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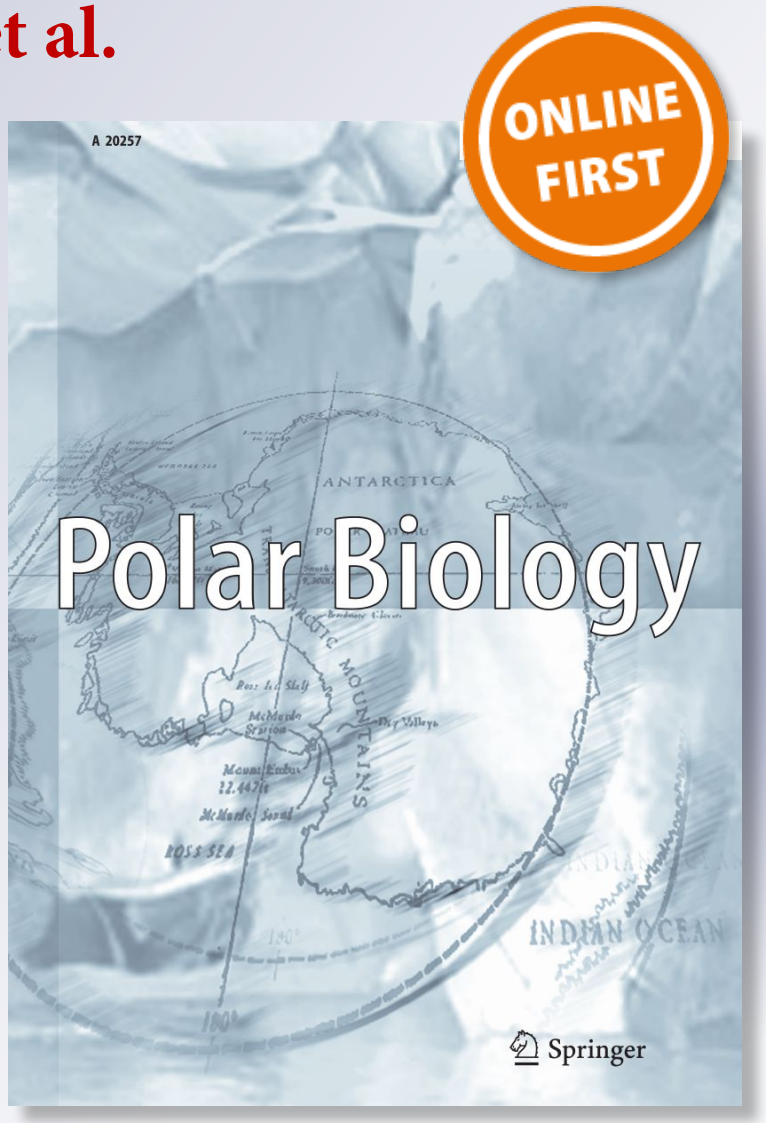

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ORIGINAL PAPER

Hydrocarbon-contaminated Antarctic soil: changes in bacterial community structure during the progress of enrichment cultures with different *n*-alkanes as substrate

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Abstract

Hydrocarbon contamination in soils from extremely cold areas, such as those from Antarctica, requires the development of specific remediation strategies for cleaning up anthropogenic pollution. Previous reports evidenced that after *on-site* biostimulation process of gasoil-contaminated Antarctic soils, 20% of the initial hydrocarbons remained undegraded (mainly C11–C14 *n*-alkanes). In the present work, these *n*-alkanes were added as sole carbon and energy source to enrichment cultures inoculated with the previously treated soil (biostimulation) as microorganism's source to investigate changes occurring in the bacterial community structure. Three subcultures (8, 16, and 24 days) were performed from each enrichment culture. Changes in bacterial communities among different cultures and its subcultures were evidenced by Denaturing Gradient Gel Electrophoresis (DGGE). Results showed that even differences of one C in the alkane chain-length led to different community structures that evolved divergently from the original one. Clusters analysis showed that while samples grouped mainly by culture time, substrate-dependent differences were also evident. Isolation of biological tools for bioremediation from the cultures showed that Pseudomonadaceae members were omnipresent, whereas *Rhodococcus* spp. were obtained in cultures with the longest chain-length substrates. Results provided evidence about the presence of certain substrate preference of soil bacteria (even when substrates differed only in one C-atom of their chain-length), leading to different community structures. A collection of psychrotolerant hydrocarbon degrading/tolerant strains was obtained, representing a valuable tool for the design of a bioaugmentation strategy as a second, more specific stage, targeting the remnant hydrocarbons after a first bioremediation process involving biostimulation.

