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Continuous bioreactors enable high-level bioremediation of diesel-contaminated seawater at low and mesophilic temperatures using Antarctic bacterial consortia: Pollutant analysis and microbial community composition☆

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ABSTRACT

In 2020, more than 21,000 tons of diesel oil were released accidently into the environment with most of it contaminating water bodies. There is an urgent need for sustainable technologies to clean up rivers and oceans to protect wildlife and human health. One solution is harnessing the power of bacterial consortia; however isolated microbes from different environments have shown low diesel bioremediation rates in seawater thus far. An outstanding question is whether Antarctic microorganisms that thrive in environments polluted with hydrocarbons exhibit better diesel degrading activities when propagated at higher temperatures than those encountered in their natural ecosystems.

Here, we isolated bacterial consortia, LR-30 (30 °C) and LR-10 (10 °C), from the Antarctic rhizosphere soil of *Deschampsia antarctica* (Livingston Island), that used diesel oil as the only carbon substrate. We found that LR-30 and LR-10 batch bioreactors metabolized nearly the entire diesel content when the initial concentration was 10 (g/L) in seawater. Increasing the initial diesel concentration to 50 gDiesel/L, LR-30 and LR-10 bioconverted 33.4 and 31.2 gDiesel/L in 7 days, respectively. The 16S rRNA gene sequencing profiles revealed that the dominant bacterial genera of the inoculated LR-30 community were *Achromobacter* (50.6%), *Pseudomonas* (25%) and *Rhodanobacter* (14.9%), whereas for LR-10 were *Pseudomonas* (58%), *Candidimonas* (10.3%) and *Renibacterium* (7.8%). We also established continuous bioreactors for diesel biodegradation where LR-30 bioremediated diesel at an unprecedent rate of (34.4 g/L per day), while LR-10 achieved (24.5 g/L per day) at 10 °C for one month. The abundance of each bacterial genera present significantly fluctuated at some point during the diesel bioremediation process, yet *Achromobacter* and *Pseudomonas* were the most abundant member at the end of the batch and continuous bioreactors for LR-30 and LR-10, respectively.

1. Introduction

Fuel hydrocarbon contamination is ubiquitous across the planet due to its extensive use as an energy source. In particular, refined oil such as diesel consist in a mixture of aliphatic and aromatic compounds, some of which fall into the category of polycyclic aromatic hydrocarbons (PAHs)—highly toxic, persistent, and recalcitrant agents (Patel et al., 2020). Although PAH compounds (naphthalene, phenanthrene,

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anthracene, and pyrene) represent a small fraction of the diesel mixture—2-4000 mg per liter of diesel (de Souza and Corrêa, 2016)—these carcinogenic compounds, aromatics (45%), and *n*-alkanes (65%) have been detected in remote sites of the globe like the Antarctic Continent (Cabrerizo et al., 2016; Mazzera et al., 1999). One source of hydrocarbon pollution in water bodies is diesel spills, during transportation and use of diesel fuel across the oceans, rivers, and lakes (Glyaznetsova et al., 2021; Karl, 1992; Lytle and Peckarsky, 2001). Physical and chemical remediation procedures have proven to be effective remediating technologies, some of which include the use of booms, *in-situ* controlled burning, skimmers to remove the floating hydrocarbons, and adding surfactants to disperse pollutants (Doshi et al., 2018; Fingas, 2013). Nevertheless, these approaches require further treatment steps that lead to additional operating costs and the generation of PAH-related pollutants if fuel oils are burned *in-situ* (Evans et al., 2001; Fritt-Rasmussen et al., 2023).

Microbial bioremediation is a complementary sustainable technology to physical procedures to degrade diesel oils (Azubuike et al., 2016; Imron et al., 2020). Currently industrial technologies supplement external nutrients (biostimulation) along with the addition of hydrocarbon-consuming bacteria (bioaugmentation) to remediate diesel spills in aquatic bodies resulting in good diesel degradation efficiencies at mesophilic temperatures (Khalid et al., 2021; Omokhagbor Adams et al., 2020). However, seawater surface temperatures decrease at high latitudes; together with the high salt content of the ocean, they create adverse conditions that prevent the propagation of microbes in these cold environments resulting in slow hydrocarbon degradation rates (Murphy et al., 2021; Shukor et al., 2009). Additionally, high levels of diesel fuel inhibit growth and biomass synthesis of microorganisms, resulting in reduced bioremediation activities (Aini Dahalan and Ayshah, 2019; Murphy et al., 2021; Shukor et al., 2009). Thus, searching for bacteria that can improve diesel bioconversion efficiency and developing bioprocesses that can be implemented near the polluted site is imperative. In this context, the highest reported biodegradation rates of diesel-polluted seawaters have been achieved in batch bioreactors at laboratory (1.6 gDiesel/L per day) (García-Cruz et al., 2019) and pilot-scale (Nikakhtari et al., 2010) . Bioreactors enable controlled environments and good mixing conditions, which accelerate the fuel bioremediation process (Gargouri et al., 2011; Tekere, 2019). At the industrial scale, large volumes of wastewater effluents contaminated with diesel fuels are successfully treated using continuous bioreactors (>98.7%) (Barash and Perrin, 2009), but this technology has not been implemented for diesel polluted seawater.

Microorganisms described as metabolizing hydrocarbons of the diesel mixture at low temperatures were mostly isolated from cold seawater and regions (Abdulrasheed et al., 2020; Huang et al., 2013; Murphy et al., 2021; Zhou et al., 2021). Despite their cold natural origin, these microbes performed a slow diesel bioconversion at low temperatures (4–10 °C) due to the repression of hydrocarbon-converting and energy-generating enzymes along with transporter proteins (de Maayer et al., 2014; Koh et al., 2017; Nedwell and Rutfer, 1994), making this method less efficient than at mesophilic conditions thus far. This is also because the most frequently isolated bacteria colonizing cold environments are classified as psychrotolerant (Pacheco et al., 2019; Dsouza et al., 2015; Hoover and Pikuta, 2010) with optimal growth temperatures above 20 °C (de Maayer et al., 2014). Further, genomic analyses have revealed that Antarctic diesel-degrading bacteria harbor genes encoding oxygenase enzymes (monooxygenases and dioxygenases) (Bej et al., 2010; Dsouza et al., 2015; Jurelevicius et al., 2012; Kube et al., 2013). These genomic features may be the results of many Antarctic islands being polluted with hydrocarbons (*n*-alkanes and PAHs) (Cabrerizo et al., 2016, 2012) due to human activities e.g., military and research bases, transportation, and tourism, where diesel is an important fuel for daily life including heating. Likewise, natural sources like marine sediments and brown-coal deposits are also inputs of hydrocarbons in Antarctic islands (Cripps, 1992; Sutilli et al., 2019).

Given that a large portion of Antarctic soil is poor in nutrient availability and mostly covered by ice (Bölter, 2011), these harsh conditions are exacerbated as Antarctic islands are constantly exposed to sea spray that may contribute to the accumulation of salt and pollutants of both natural and anthropogenic origins. Despite these impediments, the rhizosphere of the Antarctic vascular plant *Deschampsia antarctica* contains a high diversity and abundance of microorganisms with high metabolic potential for biotechnological applications (da Silva et al., 2017; Flocco et al., 2009; Guajardo-Leiva et al., 2022). Specifically, hydrocarbon pollutants have been detected in these plants on Livingston island (Cabrerizo et al., 2016), making them a suitable niche for microorganisms to adapt and evolve towards hydrocarbon-metabolizing capabilities.

Based on this, we aimed to isolate bacterial consortia from rhizosphere soil of the grass *D. antarctica* contaminated with hydrocarbons that show the ability to consume diesel oil as the only carbon source in seawater. As these microbes thrive in extremely cold conditions, we reasoned that these Antarctic bacterial consortia might degrade diesel oil at a high rate when grown at an elevated temperature (10 °C) but also at mesophilic conditions (30 °C) when supplemented with inorganic nutrients in bioreactors. We investigated the diesel conversion using batch and continuous bioreactors. Additionally, changes in the relative abundance of bacterial consortia were also recorded throughout diesel bioconversion to link process parameters and bioreactor configuration with community composition.

2. Material and methods

2.1. Sampling site

Deschampsia antarctica plants and their respective rhizosphere soil were collected during the Antarctic scientific expedition ECA57 from the Byers Peninsula on Livingston Island in Antarctica, organized by the Chilean Antarctic Institute -INACH, 2019. The *D. antarctica* plants were taken randomly and with a depth of 0–5 cm in the roots' area from a mantle located at the following coordinates: 62°40′20.6″S 60°54′37.6″W (Fig. 1). The samples were collected in sterile aluminum folia and placed in a sterile plastic bag and frozen until processing.

2.2. Culture medium

The culture medium consisted of a salt mineral M9-medium, containing the following chemicals (g/L): $Na_2HPO_4 \cdot 7H_2O$, 12.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 3.0. This medium was sterilized (autoclaved) and supplemented with filter sterilized $MgSO_4{\cdot}7H_2O$ (0.12 g/L) and 1 mL/L of a trace element solution consisting of (g/L): $CaCO_3$, 2.7; $FeSO_4 \cdot 7H_2O$, 6; $MnSO_4 \cdot H_2O$, 1.16; $ZnSO_4 \cdot H_2O$, 2.0; $CuSO_4 \cdot 5H_2O$, 0.33; H_3BO_3, 0.08; $CoSO_4 \cdot 7H_2O$, 0.37). All experiments were carried out with this medium with different concentrations of hydrocarbons or diesel fuel in seawater or distilled water as indicated.

2.3. Diesel enrichment experiments for bacterial consortia isolation

To enrich diesel-degrading bacteria from the rhizosphere soil of *D. antarctica*, 2 g of soil were added to two sterile 250 ml flasks with 50 ml mineral medium (M9), Then, the M9-medium was supplemented with 5 (g/L) of commercial diesel fuel (COPEC, Chile), as the sole carbon and energy source. The flasks were incubated in a rotary shaker (INFORS HT, Switzerland) set at 30 °C and a vertical refrigerator (Ursus Trotter, Chile) set at 10 °C with an orbital shaker (Thermo Scientific™, USA) both set at 180 rpm for two weeks. Subsequently, an aliquot of the culture broth (1 mL) was inoculated into new flasks with fresh diesel fuel (10 g/L) and M9-medium and incubated under the conditions described above. Cultures were refreshed twice, and two diesel-degrading consortia were established, LR-30 and LR-10, for their ability to propagate in diesel oil at 30 and 10 °C, respectively.

