ORIGINAL RESEARCH

Characterization of hydrocarbon degrading bacteria at EPEA station, South Atlantic coast

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ABSTRACT. Hydrocarbon degrading bacteria (HDB) were monitored since 2006 to 2018 at the 'Estación Permanente de Estudios Ambientales' (EPEA), in order to analyze its abundance and the potentiality to metabolize these pollutants. The presence of HDB was detected with counts values ranging between 10^3 and 10^5 UFC ml^{-1}. A slight increase was observed over time, which could be linked to changes in marine temperature reported within the last years. Thirty-six HDB were tested for growth on various hydrocarbons and some of them showed a broad biodegradation profile. Moreover, from phenanthrene (Phe) enrichment cultures, five strains were phylogenetically identified as *Halomonas* sp. E1, E2 and E3; *Rhodococcus* sp. E4 and *Pseudomonas* sp. E5. Complete Phe degradation was demonstrated for E4 and E5 strains, while E1, E2, E3 and E4 strains displayed surfactant production. This study contributed with the first knowledge about the intrinsic hydrocarbon biodegradation potential by bacterial communities at EPEA. Some of the strains exhibited physiological properties that might have ecological significance on environmental alterations as the presence of pollutants. Particularly, *Rhodococcus* sp. E4 could be an alternative for microbial selection in the degradation of polycyclic aromatic hydrocarbons. Further studies are needed to evaluate the impact of the climate change on microbial-mediated detoxification processes.

Key words: PAH, bioremediation, biosurfactant.

Caracterización de bacterias degradadoras de hidrocarburos en la estación EPEA, costa del Atlántico Sur

RESUMEN. Las bacterias degradadoras de hidrocarburos (BDH) fueron monitoreadas desde 2006 a 2018 en la Estación Permanente de Estudios Ambientales (EPEA), con el fin de analizar su abundancia y la potencialidad de metabolizar estos contaminantes. La presencia de BDH se detectó con valores de recuento que oscilaron entre 10^3 y 10^5 UFC ml^{-1}. Se observó un ligero aumento a lo largo del tiempo, que podría estar relacionado con cambios en la temperatura marina reportados en los últimos años. Se analizaron 36 BDH para determinar su crecimiento en varios hidrocarburos y algunas de ellas mostraron un perfil de biodegradación amplio. Además, a partir de cultivos de enriquecimiento con fenantreno (Phe), se identificaron filogenéticamente cinco cepas como *Halomonas* sp. E1, E2 y E3; *Rhodococcus* sp. E4 y *Pseudomonas* sp. E5. Se demostró una degradación completa de Phe para las cepas E4 y E5, mientras que las cepas E1, E2, E3 y E4 mostraron producción de surfactante. Este estudio contribuyó con el primer conocimiento sobre el potencial intrínseco de biodegradación de los hidrocarburos por las comunidades bacterianas en EPEA. Algunas de las cepas exhibieron propiedades fisiológicas que pueden tener importancia ecológica sobre alteraciones ambientales como la presencia de contaminantes. En particular, *Rhodococcus* sp. E4 podría ser una alternativa para la selección microbiana en la degradación de hidrocarburos poliaromáticos. Se necesitan más estudios para evaluar el impacto del cambio climático en los procesos de desintoxicación mediados por microbios.

Palabras clave: PAH, biorremediación, biosurfactante.

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INTRODUCTION

Despite the flow of water masses and the dilution power of marine waters, oil pollution caused by industrial and vessel activities has a significant impact on these systems. It is broadly recognized that hydrocarbons contamination has damaged oceans, seas and coastal zones and represents a continuous threat to the marine environment sustainability (McGenity et al. 2012). Main sources of oil pollution in open oceans and coastal waters occur by accidental spills and deliberate discharge of ballast, wash waters from oil tankers, and bilge waste discharges, producing contamination and severe adverse effects on the ecosystem (Etkin 2010). Normal shipping operations account for over 70% of the hydrocarbons entering the sea from marine transportation (Nievas et al. 2006). According to the International Maritime Organization (IMO), the shipping industry that fulfils more than 90% of trade across the world with the help of around 90,000 marine vessels contributes heavily to global pollution and climate change (Jägerbrand et al. 2019). Likewise, marine environments are especially vulnerable to oil spills because they are poorly contained and difficult to mitigate.

As a result of oil contamination in marine ecosystems, adverse effects have been observed on aquatic organisms at sub-lethal concentrations (McGentity et al. 2012). Hydrocarbons can become dangerous fundamentally in the event that they enter the food chain, since several compounds as polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic and carcinogenic (Perelo 2010). Hydrocarbons have also a natural potential for bioaccumulation in marine organisms with possible transfer to humans via seafood and are therefore considered as substances of potential human health hazards (Mrozik et al. 2003).