Keywords Bioremediation · Enrichment cultures · *n*-Alkanes · Antarctica · Bacterial community structure

Introduction

Soil represents one of the most significant organic matter reservoirs of the planet and constitutes an important habitat for microbial communities. Whitman et al. (1998) reported estimations for the total number of prokaryotic cells on the earth being around 5×10^{30}, and 2.6×10^{29} were soil inhabitants. Microbial communities are key participants in the nutrients cycling in soils (Falkowski et al. 2008) by degrading complex molecules from plants and animal remains and transforming them into small organic molecules and inorganic nutrients.

Crude oil-derived hydrocarbons are one of the most common contaminants, and their impact is seen all around the planet, even in isolated regions such as the Arctic and Antarctica (Camenzuli and Freidman 2015).

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Hydrocarbon-contaminated soils are also inhabited by complex microbial communities, in which bacteria play a vital role. Such communities are supposed to have been derived from those present in the pristine soils before the contamination event, evolving under the selection pressure represented by the presence of contaminants (Hamamura et al. 2006). Soils, sands, and sediments all around the world contain naturally occurring hydrocarbon-degrading microorganisms, and their number dramatically increases under hydrocarbon pollution (Bragg et al. 1994; Harayama et al. 2004; Head et al. 2006). These adapted communities represent a source of catabolic capabilities (mainly due to bacteria and fungi) that can be used for the design of bioremediation processes (Watanabe 2001), either based on biostimulation or bioaugmentation.

We have observed that, after a bioremediation process based on biostimulation, 10–20% of the contaminants present in a portion of Antarctic diesel-contaminated soil remained in the soil (Ruberto et al. 2009; Martínez Álvarez et al. 2017). Studies performed with gasoil contaminated soils from Carlini Station (25 de Mayo Island, South Shetlands, Antarctica) showed that the fraction persisting after the biostimulation treatment was composed mainly by lineal *n*-alkanes ranging between C11 and C14 (Martínez Álvarez et al. 2015). Bacteria able to degrade *n*-alkanes ranging from C5–C7 to C17 have soluble cytochrome P450 and/or membrane integrated non-heme iron monooxygenases, like AlkB (Rojo 2009; Austin et al. 2011). These enzymes represent key catabolic elements that initiate hydrocarbon chain oxidation (Wang and Shao 2013).

Based on these observations, we hypothesized that a microbial association (mixed culture) able to specifically degrade these compounds could be applied as inoculum (bioaugmentation) in a second bioremediation stage, after biostimulation, targeting directly the remaining fraction of contaminant compounds.

In this sense, it was observed that, when biodegradation involves complex hydrocarbon mixtures, the co-existence and synergy of different and metabolically complementary microorganisms result in a more effective process than those depending on the activity of one isolated microorganism (Richard and Vogel 1999; Cerqueira et al. 2011).

Enrichment cultures are commonly used tools to obtain degrading bacteria with specific metabolic capabilities, such as the degradation of toxic compounds (Hilyard et al. 2008; Udgire et al. 2015). Although in some cases the main aim is the isolation of single strains with a certain degrading capability, in other cases the enrichment culture is used to obtain contaminants-degrading microbial associations or consortia (El Hanafy et al. 2016). It is known that these microbial associations frequently have the ability to metabolize a wider spectrum of compounds than the isolated strains and, for this reason, they constitute a more versatile biological tool for bioremediation processes (Zuroff and Curtis 2012; Minty et al. 2013; Mee et al. 2014). As bioremediation is a biological process to be applied under partial or non-controlled environmental conditions, its robustness, among other features, is crucial. For this reason, mixed cultures are expected to have some properties that are attractive for a biotechnological context: stability, functional robustness and the ability to perform complex tasks that are not possible for isolated microorganisms. However, for a bacterial association, stability (to keep its structure constant) is a characteristic that seems to be hard to achieve. Also, as was stated by Jousset et al. (2011), resilience (the ability of a community to quickly recover after a perturbation), provides its members with an additional biotechnological feature. In this sense, Rittmann et al. (2006) suggested that, for full-scale biotechnological environmental-related processes, the focus should be put on microbial communities rather than on a "superbug" for the solution of different problems.