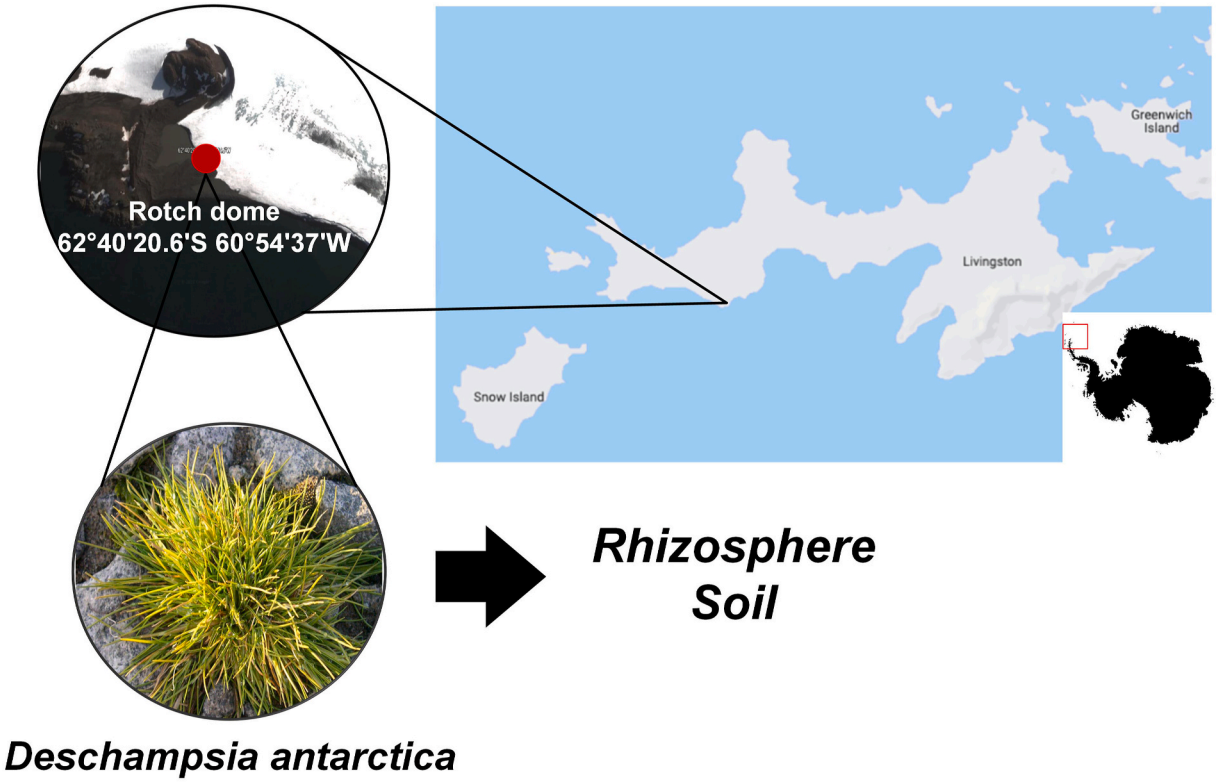

Fig. 1. Map and coordinates of the sampling site of rhizosphere Antarctic soil at Livingston Island.

2.4. Growth tests of the bacterial consortia on PAHs and alkanes in flask cultures

Inoculum of LR-30 and LR-10 consortia was prepared by growing each community in M9-medium as described above for 4 days. All cultures started with a bacterial concentration of ~2.0 × 10^4 (CFU/mL). Different aromatics and *n*-alkenes (pyrene, anthracene, phenanthrene, naphthalene, xylene, hexadecane, dodecane, heptane, and hexane) were provided as the only carbon substrate (100 mg/L) in 125 mL shaking flask containing 20 mL solution of M9-destilled water. The flasks were incubated at 10 °C (LR-10) and 30 °C (LR-30) as described above in an orbital shaker set at 180 rpm for 7 days. All experiments were carried out in triplicates.

2.5. Biomass quantification

Viable cell numbers of bacterial consortia were measured as colony forming units (CFU/mL) by using 10-fold serial dilutions of culture broth, which was pippeted onto a sterile plate-containing agar-rich lysogenic broth (LB) media. Additionally, cell dry mass of bacterial communities was recorded as volatile suspended solids (VSS/L) according to the Standard Method for the Examination of Water and Wastewater (APHA, 2012). Briefly, 10 mL of culture broth was centrifuged for 10 min at 7,000*g* at room temperature, the supernatant discarded, and the cell pellet resuspended in a 0.9% NaCl solution. This centrifugation step was repeated two times for washing the cells. Subsequently, the resuspended cells were vacuum filtered through a pre-weighted 0.45 μm glass microfiber filter. The filter was placed into a porcelain crucible and heated to 550 °C in a muffle furnace for 2 h. Finally, the filter was placed into a desiccator to cool to room temperature and weighed in a balance to calculate VSS lost in ignition.

2.6. Diesel degradation capacity of bacterial consortia in flask cultures

Flask culture experiments were carried out to evaluate the growth profile of the isolated consortia in seawater collected from mainland Chile (33.665°S, 71.638°W, Santo Domingo, Chile), supplemented with M9 mineral medium, hereafter M9-seawater. Another round of experiments used M9-distilled water using a wide range of diesel concentrations as substrates. Inoculum was prepared by growing each community in M9-medium with 1 (g/L) of diesel, trace elements (1 mL/L), and magnesium sulfate 1 (mL/L) as described above, for 7 days. All cultures started with a bacterial concentration of ~2.0 × 10^4 (CFU/mL) in 125 mL sterile flasks with 20 mL of M9-medium. The flasks were incubated at 10 °C (LR-10) and 30 °C (LR-30) as described above in an orbital shaker set at 180 rpm for 7 days. All experiments were carried out in triplicates.

2.7. Batch bioreactors for diesel degradation in seawater

An inoculum was prepared in flask cultures for each community in M9-seawater supplemented with 10 gDiesel/L. Bacterial consortia were inoculated to the bioreactors to achieve an initial cell concentration of ~2.0 × 10^4 (CFU/mL) within a 3 L vessel (1.5 L working volume) jacketed bioreactor (LiFlusGx, Biotron Inc, Korea) with M9-seawater containing an initial concentration of 10 or 50 (g/L) diesel as sole carbon source, trace elements (1 mL/L), and magnesium sulfate solutions (1 mL/L). The temperature for the LR-30 consortium was maintained at 30 °C by circulating warm water. For the cultivation of the LR-10 consortium, the temperature was regulated at 10 °C by passing a coolant fluid through the reactor jacket using a chiller (Julabo F500, Germany). The aeration rate was set at 1.5 (L/min) (1 vvm) using a mass flow controller. Dissolved oxygen was maintained >20% saturation by controlling the agitation speed up to a maximum of 800 rpm. The fermentation time for the consortia was 7 days. Every 24 h, samples were taken to determine the biomass formation, residual diesel, and DNA for microbial community composition during the diesel degradation process. The bioreactors were carried out in duplicates.

2.8. Continuous bioreactors for diesel degradation in seawater

Each bacterial consortia were grown in 500 mL flasks with 100 mL of M9-seawater medium amended with 10 gDiesel/L and cultivated on a rotary shaker for 7 days at 180 rpm at 10 °C (LR-10) and 30 °C (LR-30). The cultures were used as inoculum for inoculating the bioreactors to achieve an initial cell concentration of ~2.0×10^4 (CFU/mL). The consortia were cultured in a BIOSTAT® B plus bioreactor (Sartorius Biotech, Germany) equipped with a 10-L vessel (5-L working volume) supplemented with M9-seawater supplemented with 50 (g/L) of diesel for the batch phase. After inoculation a 14-day batch cultivation was carried out to form biomass. After that day, the continuous feeding started with a set hydraulic retention time (HRT) of 1.28 and 1.53 (days) for bioreactors run at 30 °C and 10 °C, respectively. The feed consisted of a solution of M9-seawater, trace elements (1 mL/L), and magnesium sulfate (1 mL/L) solutions (bottle 1) and pure diesel fuel (bottle 2). The flow rate of each bottle was calculated to attain a feeding diesel concentration of 50 (g/L) for each imposed dilution rate. The aeration rate was 5 L/min (1 vvm). Dissolved oxygen (DO) level was maintained at 20% saturation by controlling agitation speed up to 800 rpm. The pH was automatically controlled at 6.8 ± 0.1 using 3M NaOH and H_2SO_4. The working volume was kept constant by removing the culture through a peristaltic pump. Samples were taken during the continuous process to determine residual diesel, cell dry mass, biosurfactant production screening, and DNA for bacterial community composition determination. Continuous bioreactors were performed in duplicates.

2.9. Hydrocarbon characterization from soil and diesel quantification via GC-MS

Hydrocarbon extraction from the rhizosphere of *D. antarctica* was carried out as previously described (Haleyur et al., 2016). Briefly, 2 g of rhizosphere soil were transferred into 50 mL screw-top centrifuge tubes containing pre-dried sodium sulfate (to reduce soil moisture). After mixing, 15 mL of acetone:hexane (1:1) solution was added to the tube. The mixture was vortexed for 1 min and placed in an orbital shaker (Thermo Scientific™, USA) set at 160 rpm for 10 min at room temperature. The tubes were centrifuged at 4696 *g* for 5 min. The supernatant was transferred into a clean 50 mL centrifuge tube. The remaining pellet was subjected to a second extraction in 10 mL of the acetone:hexane mixture. Supernatants from both extractions were pooled, and the volumes were adjusted to 25 mL using the acetone:hexane solution. The mixture was transferred to a clean 50 mL tube through 0.22 μm filters using a syringe. Then the solvent mixture was evaporated to a final volume of 1 mL in a fume chamber. Afterwards, the solution was centrifuged at 4696 *g* for 5 min. An aliquot (50 μL) was transferred to a 2 mL microcentrifuge tube with 950 μL of hexane, mixed, and the samples were finally placed in vials and stored at 4 °C until analysis.

Different concentrations of phenanthrene, anthracene, pyrene, naphthalene, dodecane, and diesel fuel were used as pattern compounds to record the retention time and obtain calibration curves for each compound using the gas-chromatography mass spectrometry (GC-MS) method described elsewhere (Imron et al., 2019). The detection limits of diesel, *n*-alkane, and aromatic compounds were 5, 0.1, 0.5 mg/L, respectively. All measurements reported here were above these detection limits. The quantification limit was calculated as the average of the blank plus 10 times the standard deviation of the field blanks resulting in 15.2, 0.3, 1.5 mg/L for diesel, *n*-alkane, and aromatics, respectively.