Biodegradation by natural microbial populations is the most basic and reliable mechanism by which thousands of xenobiotic pollutants, e.g., hydrocarbons, are removed from the environment (Cappello et al. 2007). Microbial communities not only play a central role in the main biogeochemical cycles but also in the global recycling of pollutants (Falcón et al. 2008). Autochthonous hydrocarbon-degrading microorganisms living in marine systems would be better adapted to restore the hydrocarbon contamination in seawater. In general, bacteria have great adaptability to diverse environmental conditions, fast population growth and metabolic versatility (Deng et al. 2014). Therefore, the understanding of the microbial community and its catabolic activity are essential for the assessment of its biodegradation potential and for the effective remediation of contaminated areas (Muangchinda et al. 2015; Shi et al. 2019). These studies are also important in the context of the climate change. Despite the importance of microbes in the process of global recycling of anthropogenic pollutants, the potential interactions of ocean acidification, UVR, temperature, anthropogenic pollutants, and marine microbial communities have been largely ignored. It has recently been demonstrated that such interactions could alter microbial-mediated detoxification processes (Coelho et al. 2013; Louvado et al. 2018; Cabral et al. 2019).

The permanent coastal station EPEA (Estación Permanente de Estudios Ambientales) is one of the foundation stations of ANTARES, a network of time series stations along South America (www.antares.ws) located in the coastal waters of Argentina, 27 nautical miles south from Mar del Plata harbor. The main objective of EPEA time-series is to understand the annual and inter-annual dynamics of environmental variables and all components of plankton and follow possible long-term changes. This station is affected by the increasing vessel traffic causing oil pollution in the sea.

The aims of this research was to monitor the hydrocarbon degrading bacteria (HDB), isolated from EPEA station since 2006 to 2018, in order to analyze its abundance over time and the potentiality to metabolize these pollutants. Autochthonous bacteria capable of degrading the polyaro-

matic hydrocarbon phenanthrene (Phe) were also selected and identified, characterizing their biodegradation capacity and emulsifying activity. Microorganisms of several genera like *Rhodococcus*, *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Acinetobacter* and *Mycobacterium* have been previously identified as PAH-degraders, and complete PAH mineralization has been demonstrated for both low- and high-molecular-weight PAHs (Johnsen et al. 2005; Ghosal et al. 2016). In addition, some PAH-degrading bacteria display strategies to improve hydrocarbon accessibility, such as biosurfactant production (Pedetta et al. 2013).

MATERIALS AND METHODS

Study area and sampling

Sampling was performed during 46 research cruises (Table 1) carried out by research vessels (INIDEP) from 2006 to 2018 at the EPEA station, located at 38° 28′ S and 57° 41′ W in the Atlantic Ocean (Figure 1). Surface water samples were collected with a bucket and transferred to sterile plastic containers and stored at 4 °C. In order to

Table 1. Salinity and temperature values during EPEA research cruises.

Research cruise	Salinity	Temperature (°C)	Month/ year	Research cruise	Salinity	Temperature (°C)	Month/ year
CC0906	33.602	13.467	10/2006	EH0613	33.765	15.383	12/2013
CC1206	33.548	15.913	11/2006	OB0214	34.260	19.170	03/2014
OB0107	33.661	19.483	01/2007	AH0215		23.177	02/2015
CC0407	33.756	11.259	07/2007	AH0315	33.765	17.430	04/2015
CC0607		12.467	10/2007	AH0515	33.804	10.982	09/2015
OB0108	33.793	11.804	10/2008	AH0216	33.880	17.910	04/2016
OB0408	33.405	14.337	12/2008	AH0516	33.836	10.919	09/2016
OB0109	33.524	20.185	01/2009	AH0716	33.762	12.215	10/2016
CC0109	33.538	20.239	02/2009	EH0117	33.966	21.170	02/2017
OB0409	33.804	20.533	03/2009	AH0217	34.099	17.850	05/2017
OB0609	34.112	18.124	04/2009	AH0317	34.068	16.726	06/2017
CC0809	34.027	13.895	06/2009	AH0417	33.863	11.441	08/2017
CC0909	34.075	12.663	07/2009	AH0617	33.663	12.274	09/2017
CC1109	34.053	10.325	08/2009	AH0817	33.608	15.570	11/2017
CC0110	33.970	10.313	08/2010	EH0118	34.064	19.990	01/2018
CC0510	33.710	11.247	10/2010	VA0318	34.313	19.648	04/2018
CC1010	33.652	19.075	12/2010	AH0218	34.208	17.217	05/2018
CC0311	33.631	21.590	01/2011	AH0318	34.105	14.151	06/2018
OB0611	34.034	13.468	06/2011	AH0418	33.848	11.844	07/2018
OB0212	33.670	11.759	10/2012	VA1218	33.888	10.845	08/2018
OB0413	34.065	12.026	07/2013	AH0518	33.982	10.894	09/2018
CR0113	33.907	10.652	08/2013	VA1318	33.888	13.051	10/2018
OB0513	33.853	9.942	09/2013	AH0718	33.608	15.570	12/2018