Also, previous non-published own observations showed that submerged cultures containing hydrocarbons with a similar chemical structure (i.e., *n*-alkanes differing only in one C in its chain length) could evolve to states exhibiting different colours and cellular aggregations, suggesting the presence of different microbial communities or behaviour.

All these antecedents lead to the question about how to obtain a hydrocarbon-degrading bacterial association exhibiting features suitable for use in environmental biotechnology. The present work was focused on the analysis of changes occurring in the structure of an Antarctic hydrocarbon-contaminated soil bacterial community when it was growing in enrichment cultures with closely related *n*-alkanes (*n*C11, *n*C12, *n*C13, and *n*C14) as sole carbon and energy source.

Materials and methods

Soil sampling and enrichment cultures

The soil used in this study was taken from the surroundings of the diesel storage tanks at Carlini Station (Fig. 1), 25 de Mayo island (King George Island), South Shetlands, Antarctica (62°14′18″S 58°40′04″W). The soil was first subjected to a 40 day-length biostimulation treatment in the field which decreased its hydrocarbons content from 2180 mg/kg to around 500 mg/kg, representing 20% of the initial concentration. Briefly, gasoil contaminated soil was sieved (10 mm mesh) to remove large stones and gently mixed using a rotative drum. Most of this material showed a particle size that was retained in a 5 mm mesh (27%), 1 mm mesh (65.5%), 0.5 mm mesh (5.5%). Sieved and homogenized soil (830 kg) was divided into two fractions building identical 0.53 m^3 biopiles (truncated pyramids). One biopile was used as a

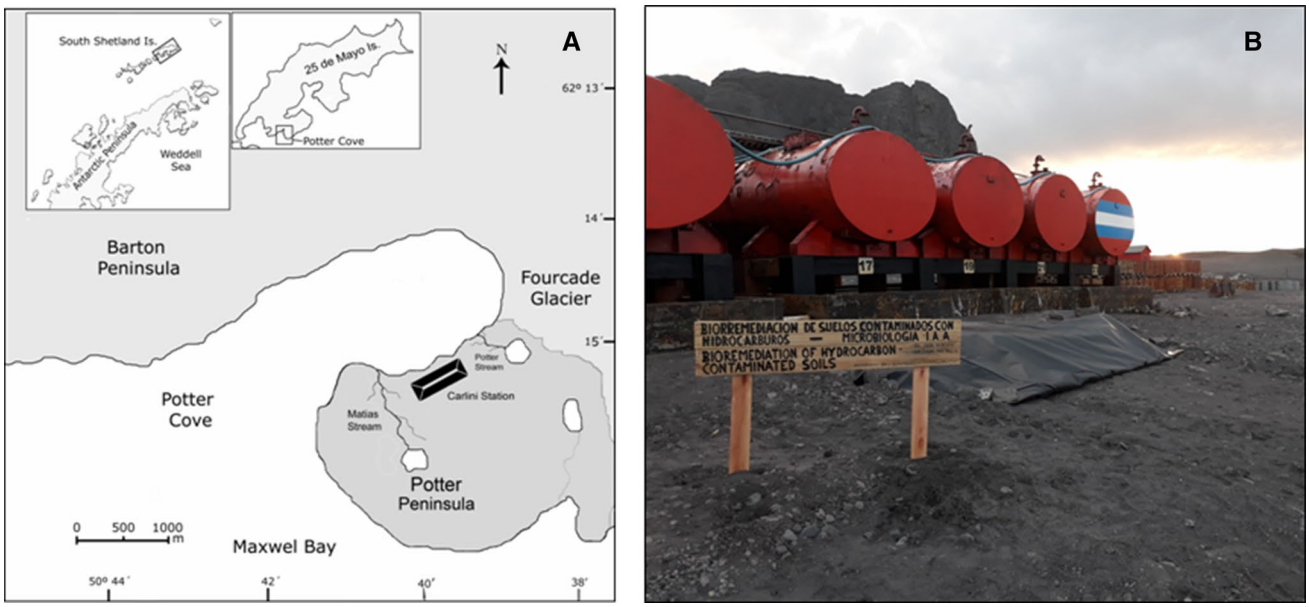

Fig. 1 Map showing location of soil sampling. A: geographical location of Carlini station. B: Area of fuel-storage tanks where the contaminated soil was obtained