Prior to injection (1,3,5-trichlorobenzene) was added to samples to a final concentration of 40 (μg/mL) as internal standard. Samples were then injected into a GC-MS device YL 6900 (Young Instruments, Korea) equipped with a Column: HP-5MS (30m × 0.25 mm, 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.0 (mL/min). The inlet temperature was 250 °C and set in a split ratio 5:1. The following GC oven temperature program was used: 50 °C isotherm for 5 min, increased by 5 °C–300 °C for 1 h and held for 15 min. The mass spectrometer ionization source was set at 230 °C; mass quadrupole analyzer at 150 °C in scan mode. To corroborate the chemical structure of each hydrocarbon, a NIST library was used to compare the results of mass compatibility (NIST 17 Mass Spectral Library).

2.10. Diesel quantification from culture broth

To corroborate the residual diesel concentration recorded by GC-MS, gravimetric measurements were also carried out using *n*-hexane extraction as described (Silva et al., 2018). The remaining diesel in flask and from the bioreactor were extracted with *n*-hexane at a culture broth/n-hexane ratio (1:2) into 50 mL screw-cap centrifuge tubes. The broth/*n*-hexane mixture was mixed in an orbital shaker at 180 rpm for 30 min at room temperature. Next, the mixture was centrifuged at 6000 *g* to separate the organic from the aqueous layer. The organic phase was removed and placed in a 50 mL centrifuge tube. The remaining aqueous phase was subjected to a new extraction with *n*-hexane. The two extractions were pooled and placed in glass Petri dishes, and the solvent evaporated in a fume hood for two days. The final weight was recorded in an analytical balance which corresponded to the extracted aliphatic and aromatic compounds of the diesel mixture that were not metabolized by microorganisms. The percentage of diesel degradation was calculated as described elsewhere (Chen et al., 2013). Abiotic control values were estimated in the same way as the residual diesel from culture broths and subtracted from the residual mass to compensate possible evaporation (McFarlin et al., 2018).

2.11. Ammonium, nitrate, and phosphorous quantification

The ammonium concentration in cell-free supernatant was measured by a photometric test (LCK 303 kit, Hach Lange, Danaher, USA). NO_3^--N was determined using seawater by the standard method 4500-B, ultraviolet spectrophotometric screening approach, recorded in a HACH DR3900 spectrophotometer. The phosphate concentration in seawater was measured using a phosphate colorimetric assay kit (MAK030, Sigma-Aldrich) recorded at 650 nm in a spectrophotometer.

2.12. 16S rRNA gene amplicon sequencing and analysis

Bacterial community composition was obtained by 16S rRNA gene amplicon sequencing. Twenty-five mL aliquots were sampled and centrifuged at 9000 *g*, 4 °C for 10 min (Eppendorf 5810 R, Hamburg, Germany). The supernatant was discarded, and the pellet was washed with NaCl (0.9% w/v). This step was repeated three times. DNA from each sample was purified using the DNA GeneJET Kit (Thermo Fisher Scientific, Waltham, Ma, USA). Next, DNA integrity and concentration were determined by 1% gel electrophoresis and $OD_{260/280}$ ratio spectroscopy. DNA samples were sent to Dalhousie University Integrated Microbiome Resource (IMR; imr.bio) for 16S rRNA amplicon sequencing (V4–V5 region). The 16S amplicons were generated using improved gene primers (Walters et al., 2016) according to the protocol described elsewhere (Comeau et al., 2017) and sequencing using 300 + 300-bp PE v3 chemistry on an Illumina MiSeq.

2.13. Bioinformatic analysis

Sequence data were analyzed using amplicon sequence variants (ASVs) as implemented in DADA2 v1.8 (Callahan et al., 2016). The following filtering parameters were used for 16S rRNA gene sequences (maxN = 0, maxEE = c(2,2), and truncQ = 2). Specific trimming parameters for 16S rRNA reads were truncLenc (220,150). For error rate learning, dereplication, denoising, and merging steps, we followed the DADA2 Pipeline Tutorial (1.16) https://benjjneb.github.io/dada2/tutorial.html. Multidimensional scaling (PCoA on a Bray-Curtis dissimilarity matrix) and visualization were conducted as implemented in the microeco and phyloseq R packages (Liu et al., 2021; McMurdie and

Holmes, 2013). Taxonomic classification was conducted using the SILVA v.132 database. Stacked bar charts show agglomerated abundances at the genus level for clarity.

2.14. Alpha diversity index

To calculate Faith's phylogenetic diversity (Faith's PD) (Faith, 1992), we aligned the ASVs sequences and inferred a phylogenetic tree using the DECIPHER v2.16.1 R package (Wright, 2016) and FastTree v2.1.10 (Price et al., 2009), respectively. The tree was added to the phyloseq object, and the index was calculated using the btools v0.0.1 R package.

2.15. Biosurfactants screening tests

The oil displacement test was carried out as previously described elsewhere (Hassanshahian, 2014). In a glass Petri dish containing 50 mL of water, 100 μL of sterile diesel was added on the water's surface. One mL sample of culture broth at the end of continuous bioreactor (day 55) was centrifuged at 11,000*g* for 10 min at room temperature. Then the cell-free supernatant was taken and placed into another Eppendorf tube and centrifuged again as described before. Then 10 μL of the cell-free supernatant was dropped on the surface of the diesel. The diameter of the clear zone on the surface of the oil was measured. Negative control was maintained with distilled water (without surfactant), in which no oil displacement was observed. Triton X100 was used as a positive control (Thavasi et al., 2013). Additionally, the emulsifying activity of the supernatant was determined by measuring the emulsion index (E24) at 25 °C as described elsewhere (Wang et al., 2014). Briefly, 4 mL of diesel was poured into a test tube containing 4 mL of supernatant. After being vigorously vortexed for 2 min, the tubes were kept for 24 h. The heights of emulsion, oil, and aqueous zones were measured, and the emulsion index (E24) was determined as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm).

2.16. Physiological parameters of the bacterial consortia and mass balances in bioreactors

The biomass production rate (R_x) (gVSS/L • day) in the batch bioreactor can be defined as

$$\frac{dx}{dt} = R_x = \mu \bullet x \tag{1}$$

where μ is the specific growth rate (1/day) and x is the biomass concentration (gVSS/L). By deriving Eq. (1), the maximum specific growth rate (μ_{max}) was calculated taking the total biomass formation during the exponential growth phase of the microbial community as follows,

$$\mu_{max} = \frac{\ln\left(x/x_o\right)}{t} \tag{2}$$

The biomass yield coefficient (Y_{xs}) (gVSS/gDiesel) is the ratio between the biomass production rate and substrate uptake rate (diesel as the only carbon source) (*Rs*) within the bioreactor,

$$Y_{xs} = \frac{R_x}{R_{diesel}} \tag{3}$$

rearranging Eq. (3), the diesel uptake (degradation) rate (R_{diesel}) (gDiesel/L • day) can be calculated using Eq. (4),

$$-\frac{dS}{dt} = R_{diesel} = \frac{R_x}{Y_{xs}} \tag{4}$$

On the other hand, for the continuous bioreactor with a constant volume, the mass balance for biomass production rate can be described at steady state as

$$\frac{dx}{dt} = \mu \bullet x \bullet V - \mathrm{F} \bullet x = 0 \tag{5}$$

where F (L/day) is the flow rate and V (L) is the reaction volume of the bioreactor. From Eq. (5), we calculated the imposed dilution rate D (1/day), which equals the specific growth rate (μ_{set}),

$$D = \mu_{set} = \frac{\mathrm{F}}{V} \tag{6}$$

It can be also defined the hydraulic retention time (HRT) (days) as

$$HRT = \frac{1}{D} \tag{7}$$

The mass balance for the diesel bioconsumption (biodegradation) rate in the continuous bioreactor with constant volume can be established following Eq. (8) when the system reaches the steady state,

$$-\frac{dS}{dt} = R_{diesel} = \mathrm{F} \bullet S_o - \frac{\mu \bullet x \bullet V}{Y_{xs}} - \mathrm{F} \bullet S = 0 \tag{8}$$

where, S_o is the diesel concentration (g/L) in the feeding solution and S is the diesel content in the bioreactor (g/L), thus R_{diesel} can be calculated as,

$$R_{diesel} = D \bullet (S_o - S) = \frac{\mathrm{D} \bullet x}{Y_{xs}} \tag{9}$$

2.17. Statistical analysis

A parametric statistical analysis was adopted to compare the mean of the data and calculate standard deviation. Welch's two sample *t*-test was used to determine the statistical significance between the μ_{max} obtained by consortia in M9-seawater and M9-distilled water during diesel degradation in flask cultures using GraphPad 9.0: Prism®. The means comparison of CFU/mL in different concentrations of diesel was analyzed directly through a one-way ANOVA and means separation was carried out by Tukey's multiple comparisons test (*P*-value <0.05, determined as significant) using GraphPad 9.0: Prism®. The ANOVA test was also used to check whether bacterial abundance and biomass formation of LR-30 and LR-10 were significantly different among samples in batch and continuous bioreactors during diesel bioremediation. To evaluate the correlation between emulsification index and diesel degradation Pearson's correlation coefficients were determined using GraphPad 9.0: Prism®.

3. Results

3.1. Occurrence of hydrocarbons in rhizosphere soil of D. antarctica and degradation of hydrocarbons by isolated diesel-consuming bacterial consortia

GC-MS analysis of the soil of *D. antarctica* sample confirmed the presence of *n*-alkanes, and aromatic hydrocarbons. Fig. 2 depicts the concentrations of the detected pollutants where dodecane had the highest concentration among *n*-alkanes and naphthalene for the ringed aromatic compounds. After confirming the presence of hydrocarbons in the soil samples, we inoculated Erlenmeyer flasks containing M9-medium and 10 gDiesel/L fuel with rhizosphere soil. After 2 weeks of cultivation, the medium turned a deeper brown indicating aerobic microbial growth at 10 °C and 30 °C (Supplementary material S1).