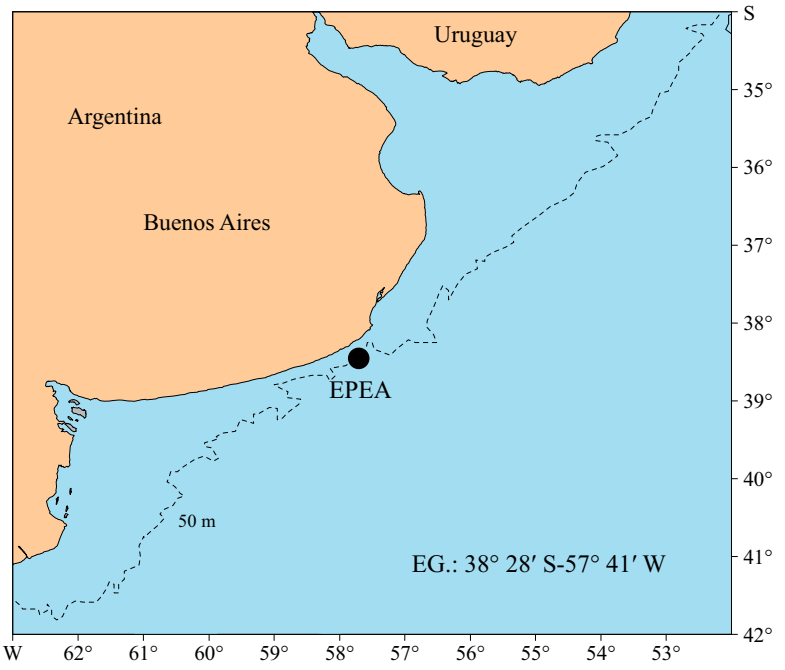

Figure 1. Location of the 'Estación Permanente de Estudios Ambientales' (EPEA).

describe the oceanography of the station, water temperature (°C) and salinity were measured with a CTD (Sea-Bird 19-01 CTD, SN 1268) by BaRDO (Base Regional de Datos Oceanográficos) of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP).

Bacterial abundance and isolation of hydrocarbon degrading bacteria (HDB)

To determine bacterial counts and to isolate pure bacterial strains, 3-fold serial dilutions were performed in saline solution (NaCl 9%, p/v). Duplicate aliquots from each dilution were spread onto mineral salts medium (MSM) agar plates (Schlegel et al. 1961), modified by the addition of NaCl 3% (w/v) and 50 ml of diesel oil as the sole carbon and energy source. MSM agar plates with no carbon source were used as negative control. Plates were incubated aerobically at 25 °C for 7-14 days, and those yielding 30 to 300 colonies were afterwards directly counted and expressed as CFU ml^{-1}. Colonies with distinct morphologies were picked and subsequently purified by repetitive streaking onto diesel oil-MSM agar. Pure cultures of final isolates selected were preserved as 10% dimethyl sulfoxide (DMSO) stocks at -80 °C.

Hydrocarbon utilization profile of the strains

Thirty-six purified strains selected from the diesel oil-MSM agar plates were tested for growth on various hydrocarbons used as sole carbon source in order to investigate their degradation potential as was reported previously (Peressutti et al. 2003). Suspensions of the strains were inoculated in MSM liquid medium supplemented with technical hydrocarbon mixtures (diesel oil, kerosene and mineral oil) and pure hydrocarbons (n-pentane, n-hexane, n-octane, n-pentadecane, n-hexadecane, dodecane, phenyldecane, cyclohexane, benzene, toluene, naphthalene, anthracene and phenanthrene). Substrates were added at 0.5%

(v/v), except for aromatic hydrocarbons which were used at 0.1% (v/v or p/v) because higher concentrations could be toxic for cellular growth. Flasks were incubated at 25 °C for 14 days and bacterial growth was evaluated by optical density at 600 nm (OD_{600}). Control cultures lacking a carbon source were performed for each isolate.

Degradation of PAH and biotic factors associated

Enrichment and isolation of PAH-degrading strains

In order to study PAH-degrading bacteria, enrichment cultures with Phe as the sole carbon and energy source were set up according to Pedetta et al. (2013). Water sub-samples (10 ml) were inoculated into 500-ml flasks containing 100 ml of MSM liquid medium supplemented with Phe (160 mg l^{-1}). Cultures were incubated aerobically on an orbital shaker at 25 °C and 150 rpm for 14 days and bacterial growth was evaluated by optical density at 600 nm (OD_{600}). Subsequently, 100 µl aliquots of cultures were spread onto MSM-Phe agarose plates (Bogardt and Hemmingsen 1992), incubated at 25 °C for 7 days and colonies of candidate Phe-degrading strain were picked up and further purified by repetitive streaking on the same fresh agar medium. Pure cultures of final isolates selected were preserved as 10% DMSO stocks at -80 °C.