control and the other one was biostimuled using an optimized C:N:P ratio (100:17,6:1,76). After 50 days of biostimulation process, a composed (5 subsamples, 400 g each) soil sample was taken from each experimental unit and kept frozen (−80 °C) for further experiments. The remaining hydrocarbon fraction showed to be composed mainly by medium chain length aliphatics ranging from C11 to C14. Design and results of this bioremediation process were described in detail in Martínez Álvarez et al. (2017).

Enrichment cultures from soil samples were prepared in 250-mL Erlenmeyer flasks containing 25 mL of saline basal medium (MSB), as previously described by Espeche et al. (1994). As source of microorganisms, 2.5 g of soil described in the previous paragraph (containing 500 mg/kg of hydrocarbons) was added to each flask. Different *n*-alkanes were used as carbon and energy source. Undecane (*n*C11), dodecane (*n*C12), tridecane (*n*C13), tetradecane (*n*C14), and a 1:1:1:1 mixture of the four compounds (Mix) were added (250 µL) to the corresponding flask. A control flask without carbon and energy source (Cl) was also prepared. Some general properties of the *n*-alkanes used as carbon source are shown in Table 1. Flasks were placed in a refrigerated orbital shaker (Infors HT Ecotron) and incubated at 15 °C with agitation (250 rpm) to assure oxygen availability. Incubation temperature was chosen considering those reported by Martínez Álvarez et al. (2017) for a covered-biopile experiment carried out during 50 Antarctic summer days. This temperature allows growth of psychrotolerant and psychrophilic bacteria from surface Antarctic soil. Three consecutive enrichment cultures were performed for each of the tested hydrocarbons as well as the mixture: from 0 to 8 days, from 8 to 16 days, and from 16 to 24 days. Subcultures were prepared transferring 250 µL of the finished culture to another flask containing fresh medium and the corresponding hydrocarbon. Inoculated volume was selected to allow microorganisms transfer from one flask to the other avoiding a significant contribution of both metabolites and remnant hydrocarbons This operation resulted in four sets of samples: set 0 (initial cultures without incubation), set 1 (day 8, samples obtained at the end of the 8-day-old culture), set 2 (day 16, at the end of the first 8-day-old subculture), and set 3 (day 24, at the end of the second 8-day-old subculture).

Table 1 Some general properties of the *n*-alkanes used as sole carbon and energy source in the liquid cultures

Compound	Chemical formula	CAS N°	Molecular weight	Solubility (mg L^{-1} 25 °C)	Log K_{ow}
Undecane (*n*C11)	$C_{11}H_{24}$	1120-21-4	156.31	0.0044 to 0.014	5.74
Dodecane (*n*C12)	$C_{12}H_{26}$	112-40-3	170.34	3.7×10–3	6.10
Tridecane (*n*C13)	$C_{13}H_{28}$	629-50-5	184.37	4.7×10–3	6.73
Tetradecane (*n*C14)	$C_{14}H_{30}$	629-59-4	198.39	3.3×10–4	7.20

At the end of each subculture, tenfold dilutions from one aliquot of the samples were plated on R2A agar (Oxoid®) and incubated at 15 °C for 15 days to obtain isolated colonies. Another aliquot was stored at – 80 °C until DNA extraction.

Bacterial community structure analysis

Changes in bacterial community structure from the cultures were analysed by denaturant gradient gel electrophoresis (DGGE). For this purpose, bacterial genomic DNA (gDNA) was extracted from 2 g of soil using the commercial kit Power Lyzer Power Soil (Mobio®), following the manufacturer's instructions. A fragment of approximately 430 bp of the V6 variable region of the 16S rRNA gene was amplified using the universal bacterial primers U968f-GC and U1401R as described in Piquet et al. (2011). The reaction mixture contained PCR buffer 1×, $MgCl_2$ 3.25 mM, formamide 1%v/v, bovine serum albumin 0.1 mg/mL, dNTPs 0.2 mM each, primer 0.2 mM each and Taq polymerase 2 U. PCR products were checked for quality and concentration by DNA gel-electrophoresis on 1%w/v agarose gels stained with GelRed™.

