | $\mu_{max}$ (day$^{-1}$) | $t_g$ (day) | $p_x$ (cells ml$^{-1}$ day$^{-1}$) |
|---|---|---|---|---|---|
| 0.25% (v/v) OBW M | $2.00 \times 10^8 \pm 1.00 \times 10^{-2}$ | $2.38 \times 10^9 \pm 6.18 \times 10^6$ | 0.335 | 2.07 | $8.20 \times 10^7$ |
| 0.5% (v/v) OBW M | $9.33 \times 10^7 \pm 1.89 \times 10^6$ | $7.68 \times 10^9 \pm 8.53 \times 10^7$ | 0.363 | 1.91 | $1.24 \times 10^8$ |
| 1% (v/v) OBW M | $2.00 \times 10^8 \pm 1{,}00 \times 10^{-2}$ | 1.71E+10 ± $1.10 \times 10^8$ | 0.094 | 7.37 | $2.76 \times 10^8$ |
| 0.5% (v/v) OBW A | $2.00 \times 10^8 \pm 1.89 \times 10^5$ | $5.09 \times 10^9 \pm 1.92 \times 10^7$ | 0.498 | 1.39 | $3.33 \times 10^8$ |
| 0.5% (v/v) OBW C | $1.70 \times 10^8 \pm 4.81 \times 10^6$ | $4.12 \times 10^9 \pm 6.07 \times 10^6$ | 0.127 | 5.46 | $1.94 \times 10^8$ |
| 0.5% (v/v) OBW D | $2.00 \times 10^8 \pm 91.43 \times 10^4$ | $3.07 \times 10^9 \pm 1.05 \times 10^9$ | 0.270 | 2.57 | $1.45 \times 10^8$ |

The reaction follows a first order kinetics. $X_0$ cell number at the beginning of the assay; $X_{max}$ max cell number at the beginning of the stationary phase of growth; $\mu_{max}$ the maximum microbial specific growth rate that is reached in this process corresponds at the exponential phase of growth; $t_g$ time in with biomass duplicates, $p_x$ cell produced per milliliter of the culture per day. *A* deep sea fishing ship; *D* dredge ship; *C* coastal fishing boat; *M* parked at the port

The cZ microbial consortium was able to grow in 0.5% (v/v) bilge oily residues from different origins (Table 2). Significant differences were found between cell growth curves ($p = 3.955 \times 10^{-12}$) at expense of OBW A, OBW C, OBW D and OBW M. Although, OBW C samples showed longer doubling time ($t_g$) than others and the answer may be in the composition and concentration of detergents, solvents and other infiltrated substances present in the OBW which could affect the microbial growth. OBW A presented the highest $\mu_{max}$ and $p_x$, maybe the quality of wastewater could impact positively over the biomass growth, since these samples proceeded from long-distance fishing vessels. Biodegradation kinetic parameters are predictive tools necessary for scaling at wastewater treatment plants, but there is a paucity of information on kinetic parameters of OBW biodegradation. Therefore, we report the first assessments on this matter in the present study.

Mixed cultures can be maintained at the laboratory by continuous growth on an enrichment mixture. However, given that large amounts of inoculum are required for using in bioremediation technologies, a large scale biomass production is necessary. The increase of biomass by subculture in rich media may involve the loss of the capacity to degrade hydrocarbons, and it is also an additional operational cost. To increase the biomass of cZ consortium, a Sequential Batch Reactor (SBR) was proposed, using OBW M (0.5% v/v) as the sole carbon and energy source. SBRs are part of unit processes, which use fill and discharge cycles in a single tank. All SBR systems have five steps in common, that follow the sequence of: filling, reaction, settle (sedimentation/clarification), draw, idle (Moreno-Andrade and Buitrón 2012). In this way, the biomass, grown and acclimated in each cycle, remains to the next until system stabilization. In this work, seven growth cycles were performed and a cellular concentration of $2.03 \times 10^{10}$ cells ml$^{-1}$ was achieved at the end of the last cycle, which represents a 67-fold increase with respect to the initial reactor ($3.00 \times 10^8$ cells ml$^{-1}$) (Fig. 4). The statistical analysis of the different cycles determined significant differences between the initial reactor and reactor number 5 ($p = 1.19 \times 10^{-8}$). No significant differences were observed between the subsequent reactors, which could be inferred as the stabilization of the bacterial consortium. This operation allowed an interesting increase in the biomass of the cZ consortium at the expense of bilge wastewater as the only source of carbon, without the addition of a rich carbon source. Otherwise, it could imply an additional step and a possible loss in the hydrocarbon degrading capacity by the microbial consortium. The increase in the initial concentration of the only carbon source (hydrocarbons) lead to an increase in the biomass content (data not shown), therefore, the increase in biomass in the SBR contributes to the degradation rate of oil from OBW.