Molecular identification of the bacterial isolates

Molecular identification was carried out by analyzing 16S rDNA gene sequencing. Genomic DNA from each isolate was extracted according to Wilson (2001), and its quality checked in a 0.8% agarose gel electrophoresis after staining with SyberSafe (Invitrogen, Argentina). PCR amplifications were performed by using the universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT-3') (Devereux and Willis 1995). The reaction mixture contained: extracted DNA 1 µl, GoTaq DNA polimerase (Promega) 5 UI/µl, buffer 5X, BSA 10 mg/ml, dNTPs 2.5 mM, each primer 0.5 µl and sterile double-distilled water was added up to the end volume of 25 µl. The program used for the amplification was: 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C; and a final elongation of 15 min at 72 °C (Olivera et al. 2005), in a thermocycler (Life Express, TC-96/T/H.a). PCR products were electrophoresed on 1.5% (w/v) agarose gels containing SyberSafe and visualized with UV in GelDoc EQ (Bio-Rad, Hercules, CA, USA. DNA fragments of expected size (1.3 kbp) were eluted, purified, and sequenced commercially at INTA Castelar (Argentina) by using the primers F63 (5'-CAGGCCTAACACATGCAAGTC-3') and F530 (5'-GTGCCAGCMGCCGCGG-3'). The resulting sequences of the amplified fragments were compared against sequences contained within Public Database (NCBI/BLAST). Then, sequences were analyzed phylogenetically with the MEGA 5.2 program (Tamura et al. 2011). Phylogenetic trees were constructed through the neighbor-joining (NJ) algorithm from a distance matrix calculated following Tamura-Nei model plus discrete Gamma distribution. Stability among the clades was assessed with the 1,000-replication bootstrap analysis. 16S rRNA sequences were deposited at the GenBank database under accession numbers MW160443 to MW160447.

Phenanthrene degradation

Biodegradation assays were conducted inoculating an exponential phase culture of each bacterial strain in 50 ml of MSM-Phe liquid medium, and a non-inoculated flask was used as abiotic control. Cultures were incubated aerobically on an orbital shaker at 28 °C and 150 rpm for 12 days. To measure residual Phe concentrations, 3-ml aliquots were withdrawn from the cultures each 48 h and subsequently extracted with 6 ml of acetonitrile. Tubes were incubated on an orbital shaker for 1 h at 25 °C and 150 rpm. After that, extracts of each culture were centrifuged (2500 g,

10 min) and supernatants analyzed by reverse-phase HPLC according to NIOSH (1998), at CNEA (Buenos Aires). Chromatographic measurements were carried out with an ACCELA 600 HPLC instrument (Thermo Scientific, USA), consisting of a quaternary pump, an autosampler and a photodiode-array detector. Column oven temperature was set at 50 °C and quantification wavelength was 254 nm. Separation was performed using a 3 µm particle C-18 column of 250 × 4.6 mm (Inertsil ODS-3; GL Science, Japan). Isocratic elution with 80% acetonitrile/20% water was performed at a flow rate of 0.9 ml min^{-1}.

Biosurfactant production

Two distinct methods were used for the screening of the biosurfactant production by isolates: (i) the drop collapse test and (ii) the emulsification assay. The drop collapse test was performed according to Jain et al. (1991) by adding 1 µl of methylene blue [0.1% (w/v)] to 20 µl of cell-free medium from saturated cultures grown in MSM-Phe. The resulting mixture was spotted onto a piece of Parafilm sheet (Pechiney Plastic Packaging, USA), and after 5 min of incubation the shape of the drop on the surface of the oil was observed. If the drop collapsed, the presence of surfactant (positive result) was indicated; if it remained beaded, absence of surfactant (negative response) was implied. Methylene blue was added for visualization purposes without influencing in droplet collapse activity. Fresh MSM-Phe medium containing either no addition or 1% sodium dodecyl sulphate was used as negative and positive controls, respectively.

The emulsification index (E_{24}) of the culture supernatant was determined by adding 2 ml of hexadecane to the same amount of aqueous supernatant. The mixture was vigorously mixed (vortex) for 2 min and kept in an incubator at 25 °C for 24 h prior to measurement. The emulsification activity was calculated as a percentage of the height of the emulsified layer divided by the total height of the liquid column (Iyer et al. 2006).

RESULTS AND DISCUSSION

Biodegradation mediated by indigenous microbial communities is the ultimate fate of the majority of oil hydrocarbon that enters the marine environment, where hydrocarbon-degrading microorganisms are ubiquitous. However, rates of biodegradation depend on abundance and metabolic ability of HDB, chemical structure of the pollutant and environmental conditions (Ron and Rosenberg 2014). Despite the EPEA station is not affected by industrial activities, the increasing maritime traffic shows the importance of monitoring HDB abundance and their degradation capability in order to predict the natural decontamination potential in this area.

Hydrocarbon degrading bacterial abundance

This study showed the presence of HDB at EPEA station with abundance values ranging between 10^3 and 10^5 UFC ml^{-1} (Figure 2). Salinity values fluctuated from 33.6 to 34.2 (Table 1) and this variation did not seem to influence on bacterial counts. Higher counts were associated, in general, to warmer months (summer) (Table 1) confirming that elevated temperatures enhanced HDB growth as reported previously (Scheibner et al. 2018). In addition, it is remarkable the slight increase of HDB counts observed over time, which could be linked to the changes in marine temperature reported within the last years (Silvestri and Berman, 2018). These authors described, through mathematical simulation models, the significant temperature changes in Southwestern Atlantic Ocean during the past years and predicted the accelerated global warming for the next decades in this area.