We then investigated whether the diesel-consuming consortia could metabolize some of the hydrocarbon compounds detected in the soil sample, where each was provided at a concentration of 100 (mg/L) as the sole carbon substrate in M9-medium flask experiments. LR-30 community grew on the aromatic pyrene, anthracene, naphthalene, and longer *n*-alkane chains at 30 °C, LR-10 flask cultures (10 °C) exhibited no growth on the PAHs pyrene and naphthalene, but it could

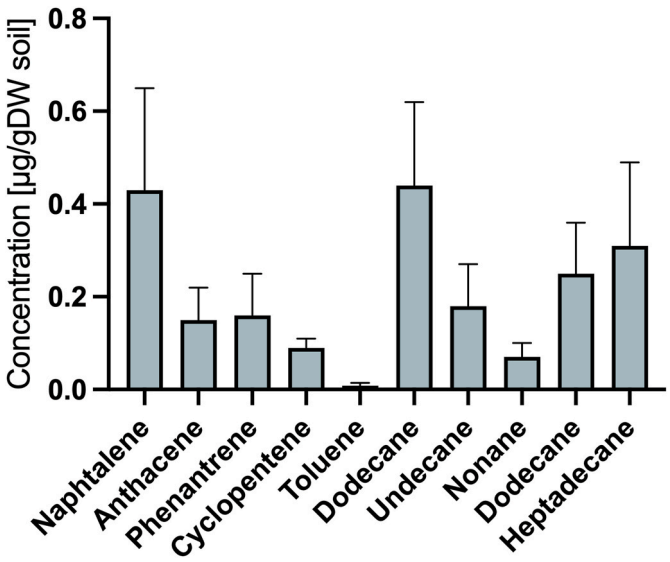

Fig. 2. Concentrations of *n*-alkenes and PAHs detected in the rhizosphere Antarctic soil at Livingston Island. The values represent mean and standard deviation from three independent samples.

utilize anthracene, phenanthrene, heptane, dodecane, and hexadecane as carbon substrates (Table 1). None of the microbial consortia propagated on hexane or xylene.

3.2. Diesel degradation capacity of bacterial consortia in distilled and seawater with different pollutant concentrations in flask cultures

When growing on diesel as the sole carbon source, LR-30 and LR-10 flask cultures exhibited similar specific growth rates of 2.8 (1/day) and 2.9 (1/day) in M9-distilled water at 30 °C and 10 °C, respectively. No significant changes were observed in the specific growth rate for LR-30 community while metabolizing diesel in M9-seawater ($P = 0.45$, Welch's test) compared to M9-distilled water, whereas LR-10 flask cultures showed a significant reduction from 2.9 to 2.7 ($P < 0.05$, Welch's test) (1/day) (Fig. 3A and B). We then measured phosphate, ammonium, and nitrate concentrations in the used seawater which contained 0.9 (mg/L), 0.4 (mg/L), and 21.5 (mg/L), respectively. As expected, neither the flask cultures without inoculation (LR-30 or LR-10 consortia) in seawater nor the experiments using M9-seawater exhibited some consumption of the initial diesel concentration after 7-day cultivation (Fig. 3A and B). In terms of biomass formation, there was a decline in colony forming unit counts (CFU/mL) of nearly 20% ($P < 0.05$) when LR-10 and LR-30 propagated in M9-seawater compared to M9-distilled water for both growth temperatures (Fig. 3A and B). Additionally, we challenged the microbial community to pollutant concentrations ranging from 10 to 200 gDiesel/L. Up to 20 (g/L) of diesel in the culture broth, each microbial consortia bioconverted ~97% of the initial content (Fig. 3C and D). For both bacterial consortia, the highest diesel degradations and biomass were obtained when the flask cultures started with 50 gDiesel/L after 7-day cultivation (Fig. 3C and D). Higher diesel contents (>100 g/L) further restricted the amount of diesel remediated, microbes only reaching less than 18% of the initial diesel oil concentration. Finally, flask cultures supplemented with 200 gDiesel/L did not show bacterial growth for either community due to inhibitory effects (Fig. 2C and D).

Table 1
Flask culture growth experiments on aromatic and aliphatic compounds by LR-30 and LR-10 consortia at 30 °C and 10 °C in M9-medium, respectively. The bacterial growth was evaluated after 7 days cultivation.

LR-30 community	LR-10 community	Growth / No growth
		Growth
		No growth
Growth	No growth	Pyrene
Growth	Growth	Anthracene
No growth	Growth	Phenanthrene
Growth	No growth	Naphthalene
No growth	No growth	Xylene
Growth	Growth	Hexadecane
Growth	Growth	Dodecane
Growth	Growth	Heptane
No growth	No growth	Hexane

3.3. Microbial community composition and diesel degradation in M9-seawater using batch bioreactors at mesophilic temperature

At this stage, we carried out batch bioreactors to achieve better mixing and controlled parameters within the process. We evaluated microbial composition and diesel degradation kinetics with two initial diesel concentrations, 10 and 50 (g/L). First, the diesel consumption rate for the LR-30 community was 1.7 (g/L per day) when the batch bioreactor was supplemented with 10 gDiesel/L, consuming 98% of the pollutant after 7 days (Fig. 4A). 16S rRNA sequence analyses revealed that the inoculated LR-30 consortium is mostly composed of the genus *Achromobacter* (50.6%), followed by *Pseudomonas* (26%), *Rhodanobacter* (14.8%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (3.3%), *Demetria* (2.0%), and *Chryseobacterium* (1.1%) (Fig. 4B). LR-30 batch bioreactors showed a considerable decline in the amount of *Pseudomonas* on day 4 of cultivation from 25% to 5.6% ($P < 0.05$) before finally setting as the third most abundant bacteria (10.6%) at the end of the process when growing on 10 (gDiesel/L). In contrast, *Achromobacter* was the most abundant genera and registered a significant decrease in relative abundance on day 4 ($P < 0.05$), before finally spiking at 48.3% on day 7 (Fig. 4B). The highest increase was recorded for *Rhodanobacter* which accounted for 24.7% bacterial abundance ($P < 0.05$) by the end the process. We also inspected the degradation of *n*-alkenes and PAH compounds of the diesel mixture summarized in Fig. 4C. The LR-30 community metabolized a large fraction of the aliphatic compounds (>90%), yet it could not consume phenanthrene and xylene from the aromatic fraction and exhibited a consumption of nearly 50% of the initial amount of naphthalene and pyrene at the end of the process (Fig. 4C).

For LR-30 batch bioreactors supplemented with a higher diesel concentration (50 gDiesel/L), the diesel consumption rate had two values, between 0 and 4 days (7.6 g/L day) and 4–7 days (1.0 g/L day) (Fig. 3D). On day 5, nitrogen was found under the detection limit (Fig. 4D). LR-30 batch bioreactors degraded 33.4 (g/L) of diesel oil, displaying an overall conversion rate of the pollutant of 4.8 (g/L per day) (Fig. 4D). Fig. 4E illustrates that *Achromobacter* was stable until day 3 and significantly declined on day 5–36.7% ($P < 0.05$), recovering to nearly the same relative abundance (49.3%, $P = 0.95$) as that detected at the beginning of the bioconversion (Fig. 3E). *Rhodanobacter* saw an increment from 14.9% to 24.7% ($P < 0.05$), making it the second most abundant genera on day 7. *Pseudomonas* reduced its proportion significantly throughout the process attaining their lowest value on day 4 (5.6%, $P < 0.05$) (Fig. 4E). *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genera were stable until day 5 ($P = 0.30$) and displayed a considerable increment on day 6 ($P < 0.05$). Interestingly, the most abundant initial members of the LR-30 community, *Achromobacter*, *Pseudomonas*, *Rhodonobacter*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* fluctuated throughout the diesel remediation process to varying degrees (Fig. 4E). However, they were among the community's top four most abundant members, all belonging to the phylum Proteobacteria (Fig. 4E, Supplementary S2). The degradation of hydrocarbons by the LR-30 community was affected when increasing the initial diesel concentration from 10 to 50 (g/L) since some *n*-alkanes

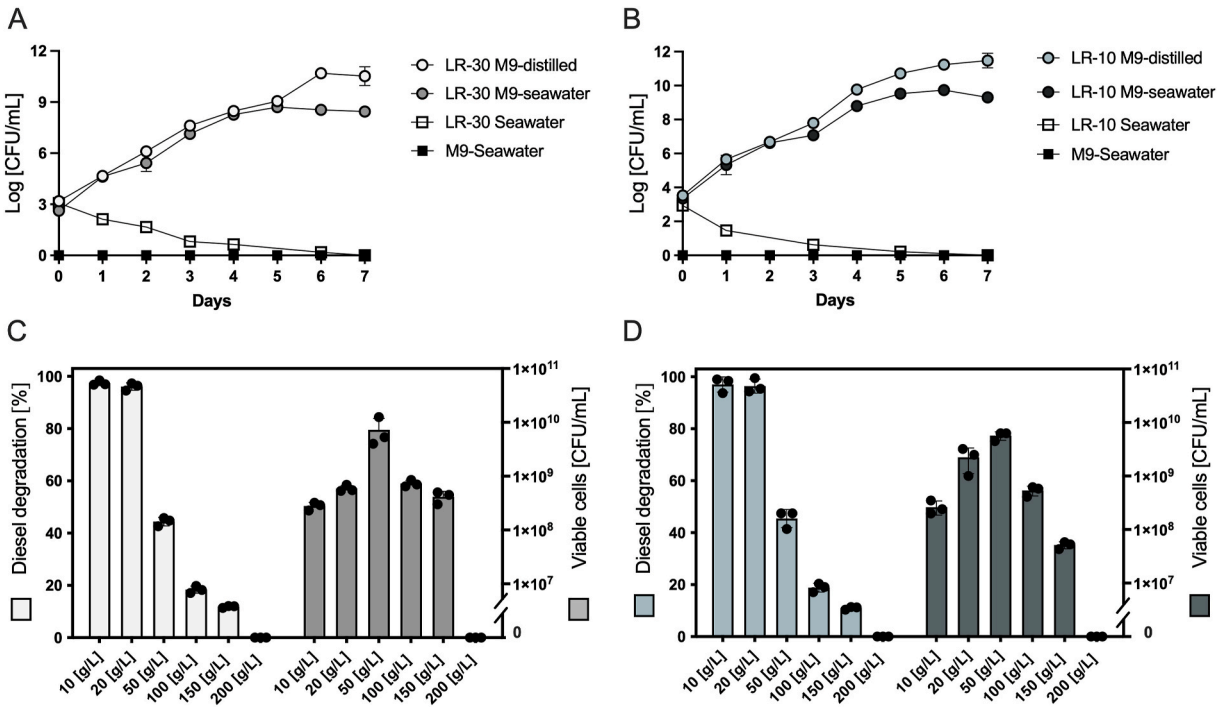

Fig. 3. Growth profiles of the LR-30 and LR-10 consortia in M9-seawater and M9-distilled amended with diesel in flask cultivation. A) Growth comparison of LR-30 growing on 10 gDiesel/L in M9-seawater and M9-distilled water at 30 °C. Additionally, seawater was supplemented with and without M9 medium to evaluate growth on diesel of the native microbes of seawater at 30 °C. Inoculation of the LR-30 community in seawater supplemented with diesel and without M9 medium was also evaluated at 30 °C. B) Growth comparison of LR-10 growing on 10 gDiesel/L in M9-seawater and M9-distilled water at 10 °C. Additionally, seawater was supplemented with and without M9 medium to evaluate growth on diesel of the native microbes of seawater at 10 °C. Inoculation of the LR-10 community in seawater supplemented with diesel and without M9 medium was also evaluated at 10 °C. Diesel degradation efficiency and maximum biomass formation using varying concentrations of diesel fuel in M9-seawater by C) the LR-30 and D) LR-10 consortia after 7-day cultivation. C11: Undecane; C12: Dodecane; C13: Tridecane; C14: Tetradecane; C15: Pentadecane; C16: Hexadecane; C17: Heptadecane; C18: Octadecane; C19: Nonadecane; C20: Icosane; C21: Heneicosane; C25: Pentacosane. Values represent the mean and standard deviation from three independent experiments.