## Conclusions

This work reports the generation of a microbial consortium arising from oily bilge wastewaters (OBW), a harsh, toxic and chemically complex

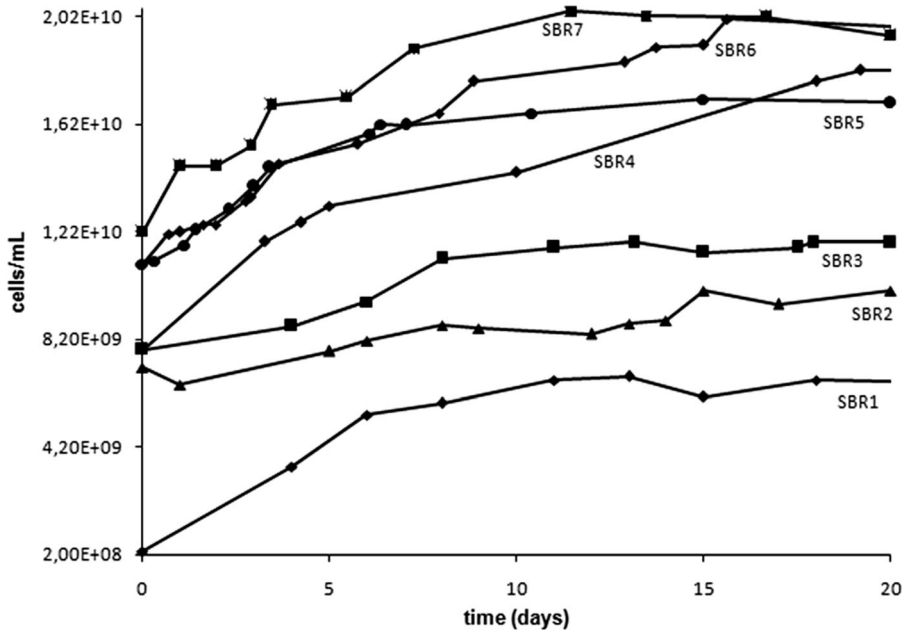


**Fig. 4** Biomass increase of the cZ consortium in SBRs. CZ consortium was cultivated at an initial $OD_{600nm}$ of 0.1 in MS medium and BW M as the only carbon and energy source. Seven cycles of SBR were done. Growth was followed by measurement of $OD_{600nm}$ over time. $OD_{600nm}$ values were expressed as cell/mL using the following equation $y = 2 \times 10^9 x$ ($R^2 = 0.9051$); were $y$ = number of cells/mL and $x = OD_{600nm}$ as described in "Material and methods" section. The values are the averages of triplicate samples

contaminant in the marine environment. After several rounds of consortium establishment and evidenced of its stability and robust growth across oily bilge wastes of different origins tested, the consortium bacterial composition by 16S rRNA gene sequencing of the selected assemblage of autochthonous bacteria collectively thriving and achieving the degrading the hydrocarbons of oily bilge wastewaters. The composition of the cZ consortium microbiome showed the predominant bacterial types classified as *Marinobacter* spp., *Alcanivorax* spp., *Parvibaculum* spp., Flavobacteriaceae, Gammaproteobacteria PYR10d3, *Novispirillum* spp., Xanthomonadaceae as main components, followed by *Thalassospira* spp., *Shewanella* spp., Rhodospirillaceae, and Gammaproteobacteria, Rhodobacteriaceae and *Achromobacter* spp. The selected assemblage of autochthonous bacteria thrives and collectively achieves the degradation of the aliphatic and aromatic hydrocarbons of OBW. With five cycles of SBR the microbial consortium biomass was enriched 67-fold employing OBW as the only carbon and energy source. The present study allowed the identification of organisms which, isolated or associated in consortium, could potentially be explored in biotechnological processes for biodegradation of hydrocarbon pollutants present in wastewaters. Microorganisms that are clearly abundant, in some cases, are accurately classified as belonging to genera or species of hydrocarbon degraders or biosurfactant producers well described in the marine environment. However, other abundant members cannot be classified within a specific genus or species, which would indicate that, in the enriched consortium, such crucial members are very likely to come from bacterial species not yet described. This study highlights that the indigenous emerged microbial community from oily bilge wastewaters is intrinsically diverse and, notably, remarkable biodegradation rates could be achieved without any nutrient or surfactant addition, which are desirable features that might be of advantage for convenient application costs and feasibility on site. More research is needed to obtained kinetic parameters and thus be able to model a possible batch treatment for OBW.

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**Code availability** Not applicable.

**Compliance with ethical standards**

**Conflict of interests** The authors declare that they have no conflict of interest.

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