Rising temperatures directly and indirectly impact pelagic microorganisms and aquatic food webs leading to changes in the structure and functioning of marine ecosystems. In this sense,

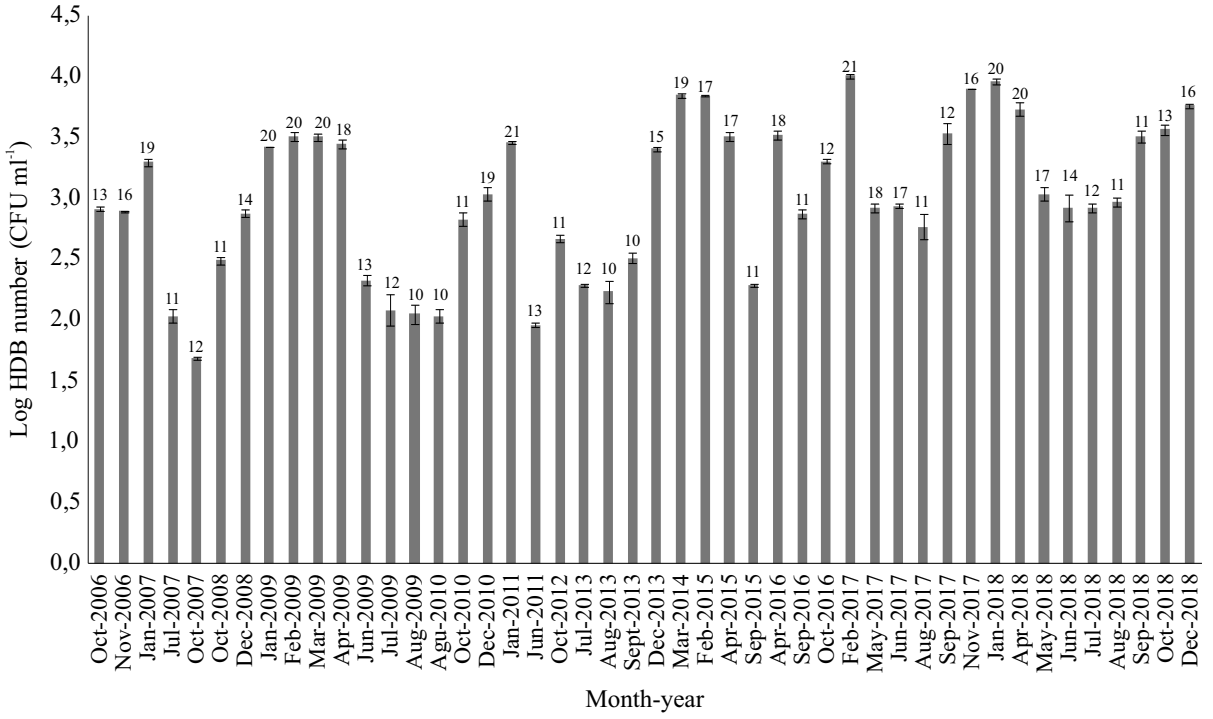

Figure 2. Abundance of hydrocarbon degrading bacteria –HDB (in duplicate) during EPEA research cruises, since 2006 to 2018. Temperature values (°C) are indicated over the bars. Values are means ± standard deviations for two replicates.

warming-induced increases in bacterial activities (abundance, production and respiration) result in higher processing of organic matter affecting carbon flow into the microbial food web (Scheibner et al. 2018). Even more, changes in oceanic temperature along with pH and UVR could disrupt key microbial-mediated services in marine ecosystems, like bacterial pollutant detoxification processes (Coelho et al. 2016). Therefore, the study of microbial communities is crucial to understand the consequences of these continuing global and local anthropogenic distresses on health and function of marine ecosystems.

Finally, samples corresponding to AH0617, AH0518 and VA1318 research cruises showed water temperatures quite low (between 11 and 13 °C) associated to high HDB counts, suggesting that other factor/s could also be impacting on the HDB counts besides water temperature.

Carbon-source utilization

Most of the strains were able to grow on technical hydrocarbon mixtures derived from oil distillation as kerosene, gasoil and mineral oil (Table 2). These substances are usually released from vessels either by accidental spills or deliberate discharge (Nievas et al. 2005). On the other hand, alkanes are major crude oil components and despite their low water solubility various microorganisms have the ability to utilize them as substrate using different uptake strategies followed by specific metabolic pathways (Guibert et al. 2016). In general, the analyzed isolates were able to grow on alkanes assayed as sole carbon source, especially medium chain n-alkanes (C12-C16) but only one strain used cyclohexane.

Polyaromatic hydrocarbons are resistant to biodegradation because of their chemical stability, low water solubility and high recalcitrance

Table 2. Utilization of hydrocarbons as carbon substrate.