(C13–C18) were detected with 60% degradation. Likewise, PAH agents were also detected with incomplete bioconversion (Fig. 3F).

3.4. Microbial community composition and diesel degradation in seawater using batch bioreactors at low temperature

LR-10 batch bioreactors could biotransform diesel at a rate of 1.9 (g/L per day) when degrading 10 gDiesel/L at 10 °C, remediating 99% the initial diesel concentration (Fig. 5A). Amplicon sequencing results of the inoculated LR-10 biomass in batch bioreactors present a different microbial composition to that encountered for the LR-30 community as *Achromobacter* only represented 0.06% of the relative abundance. The dominating bacterial genera were *Pseudomonas* (58%), *Candidimonas* (10.3%), *Rhodanobacter* (8,6%), *Renibacterium* (7,8%), *Pseudoarthrobacter* (4.6%), and *Frateuria* (3.9%) (Fig. 5B). We found that *Pseudomonas* increased to 71% of the bacterial content on day 4 ($P < 0.01$), which then significantly declined to 44% ($P < 0.01$) and kept steady until the end of the process ($P = 0.68$) when consuming an initial diesel content of 10 (g/L). Conversely, *Rhodonobacter* had an oscillating behavior and reached 21.1% abundance after 7-day culture, whereas *Candidimonas* accounted for 10% of the LR-10 microbial population, maintaining this level during the entire diesel degradation process ($P = 0.43$) (Fig. 5B). GC-MS analyses show that most *n*-alkenes were consumed (80% for C10 to C21) by the LR-10 community while just a fraction of phenanthrene (25.9%) was degraded (Fig. 5C). No degradation was recorded for pyrene, xylene, and naphthalene (Fig. 5C).

LR-10 batch bioreactors carried out with 50 (gDiesel/L) metabolized diesel at a rate of 4.8 (g/L per day) and on day 6 nitrogen was detected as the limiting nutrient (Fig. 5D). Sequencing analysis showed that *Pseudomonas* was also the most prominent genera by the end of the process like LR-10 batch bioreactor consuming 10 gDiesel/L, yet it represented nearly 75.3% of the relative abundance along with genera *Rhodanobacter* (12.1%), *Renibacterium* (6.4%), and *Pseudarthrobacter* (2.6%) (Fig. 5E). This significant variation in bacterial genera abundance was consistent with the fluctuation in values computed for Faith's phylogenetic diversity index, which ranged from 37.1 to 27.3 (Supplementary material S3). The degradation of *n*-alkenes was inhibited as the quantified concentrations reached less than 60% degradation, except for C12 and C13 that attained more than 90% degradation (Fig. 5F). No degradation was observed for pyrene, xylene, and naphthalene.

3.5. High-level continuous biodegradation of diesel polluted seawater by LR-30 community using continuous bioreactors at mesophilic temperature

We evaluated the effectiveness of diesel bioremediation by the LR-30 community establishing continuous bioreactors fed with 50 (gDiesel/L) in M9-seawater with a dilution rate set at 0.78 (1/day, HRT = 1.28 day). For the first 14 days, we ran the bioreactor in batch mode to form biomass before switching to continuous operation (Fig. 6A). At this point, LR-30 continuous bioreactor synthesized a dry cell mass of 9.8 (gVSS/L), which started to decay significantly within the first 10 days ($P < 0.01$) of feeding and then spiked to 9.1 (gVSS/L), whereby this value did not show significant variations ($P = 0.07$) for the last 12 days of the continuous bioreactor operation. Nitrogen was found to be the limiting nutrient since the beginning of the feeding phase (Fig. 6A). We also quantified the emulsification index (EI24), which is a good measurement of how well the produced biosurfactants emulsify hydrophobic agents. The EI24 increased considerably from 43% at the end of batch phase, to 67% on day 55 of continuous bioreactors accompanied by an enhanced diesel bioconversion (Fig. 6A). The remained diesel stabilized

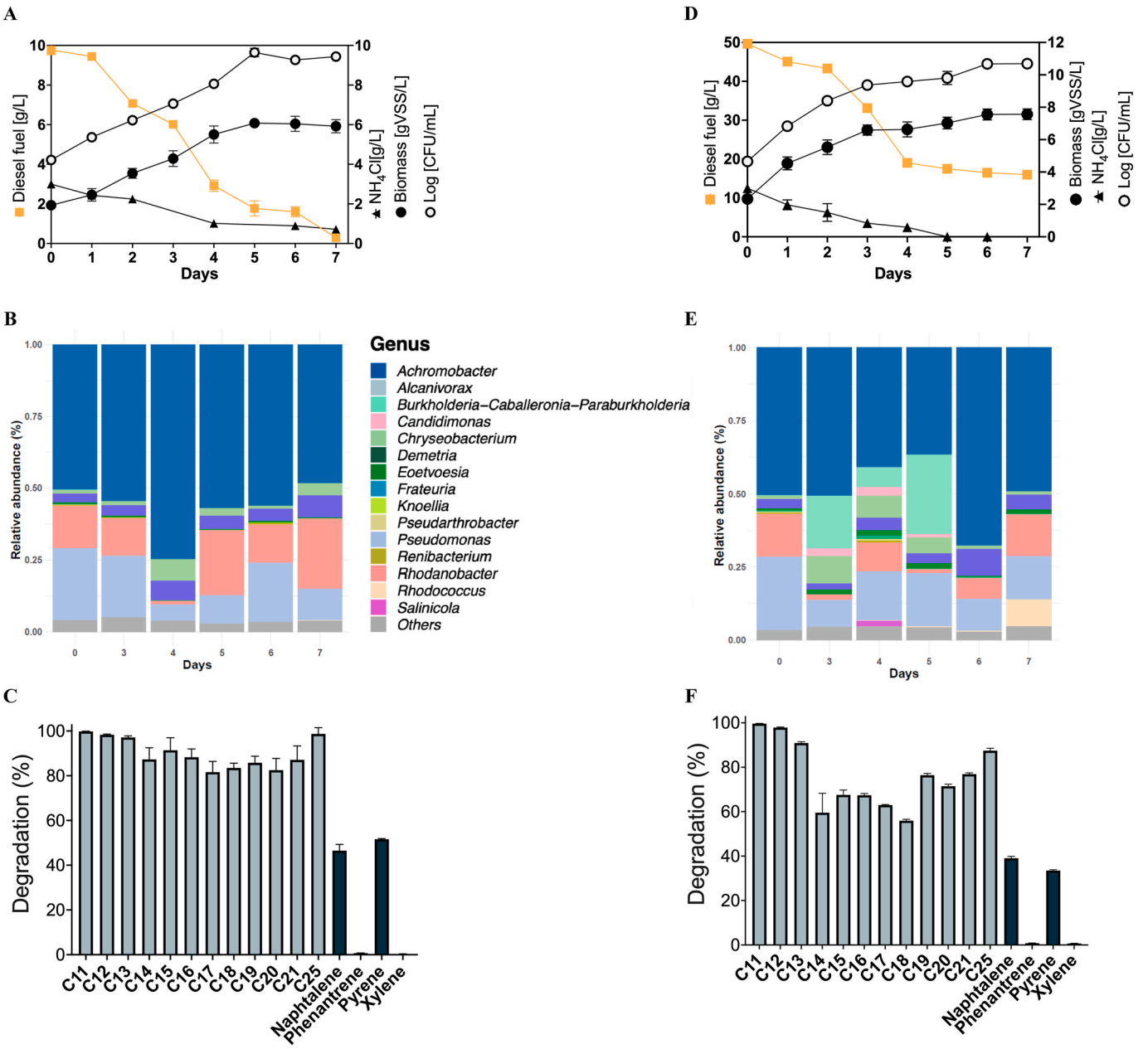

Fig. 4. Bacterial community composition, diesel biodegradation and growth profiles, and degradation efficiency of hydrocarbons of the diesel mixture by LR-30 community under mesophilic conditions in batch bioreactors. Diesel degradation and biomass formation kinetics in M9-seawater supplemented with A) 10 gDiesel/L and D) 50 gDiesel/L at 30 °C. LR-30 bacterial community composition through the diesel mineralization process amended with B) 10 gDiesel/L and E) 50 gDiesel/L. Hydrocarbon degradation efficiency after 7-day cultivation by LR-30 community supplemented with C) 10 gDiesel/L and F) 50 gDiesel/L. C11: Undecane; C12: Dodecane; C13: Tridecane; C14: Tetradecane; C15: Pentadecane; C16: Hexadecane; C17: Heptadecane; C18: Octadecane; C19: Nonadecane; C20: Icosane; C21: Heneicosane; C25: Pentacosane. Values represent the mean and standard deviation from two independent batch bioreactors.

within the continuous culture on day 27— after 13 hydraulic residence times (HRT)—attaining a constant diesel bioconversion of 93% at an unprecedent rate of 34.4 (g/L per day) for a month (Fig. 6A). We also performed oil displacement tests of the supernatant on day 55 of the LR-30 continuous bioreactor, which presented a diameter of 8.4 cm, presenting a lower value to that obtained with the industrial surfactant Triton X100 (9.0 cm) (Fig. 6B).

Microbial composition significantly fluctuated during initial volume exchanges of the continuous bioreactor (Fig. 6C). For instance, *Achromobacter*, initially the community's main bacterial genus, was overtaken by *Pseudomonas, Rhodococcus and Brevundimonas* during this period. Furthermore, when the culture reached a stable diesel bioconversion rate on day 27, *Achromobacter* increased drastically from less than 0.1% to 12% of the microbial content, showing an abundance of 33.7% on day 55 (Fig. 6C). Another significant distinction was that *Stenotrophomonas* (29.4%) was now the second most abundant bacterial genera exceeding *Pseudomonas* (4.4%). Finally, *Alcanivorax* (18%) and *Salinicola* (13.1%) saw a considerable increment in abundance within the continuous bioconversion process (Fig. 6C), whereby these bacteria were not detected by 16S rRNA sequencing within LR-30 batch bioreactors (Fig. 5B, D). The Faith's phylogenetic diversity index also significantly varied ($P < 0.001$) and the samples did not group (PCoA analysis) for the LR-30 community through the continuous remediation procedure (Supplementary material S3, S4). The results in Fig. 6D show that more than 95% of all initial concentrations of quantified *n*-alkanes and the PAHs, anthracene, and pyrene, were metabolized using LR-30

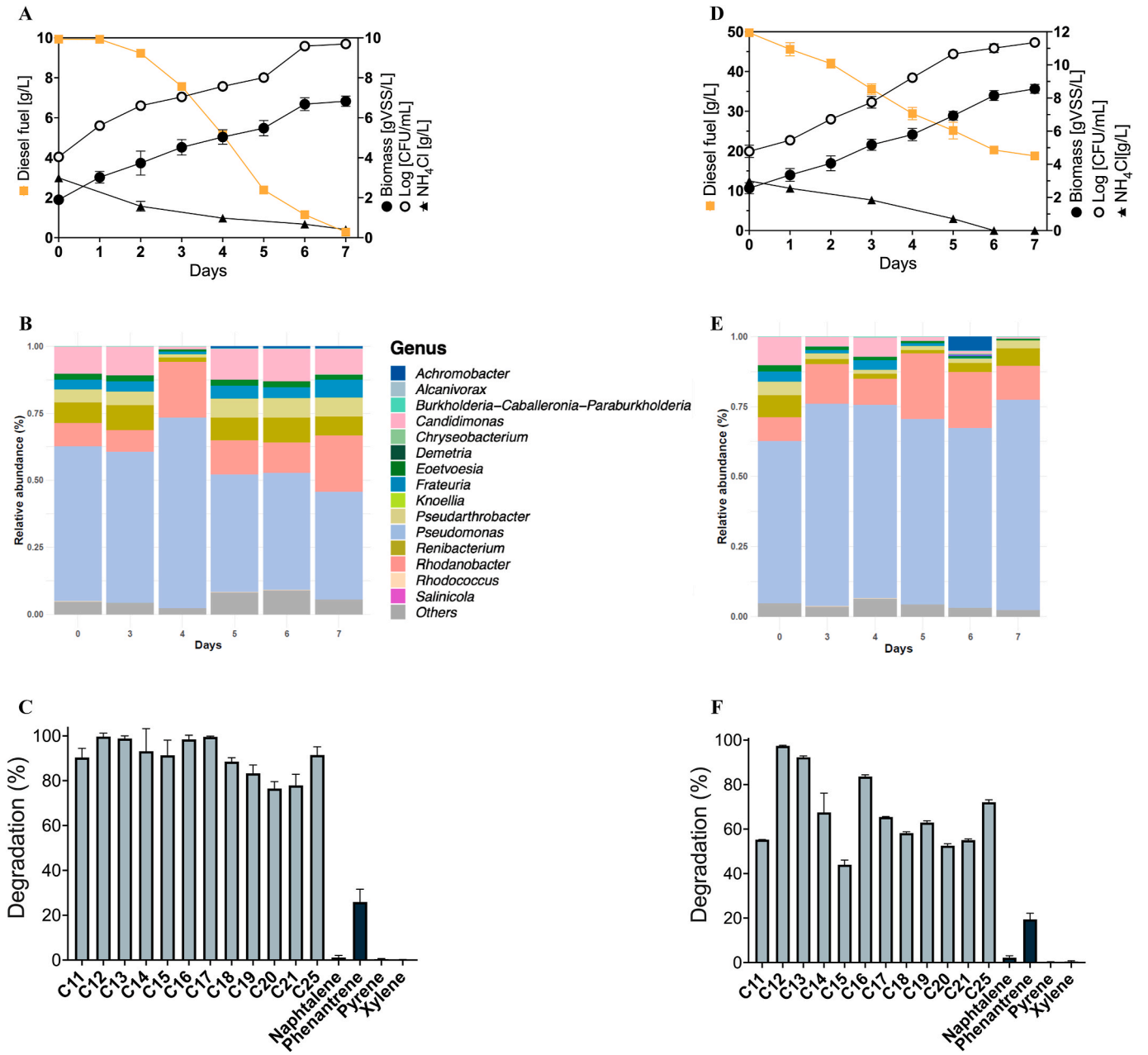

Fig. 5. Bacterial community composition, diesel biodegradation and growth profiles, and degradation efficiency of hydrocarbons of the diesel mixture by LR-10 community at low temperature in batch bioreactors. Diesel degradation and biomass formation kinetics in M9-seawater supplemented with A) 10 gDiesel/L and D) 50 gDiesel/L at 10 °C. LR-10 bacterial community composition through the diesel mineralization process amended with B) 10 gDiesel/L and E) 50 gDiesel/L. Hydrocarbon degradation efficiency after 7-day cultivation of LR-10 community supplemented with C) 10 gDiesel/L and F) 50 gDiesel/L. C11: Undecane; C12: Dodecane; C13: Tridecane; C14: Tetradecane; C15: Pentadecane; C16: Hexadecane; C17: Heptadecane; C18: Octadecane; C19: Nonadecane; C20: Icosane; C21: Heneicosane; C25: Pentacosane. Values represent the mean and standard deviation from two independent batch bioreactors.

continuous bioreactors. As observed for LR-30 batch bioreactors, the bacterial community did not degrade xylene and phenanthrene in continuous bioreactors (Fig. 6D).

3.6. *High-level continuous biodegradation of diesel polluted seawater by LR-10 community using continuous bioreactors at low temperature*

As for the LR-30 community in continuous bioreactors, the LR-10 community had an initial batch phase of 14 days (Fig. 7A). Within the feeding phase, biomass formation and diesel consumption stabilized earlier in the LR-10 compared to the LR-30 continuous bioreactors, after only 11 days of feeding (Fig. 7A). Nitrogen was the limiting nutrient at the end of the batch phase and it remained at that level through the diesel feeding stage. On day 25, LR-10 continuous bioreactors formed 6.1 (gSSV/L) of biomass and metabolizing 80% of the diesel content at a constant rate of 24.5 (g/L day) at 10 °C for the next 30 days (Fig. 7A). Another key feature of the LR-10 community was the strong correlation between the emulsification index and diesel degradation, showing a *Pearson* coefficient of 0.93 ($r^2 = 0.86$, $P < 0.0001$). The oil displacement test showed a clear diameter of 8.6 cm (Fig. 7B).

Whereas the diesel bioconversion by LR-10 continuous bioreactor was stable from day 25, this was not case for the bacterial community structure as the abundance of bacteria significantly varied within the first 20 days of feeding with *Rhodanobacter* dominating the community

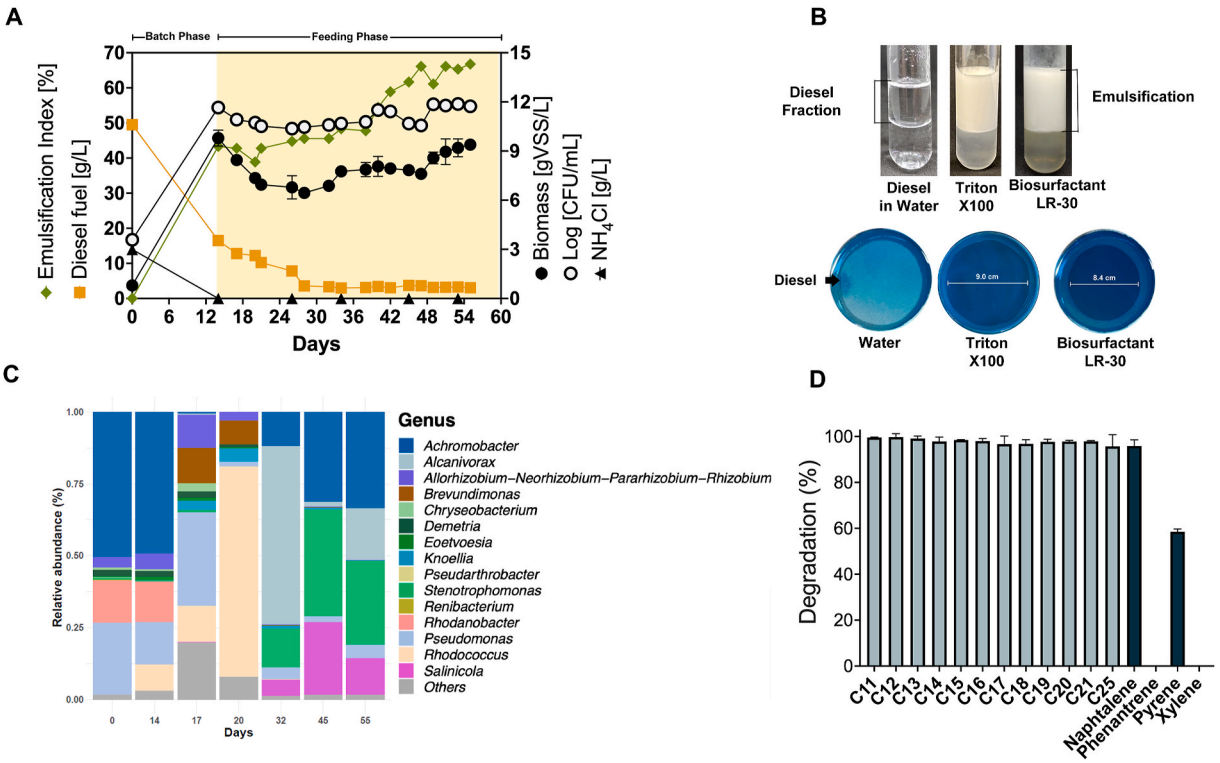

Fig. 6. Continuous biodegradation of diesel polluted seawater by the LR-30 community in continuous bioreactors under mesophilic conditions. A) Biomass production, diesel degradation, and emulsification index kinetics during diesel fuel biodegradation by the LR-30 community at 30 °C. B) Emulsification and oil displacement tests of diesel oil in aqueous solution with cell-free supernatant from the culture broth of the LR-30 community and Triton X100. C) LR-30 bacterial community composition profiles in continuous bioreactors run at 30 °C. D) Hydrocarbon degradation efficiency of the diesel mixture after 55 days cultivation of the LR-30 community in continuous bioreactors at 30 °C. C11: Undecane; C12: Dodecane; C13: Tridecane; C14: Tetradecane; C15: Pentadecane; C16: Hexadecane; C17: Heptadecane; C18: Octadecane; C19: Nonadecane; C20: Icosane; C21: Heneicosane; C25: Pentacosane. All values represent the mean and standard deviation from two independent continuous bioreactors.