	Strain																	
Hydrocarbon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Kersosene	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Diesel oil	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Mineral oil	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-
n-Pentane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n-Hexane	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-
n-Octane	-	-	-	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-
n-Pentadecane	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+
n-Hexadecane	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
n-Dodecane	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+
Phenyldecane	-	-	+	-	+	+	-	-	+	-	-	-	+	-	+	-	-	-
Cyclohexane	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Benzene	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Toluene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Naphtalene	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Anthracene	-	+	-	+	+	+	+	-	-	+	-	-	+	-	-	-	-	-
Phenantrene	-	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	-	-
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Kersosene	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
Diesel oil	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mineral oil	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-
n-Pentane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n-Hexane	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-
n-Octane	+	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	+	-
n-Pentadecane	-	-	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+
n-Hexadecane	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
n-Dodecane	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-
Phenyldecane	-	-	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-
Cyclohexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Toluene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Naphtalene	-	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	-	+
Anthracene	-	-	+	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-
Phenantrene	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	-	-	-

properties (Isaac et al. 2016). Some of the strains in this work were found to metabolize naphatalene anthracene and phenanthrene. These compounds are among the 16 PAHs priority pollutants according to the US Environmental Protection agency (EPA), and are considered some of the most noxious compounds in the water-soluble fraction of oil (Abo-State et al. 2018). Finally, a few isolates (strains 6, 13, 25 and 26) showed a broad biodegradation profile using most of the 16 HC assayed. The carbon-source utilization experiment conveys some useful information showing the potential of the selected strains for marine decontamination, and even some of them could be considered as possible candidates for biotechnological approaches.

Isolation and characterization of phenanthrene degrading bacteria

PAHs are among the most persistent organic pollutants in the environment, and during the last years there has been increasing concern about contamination of these compounds in marine systems owing to their detrimental biological effects, toxicity and carcinogenicity (Haritash and Kaushik 2009). Although it is well known that bacterial degradation plays an important role in PAHs removal from marine environments (Dong et al. 2015; Sakshi Sing and Haritash 2020), nowadays most studies have reported on PAH-degrading bacteria isolated from PAH-contaminated soil or sediments (Haritash and Kaushik 2009; Dell'Anno et al. 2020), and little is known about bacteria isolated from sea or brackish waters (Izzo et al. 2019; Govarthanan et al. 2020).

During the present study five strains named E1 to E5 were selected from enrichment cultures with phenanthrene as sole carbon source (data not shown). Phe is often used as a model substrate in studies on the environmental degradation of PAHs since its structure is found in carcinogenic compounds as benzo[a]pyrene (Gran-Scheuch et al. 2017).

Identification of bacterial isolates and phylogenetic analysis

Sequence analysis of the 16S rRNA gene of the isolates E1, E2 and E3 allowed to determine their relationship to the genus *Halomonas* (Figure 3). E1 strain was closely related to *Halomonas* sp. XJ10 (99.19 %) and also with *Salinicola* sp. ATA 24 (99.35%). Similarly, E2 strain was associated to *Halomonas* sp. CR-55 (99.26%) and *H. meridiana* RT31 (99,14%), while E3 strain was related to *H. nanhaiensis* MTA-40-2-2 and *H. sulfidaeris* M-143 with a similarity value of 99.87%. *Halomonas* are slight to moderately halophilic and oligotrophic organisms that are ubiquitous to marine and hypersaline environments, and grow under different environmental conditions. Although among halophilic bacteria, *Halomonas* sp. has been shown to utilize a wide range of readily available substrates as energy sources for its fast growth (Ali et al. 2016) and is multi-metal resistant (Govarthanan et al. 2017), to our knowledge there are few studies on PAH degrading *Halomonas* isolated from marine waters (Gasperotti et al. 2015; Corti Monzón et al. 2018; Izzo et al. 2019; Govarthanan et al. 2020). It is worth noting that *H. meridiana* and *H. sulfidaeris* strains retrieved from deep sea sediments were also previously reported as PAH degraders (Cui et al. 2008; Yuan et al. 2015).

On the other hand, strain E4 showed closest association with *Rhodococcus* sp. Voy40th18-6 and *R. erythropolis* MC15 (97.78%). Bacteria belonging to *Rhodococcus* sp. have been characterized by their enormous metabolic versatility and by the concomitant metabolic bioconversion reactions of structurally diverse HC in marine systems (Brzeszcz and Kaszycki 2018). Likewise, *R. erythropolis* and other *Rhodococcus* members are able to degrade PAHs through different catabolic pathways (Seo et al. 2009).