(Fig. 7C). However, a drastic change occurred on day 44, where *Pseudomonas* became the dominant members of the community, maintaining nearly the same microbial composition till day 55 of cultivation ($P = 0.27$) (Fig. 7C). In fact, within the LR-10 continuous bioreactor the bacterial genera *Knoellia*, *Chryseobacterium,* and *Salinicola* were now detected using amplicon sequencing at the end of the continuous bioremediating process. The Faith's phylogenetic diversity index also fluctuated ($P < 0.0001$) throughout the continuous diesel bioremediation process (Supplementary material S3). Additionally, there was a high degree of variation between samples as there is no clear clustering for the PCoA, corroborating the disparity between microbial community structures (Supplementary S4). Compared with the LR-10 batch bioreactors degrading 50 gDiesel/L, continuous bioreactors showed a better degradation of *n*-alkanes (>80%), but it converted less than 8% of the concentration of phenanthrene and naphthalene (Fig. 7D).

4. Discussion

Previous studies have reported that the soil of Livingston island is contaminated with *n*-alkanes and PHAs (Cabrerizo et al., 2012), showing higher values for medium- and long-alkanes than those detected in our study. Cabrerizo et al. (2016) also quantified PAH concentrations that are similar to those recorded for anthracene and phenanthrene and lower for naphthalene compared to samples of *D. antarctica* rhizosphere soil (Fig. 2B). The concentration of hydrocarbons can fluctuate at sites near the coast as the transport of contaminants through air and seawater is highly variable (Cao et al., 2018). Natural inputs of hydrocarbons from the erosion of coal sediments in the South Shetland Archipelago would account for only 2–10% of the hydrocarbon concentrations (Cripps, 1992; Sutilli et al., 2019). It appears that the two scientific bases on Livingston island are the main source of PAHs and medium-chain length alkenes, as diesel is the main fossil fuel for energy generation (Cabrerizo et al., 2016).

It was particularly interesting to find that LR-30 and LR-10 specialize in metabolizing specific PAHs despite sharing the same environmental niche (Table 1). For instance, the LR-10 community showed no growth on naphthalene but did on anthracene and phenanthrene. In contrast, the LR-30 community can metabolize anthracene and naphthalene but not phenanthrene (Fig. 2). Anthracene can be mineralized aerobically by several bacteria that harbor the *nah* operon such as *Pseudomonas*, *Paracoccus*, *Rhodococcus*, and *Bacillus*, among others (Abbasian et al., 2016; Jurelevicius et al., 2012; Swaathy et al., 2014; Zhang et al., 2004). The first enzymes attacking the aromatic rings are dioxygenases that can yield the corresponding dihydrodiols and the two-ring PAH naphthalene (Abbasian et al., 2016; Ghosal et al., 2016; Sanseverino et al., 1993). The first compounds undergo several enzymatic conversions by the action of dioxygenases and dehydrogenases resulting in phthalic acid, whereas naphthalene is converted in subsequent enzymatic steps into catechol and gentisate. During anthracene degradation using *Pseudomonas* and *Bacillus*, naphthalene has been detected in the extracellular milieu where it accumulates, possibly as a detoxification mechanism when the strains lack the genetic machinery for its bioconversion (Swaathy et al., 2014). Additionally, *Paracoccus* sp. Ophe1 has been reported to propagate on anthracene, yet it could not grow on naphthalene (Zhang et al., 2004). Given the multiple Anthracene degrading pathways present in bacteria (Góngora et al., 2022), it seems that microbes comprising the LR-10 community contain no genes for naphthalene degradation and the enzymatic conversion of anthracene and phenanthrene occurs through alternative aromatic routes (Arora, 2020; Magdy et al., 2022; Swaathy et al., 2014; Zhang et al., 2004). It is also important to mention that

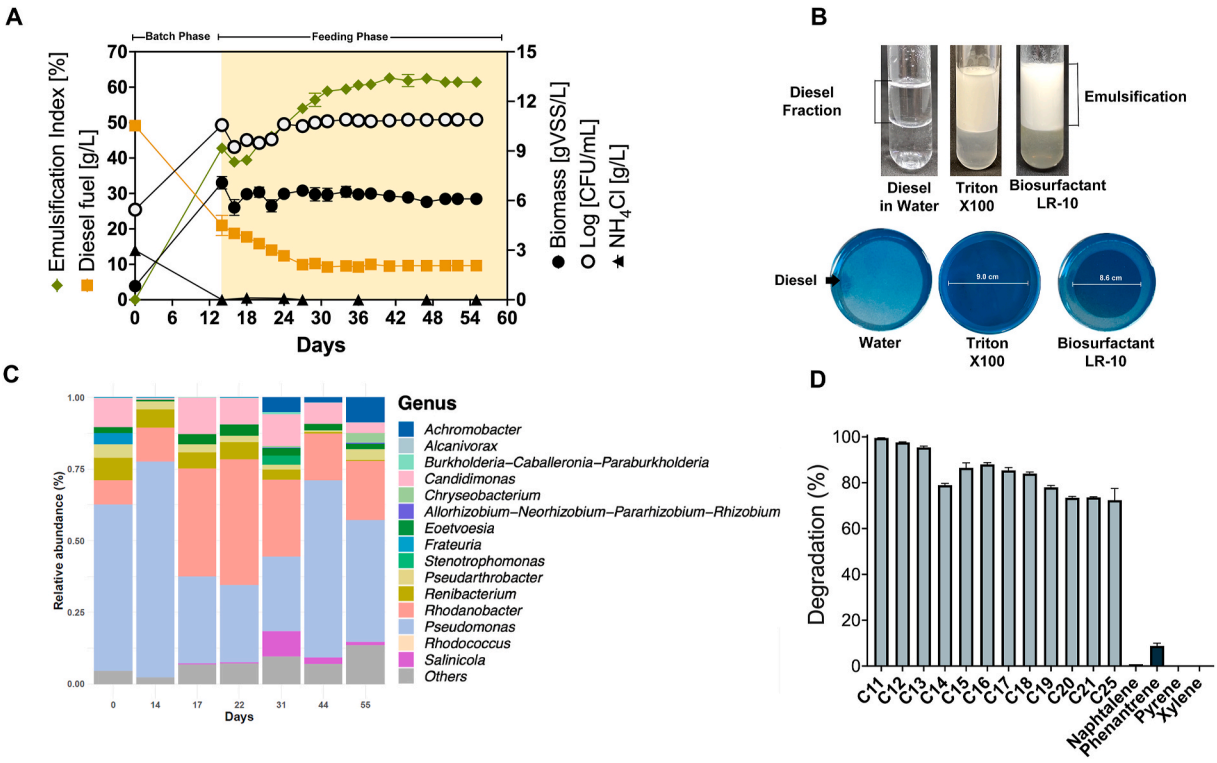

Fig. 7. Continuous biodegradation of diesel polluted seawater by the LR-10 community in continuous bioreactors at low temperature. A) Biomass production, diesel degradation, and emulsification index kinetics during diesel fuel biodegradation by the LR-10 community at 10 °C. B) Emulsification and oil displacement tests of diesel oil in aqueous solution with cell-free supernatant from the culture broth of the LR-10 community and Triton X100. C) LR-10 bacterial community composition profiles in continuous bioreactors run at 10 °C. D) Hydrocarbon degradation efficiency of the diesel mixture after 55 days cultivation of the LR-10 community in continuous bioreactors at 10 °C. C11: Undecane; C12: Dodecane; C13: Tridecane; C14: Tetradecane; C15: Pentadecane; C16: Hexadecane; C17: Heptadecane; C18: Octadecane; C19: Nonadecane; C20: Icosane; C21: Heneicosane; C25: Pentacosane. All values represent the mean and standard deviation from two independent continuous bioreactors.

Table 2
Bacterial efficiency on diesel fuel biodegradation at different temperatures and salinities in liquid phases.

Microorganisms	Cultivation mode	Temp (°C)	Salinity (g/L)	Initial Diesel (g/L)	Diesel biodegradation (%)	Incubation time (days)	Degradation rate (g/L day)	Ref.
Bacterial Consortia LR-30	Continuous bioreactor	30	Seawater	50[a]	93	55	34.4	This study
Bacterial Consortia LR-10	Continuous bioreactor	10	Seawater	50[a]	80	55	24.5	This study
Bacterial Consortia LR-30	Batch bioreactor	30	Seawater	50	66.7	7	4.8	This study
Bacterial Consortia LR-10	Batch bioreactor	10	Seawater	50	61	7	4.5	This study
Bacterial Consortia LR-30	Batch bioreactor	30	Seawater	10	98	7	1.7	This study
Bacterial Consortia LR-10	Batch bioreactor	10	Seawater	10	99	7	1.9	This study
Bacterial consortia	Batch flasks	30	Seawater	8.5	71	45	0.13	Shi et al. (2020)
Bacterial consortia	Batch flasks	25	Seawater	8.5	78.4	30	0.11	Fu et al. (2021)
Bacterial consortia	Batch bioreactor	30	Seawater	13	74	6	1.60	García-Cruz et al. (2019)
Vibrio sp. LQ2	Batch/Flasks	30	Seawater	8.5	92	7	1.12	Zhou et al. (2021)
Rhodococcus erythropolis	Batch/Flasks	15	Seawater	1	75	7	0.11	Huang et al. (2008)
P. aeruginosa NH1	Batch/Flasks	30	Seawater	6	76	28	0.16	El-Gendy and Nassar (2015)
A.beijerinckii ZRS	Batch/Flasks	30	Seawater	5	80.4	7	0.57	Huang et al. (2013)
Bacterial consortia	Batch bioreactor	25	0.5	130	91.1	10	11,8	Dutta et al. (2018)
Vibrio alginolyticus	Batch flasks	30	16	85	26	10	2.21	Imron et al. (2019)
Bacterial consortia	Batch flasks	10	25	30.5	40	7	1.75	Zakaria et al. (2021)
Unidentified bacteria	Batch flasks	30	35	8.5	29	15	0.16	Mukherji et al. (2004)
Delftia sp. NL1	Batch flasks	40	16	42.5	67	7	4.07	Lenchi et al. (2020)
Rhodococcus sp. AQ5−07	Batch flasks	10	10	8.5	58	7	0.70	Roslee et al. (2020)
Halomonas sp.	Batch/Flasks	30	35	8.5	83	12	0.59	Shi et al. (2019)
Pseudomonas sp. DRYJ3	Batch flasks	10	0.5	4.3	100	6	0.72	Shukor et al. (2009)
A. agilis E28 & *R. luteus* E60	Batch/Flasks	4	30	8.5	55	60	0.08	Michaud et al. (2004)
Achromobacter sp. HZ01	Batch/Flasks	28	30	2	95	10	0.19	Deng et al. (2014)

[a] During the feeding phase, 50 (g/L) of diesel fuel was constantly supplied to the continuous bioreactor.

naphthalene had the highest concentration among PAHs in the soil of *D. antarctica* (Fig. 2). Therefore, although the Antarctic soil contains microbes able to degrade naphthalene, as part of the LR-30 community, they are likely inhibited by the cold environment of the polar region (Góngora et al., 2022).

After supplementing the contaminated soil with diesel fuel and M9-medium in flask cultures to enrich microorganisms with the ability to use diesel as carbon substrate, we found that psychrotolerant bacteria, properly growing at 30 °C, are also present in the Antarctic rhizosphere soil. However, there is little information as to why Antarctic bacteria do not exclusively evolve towards psychrophilic growth given this continent exhibit temperatures below 0 °C most of the year. Some authors argue that they have better nutritional adaptability than psychrophilic microbes (Wynn-Williams, 1990) or that many of them are transported to the Antarctic territory via air and seawater currents (Hoover and Pikuta, 2010).

The amount of diesel degradation exhibited by LR-10 and LR-30 consortia in flask cultures surpassed previous attempts using psychrotolerant isolates, even compared to mesophilic bacteria from warmer regions (Fig. 3, Table 2). For bacteria metabolizing diesel concentrations above 20 (g/L), cell growth is considerably inhibited, accompanied by a reduction in hydrocarbon bioconversion rates (Aini Dahalan and Ayshah, 2019; Lee et al., 2006). This was not the case for LR-30 and LR-10 consortia, which consumed 97% and 98% of the pollutant when grown on 20 gDiesel/L (Fig. 3). This points to the need to explore more intensively remote environments that may harbor microorganisms with unexpected metabolic capabilities for biodegradation much more thoroughly, such as those shown in this study.

The use of stirred tank bioreactors for diesel bioremediation provides a proper mixing of the culture broth with diesel oil that in turn enabled the bacterial consortia to increase the bioconversion rate and diesel degradation yields compared to flask cultures (Table 2). This phenomenon is probably explained by the enhanced contact of cells with diesel hydrophobic compounds (Tekere, 2019) along with the appropriate oxygen levels in the bioreactor (Dutta et al., 2018; García-Cruz et al., 2019) that allow the intracellular enzymatic conversion mediated by oxygenase and dioxygenase enzymes (Abbasian et al., 2016; Ghosal et al., 2016). A study in airlift bioreactors determined that increasing diesel concentrations requires enhanced oxygen transfer rates to avoid diesel transfer rate limitations (Dutta et al., 2018). In our study the dissolved oxygen was always above 20% saturation, but when batch bioreactors started with 50 gDiesel/L for both consortia, nitrogen limitation occurred within the process given the high C/N ratio (Figs. 5A and 6A). Diesel consumption was significantly delayed from that point, explaining the two observed diesel degradation rates when nitrogen limitation occurred for LR-30 and LR-10 batch bioreactor. Many scientific reports attributed the inability of bacterial cultures to biodegrade high levels of diesel due to the accumulation of toxic hydrocarbons that inhibit the diesel uptake capacity of the cells and, as a result, bacterial growth (Abdulrasheed et al., 2020; El-Gendy and Nassar, 2015; Ho et al., 2020; Shukor et al., 2009; Xia et al., 2006). Unfortunately, we did not find studies where nitrogen was also measured throughout diesel bioconversion in batch cultivations, so it remains an open question whether the incomplete diesel bioconversion reported in the literature, it is the result of toxic compound accumulation or nutrient limitation.

Amplicon sequencing of the inoculum of LR-30 and LR-10 batch bioreactors revealed that they differ in terms of relative bacterial abundance due to the difference in growth temperature. Nevertheless, both microbial consortia share many bacterial genera, well-known hydrocarbon degraders such as *Achromobacter, Rhodanobacter, Pseudomonas, Candidimonas, and Rhodococcus*, among others (Chaudhary et al., 2021; Deng et al., 2014; El-Gendy and Nassar, 2015; Huang et al., 2008; Lee et al., 2006). Moreover, performing statistical analyses (ANOVA) of each bacterial genera during diesel degradation revealed that the abundance of most microbes fluctuated significantly at various points within batch bioreactors for both consortia (Supplementary S2). This pattern seems to be conserved for bacteria degrading hydrocarbon mixtures like diesel and crude oils (Chaudhary et al., 2021; Fu et al., 2021; Kostka et al., 2011; Yan et al., 2020), as some specialize in metabolizing specific aromatic or aliphatic compounds, changing their abundance in direct proportion to the level of the hydrocarbon agents.

During diesel bioremediation in continuous bioreactor, an increased diesel bioconversion rate was achieved compared to batch bioreactors supplemented with 50 gDiesel/L for both bacterial consortia (Table 2). These values are the highest reported for diesel bioremediation at low and mesophilic temperatures in seawater supplemented with inorganic nutrients (Table 2). LR-30 and LR-10 continuous bioreactors started with a batch phase for biomass formation (14 days). By the end of the batch phase, both bioreactors displayed an emulsification index of 43%, which started to increase over time, reaching 66 and 62% after 30-day of diesel supply for LR-30 and LR-10, respectively. These values had a direct correlation with the amount of diesel degraded as inferred from the high *Pearson* coefficient of 0.93. In other words, better emulsification indices (production of biosurfactants) were obtained within continuous rather than batch bioreactors, indicating how well these surface-active emulsifying agents interact with the water insoluble fraction of the fossil fuel (ben Ayed et al., 2015; Sun et al., 2018). Oil displacement experiments confirmed that the LR-30 and LR-10 produce biosurfactants that allow diesel dispersion and micelle formation with the hydrophobic compounds of the diesel mixture when the amount of surfactant is beyond the critical micelle concentration (CMC) (ben Ayed et al., 2015; Bordoloi and Konwar, 2009; Goldsmith and Balderson, 1989), enabling access and further incorporation into bacterial cells (Neu, 1996; Souza et al., 2014). As the emulsifying agent is biosynthesized from the substrate (diesel oil), biomass formation tends to decline most likely due to carbon flux partitioning towards surfactant biosynthesis, a trend observed for continuous bioreactors and consistent with the enhanced diesel remediation yield and rate (Figs. 6A and 7A).

Continuous bioreactors differ highly in terms of physiological characteristics, particularly growth rate, of microbial communities compared to batch bioreactors (Li et al., 2020; Ziv et al., 2013). First, batch systems promote the fastest-growing bacteria in a community, given that all nutrients are in excess at the beginning of the culture (Veldkamp, 1976; Ziv et al., 2013). Conversely, within the continuous bioreactor, all bacterial genera are replicating to the same specific growth rate (dilution rate = 1/HRT), allowing one to evaluate the proportion of bacteria established under steady-state conditions (Li et al., 2020; Ziv et al., 2013). This is worth mentioning given LR-30 and LR-10 continuous bioreactors exhibited a stable diesel degradation rate for a month; however, we found significant variations in the bacterial abundance and Faith's diversity index during this period (Figs. 6A and 7A, Supplementary material S3, S4). Notably, biomass formation for the LR-10 community was also stable for 30 days, yet variation in the bacterial abundance occurred (Fig. 7C). We believe that other factors could prevent the diesel-degrading communities from reaching steady-state regimes, as natural seawater contains bacteriophages that infect and lyse the cells under certain conditions (Brown et al., 2022; Filé et al., 2005). Additionally, these open systems are prone to external colonization of microbes—for instance fungi that are not detected by our sequencing approach (Amend et al., 2019; Gonçalves et al., 2022)—that feed on cell debris or by-products secreted by the community, and that are not necessarily specialized in hydrocarbon degradation, altering the bacterial community composition to some extent.

5. Conclusions

Our findings demonstrate that the rhizosphere of the vascular plant *D. antarctica* obtained from Livingston Island is contaminated with *n*-alkanes and PAHs and harbors microorganisms with metabolic capabilities to degrade high-level of diesel oils at low and mesophilic temperatures. For the first time, we demonstrated that bacterial consortia specialized in catabolizing hydrocarbons in high-salinity environments

are more efficient for diesel biodegradation in continuous rather than batch bioreactors when supplied with inorganic nutrients. The 16S rRNA gene sequencing results revealed that bacterial genera comprising the isolated Antarctic consortia are well-known hydrocarbon degraders. LR-30 community contains, in descending order, *Achromobacter, Pseudomonas, Rhodanobacter, and Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, whereas *Pseudomonas, Candidimonas, Rhodanobacter, Renibacterium* are the dominant members for LR-10 consortium.

LR-30 continuous bioreactors reached a diesel conversion rate of 34.4 (g/L per day) at 30 °C, whereas the LR-10 community achieved 24.5 (g/L per day) at 10 °C (Table 2). Additionally, continuous bioreactors exhibited higher emulsification activities than batch bioreactors, which appear to be directly correlated with the increased diesel remediation by the action of biosurfactant production by the consortia at the expense of biomass formation. Despite the stable diesel bioconversion rate within continuous bioreactors, the relative abundance of bacteria composing the LR-30 and LR-10 consortia fluctuated through the diesel degradation process, where steady-state conditions were never attained. These high-level continuous diesel degradations form the basis for further investigations with shorter hydraulic retention times to fully degrade the provided diesel and decipher how these parameters influence bacterial community composition during diesel bioremediation.

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Credit author statement

Y.S–B and M.O–S carried out the experimental part, analyzed the data, and prepared figures and tables. J.C–S inferred phylogenetic relationships and performed statistical analyses. C. G-M analyzed the GC-MS data and contributed to the writing of the manuscript. E.C–N obtained the samples, analyzed the sequence data, inferred phylogenetic relationships, and contributed to the writing of the manuscript. I.P–C conceived the study, supervised the study, analyzed the data, and wrote the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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