Finally, strain E5 belongs to the genus *Pseudomonas* since its 16S rDNA sequence was closely related to those of *Pseudomonas rhodesiae* 15D2 (98.20%) and *Pseudomonas* sp. SI4

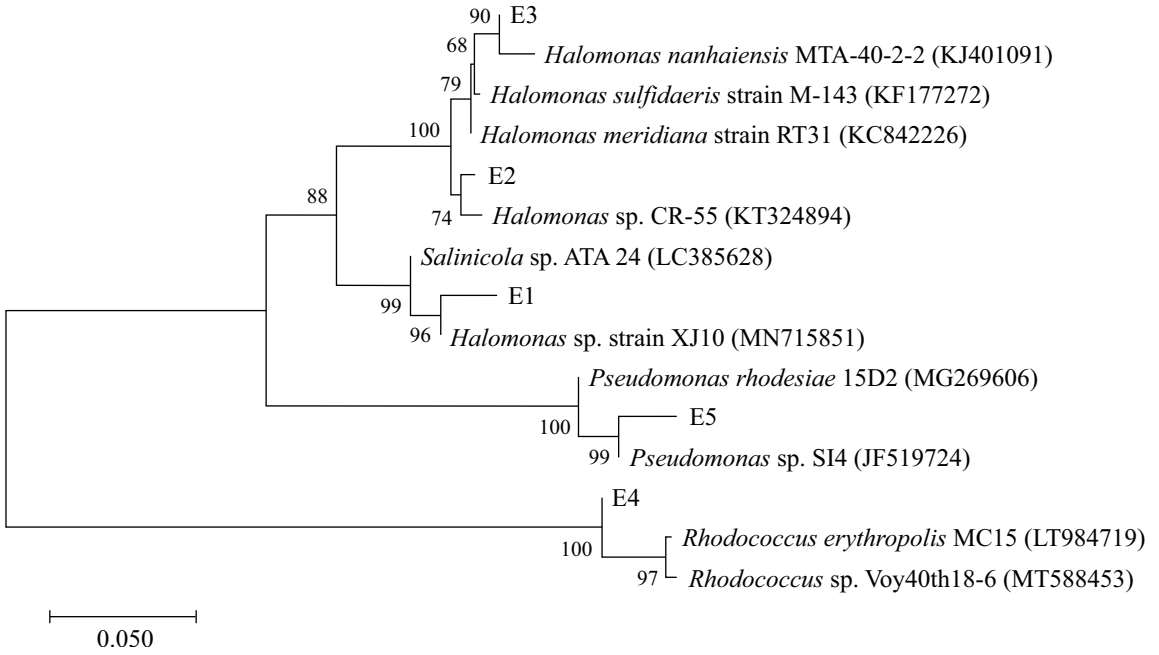

Figure 3. NJ phylogenetic tree based on an approximately 1,300 bp segment of the 16S rRNA gene sequence of PAHs degrading strains from this work and related sequences. GenBank accession numbers are given in parentheses. Only bootstrap values higher than 50% out of 1,000 replications are shown. Bar represents 0.05 nucleotide substitutions per site.

(98.20%). *Pseudomonas* sp. is one of the most studied genus and has been reported as a degrader of a wide range of organic pollutants including PAHs and other recalcitrant xenobiotics (Mulet et al. 2011). PAH degraders from coastal marine environments have been also described as bacteria associated to this genus (Isaac et al. 2016). Particularly, *P. rhodesiae* has shown ability to grow rapidly in PAHs (Kahng et al. 2002).

Phenanthrene biodegradation

The ability to estimate PAH degradation rates is essential for predicting environmental fate and for designing remediation efforts (Mallick and Dutta 2008). Biodegradation of phenanthrene used as the sole carbon and energy source was analyzed by HPLC for five isolates at an initial concentration of 160 mg l^{-1} (Figure 4). A continuous degradation curve from the initiation of the assay was observed for *Halomonas* sp. strains E1, E2 and E3, reaching a substrate disappearance between 37 to 45%. Phenanthrene concentration decreased more rapidly in the first four days for strain E1 and in the first six days for strains E2 and E3 than in later days, remaining steady after ten days of incubation. This might be attributed to the higher concentration of substrate at the beginning and the inhibited degradation of phenanthrene to some extent by metabolites later.

For *Rhodococcus* sp. strain E4 and *Pseudomonas* sp. strain E5, instead, a fast drop in Phe concentration was detected during the first 48 h, continuing with a slower decrease as the experiment progressed. The initial drop could be due to an active cellular assimilation or adsorption to the cell wall as was observed by Tian et al. (2002), who proposed that real degradation would occur, at least partially, after the early assimilation or adsorption and it should be considered during the discussion of degradation kinetics. This highly insoluble hydrocarbon was completely used by

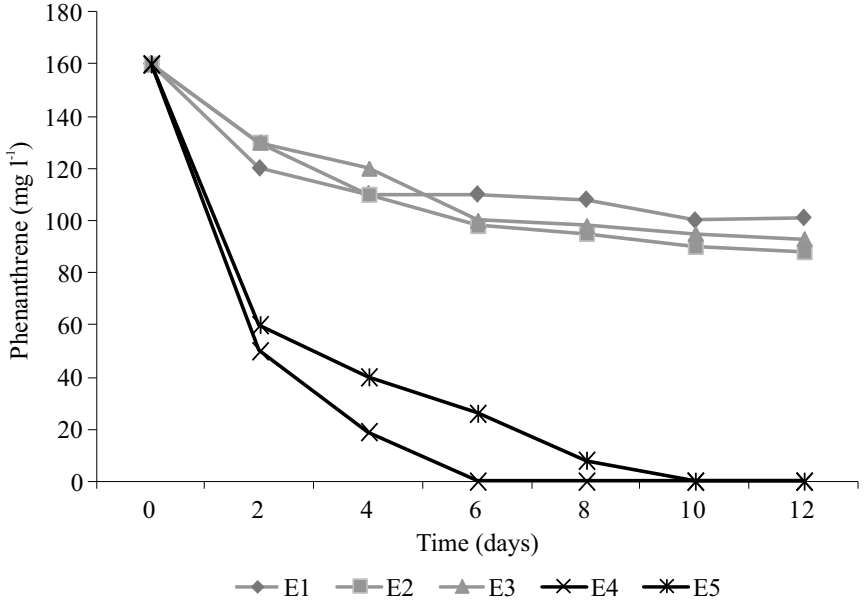

Figure 4. Degradation of phenanthrene by isolated strains.

strains E4 and E5 after 6 and 10 incubation days, respectively. By contrast, the biodegradation level of most bacteria reported in the literature was found to be below 50%, even after 10-day incubation (Song et al. 2011; Thavamani et al. 2012). Degradation rates were as follow: strains E1, E2 and E3: 4.92, 6.0 and 5.58 mg l^{-1} day^{-1}, respectively; whereas strains E4 and E5: 26.66 and 16.0 mg l^{-1} day^{-1}, respectively. These values were concomitant with OD measurements from the strain cultures (data not shown). Unlike other studies (Yuan et al. 2000) the addition of simpler carbons sources, as glucose, was not necessary to enhance PAH biodegradation rates.

Finally, degradations by *Rhodococcus* sp. and *Pseudomonas* sp. strains were around 2-3 times higher compared to *Halomonas* sp., indicating that potentials inherent in a genus and its species are also crucial considerations in the biodegradation of these aromatic compounds. Nevertheless, exhaustive information on the biodegradation of PAHs in seawaters by halophilic/halotolerant bacteria is still an emerging field in its initial stage of exploration (Ghosal et al. 2016).

Biosurfactant production assays

Many PAH-degrading bacteria have developed different strategies to overcome the low aqueous solubility of hydrocarbons (HC). One of the main HC accession processes is the production of surface-active agents (Olivera et al. 2009). Biosurfactants are amphipathic molecules secreted to the environment, which enhance solubilization and elimination of contaminants. Their action mechanism lies in the accumulation of immiscible compound at the interface, diminishing the surface tension and thus increasing their surface area; this allows a higher bioavailability that facilitates the degradation of diverse pollutants as aromatics (Batista et al. 2006). Microorganisms able to increase the degradation of hydrophobic compounds by releasing biosurfactants usually belong to the Genera *Pseudomonas*, *Halomonas*, *Bacillus*, *Rhodococcus* and *Stenotrophomonas* (Tripathi et al. 2020). In this study, biosurfactant production was determined by droplet collapsing test and hexadecane emulsification abilities of cell-free culture media. Supernatant from cultures of the five analyzed strains indicated the presence

Table 3. Emulsifying activities from isolates.

Strains	Emulsifying indexes (E_{24})	Drop collapsing
Halomonas sp. E1	50	+
Halomonas sp. E2	38	+
Halomonas sp. E3	42	+
Rodococcus sp. E4	48	+
Pseudomonas sp. E5	15	-

of surface-active compounds in both assays (Table 3), showing relatively high emulsifying indexes and positive drop collapsing activity in strains E1, E2, E3 and E4. The significant biosurfactant activity observed in *Halomonas* and *Rhodococcus* strains suggested somehow that the surfactant secreted to the medium would be involved in Phe degradation.

Various *Halomonas* species have been reported to produce abundant quantities of surface-active agents, as exopolymeric substances (EPS), which may provide a tool to scavenge hardly soluble, hydrophobic substrates, that cells could then utilize for growth in marine environments (Gutierrez et al. 2020). In addition, previous reports showed that several members of *Rhodococcus* produce biosurfactants, and even some species as *R. eritropolis* are regarded as natural reservoirs of new biosurfactants (Peng et al. 2007).

CONCLUSIONS

In this study, knowledge about the intrinsic HC biodegradation potential by native microbial communities around the EPEA station (Atlantic Coast) was first revealed. An increasing bacterial abundance associated with temperature over time was detected, indicating that further studies are needed to evaluate how climate change, anthropogenic pollution, and microbiological interactions may affect marine ecosystems in the future. In addition, some HDB isolated during this research were able to utilize technical hydrocarbon mixtures, alkanes, cycloalkanes and/or polyaromatic hydrocarbons (PAHs) as substrate, showing their potential for marine decontamination.

Finally, the five Phe-degrading bacteria selected and characterized in this study exhibited physiological properties that might have ecological significance on environmental alterations as the presence of pollutants. It is worth noting that *Rhodococcus* sp. strain E4 showed an outstanding PHA degrading capacity and significant biosurfactant activity. This strain could be exploited for biotechnological applications, as the development of cost-effective and eco-friendly technologies for the removal of PAHs in diverse marine environments.

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