DGGE molecular fingerprints of the bacterial communities were obtained using the TV400 (Scie-Pls Ltd.) system. For this purpose, PCR products (200 ng) from each sample, supplemented with 1×loading buffer (0.05% w/v bromophenol blue, 40% sucrose, 0.1 M EDTA pH 8.0, 0.5% sodium lauryl sulphate), were separated on 6% polyacrylamide gels with a 40–65% urea-formamide DNA-denaturing gradient (100% urea-formamide was defined as 7 M urea and 40% deionized formamide). The DGGE was run in TAE buffer 0.5×for 16 h at 60 °C and 100 V. An in-house standard sample (STD), prepared from independent PCR reactions using the same primers and genomic DNA obtained from Antarctic isolates as a template, was added to each gel as a reference for subsequent analysis of banding patterns. The similarity between the bacterial communities from different treatments was determined by clustering analysis after digitalization and normalization of the DGGE banding patterns using the Gelcompare II v.6.5 software (Applied Maths). The DICE correlation coefficient was used to calculate the similarities among DGGE patterns, and the dendrograms were obtained using the UPGMA clustering algorithm.

Isolation and identification of bacteria able to grow on hydrocarbons

Bacteria able to grow in the enrichment cultures using hydrocarbons were obtained, not to describe bacterial community structure, but with the aim to obtain useful biological tools for the design of bioaugmentation strategies. Isolates growing on the different hydrocarbons as carbon and energy source were obtained from R2A (Oxoid®) plates. Single morphotypes were picked and repeatedly re-streaked on the same medium until obtaining pure cultures. Isolates were checked for purity and morphological characteristics by Gram staining and preserved at – 80 °C on 30% glycerol.

Isolates were identified based on their 16S rRNA gene partial sequencing. After centrifugation of 5 mL of cultures, cell biomass was incubated (30 min) at 37 °C with 5 µl of lysozyme (300 mg/mL) and 5 µL of RNAse (20 mg/mL) followed by 30-min incubation at 50 °C with 5 µL proteinase K (10 mg/mL) and 15 µL SDS 10%w/v. After that, genomic DNA was extracted using the AxyPrep™ Blood Genomic DNA Purification Miniprep Kit (Corning Axygen®). Approximately 1500 bp of 16S rRNA gene were amplified by polymerase chain reaction (PCR) using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Each PCR tube contained 50 µL of a mixture composed by 20–50 ng of gDNA, 0.1 mM of each dNTP, 0.3 µM of each primer, 1.5 mM of Cl_2Mg, 0.3 mg/mL bovine serum albumin and 1.5 U of Taq DNA polymerase. PCR consisted of an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were examined by electrophoresis on 1% agarose gels stained with GelRed™ and visualized under UV light. PCR products were purified with the AxyPrep™ PCR Clean-Up Kit (Corning Axygen®) and sequenced at the Genomic Unit of the Biotechnology Institute of CNIA-INTA (Argentina), using a capillary automatic sequencer model ABI3130XL (Applied Biosystems, USA). Sequences were edited using BioEdit 7.0 software. Naïve Bayesian Classifier utility (Wang et al. 2007) from the RDP Release 10 was used for taxonomic placement of the isolates at the genus level, with 95% confidence. The EzBioCloud's Identify Service (Yoon et al. 2017) was used to find the nearest neighbour sequences (type strains and reference genomes) according to their 16S rRNA gene sequence similarity, to suggest most probable affiliations of the isolates obtained at the species level. Resulting sequences were deposited in GenBank under accession numbers MG825063–MG825088.

Results

Enrichment cultures

At the end of the incubation periods, enrichment cultures differed, exhibiting diverse kinds of aggregates and different colours depending on the *n*-alkane used as the substrate. These differences were slightly evident after the first 8 days of culture (end of the initial culture) and became more noticeable at the end of the further subcultures, leading to a totally different visual aspect after 24 days (set 3, end of the

second subculture). Colour, as well as the amount and size of aggregates, were the main contributors to the differences in the visual aspect. Table 2 summarizes the characteristics observed in each flask at the end of the first (set 2, 16 days) and second (set 3, 24 days) subcultures.

Bacterial community structure analysis

Duplicates (different soil samples processed and amplified independently) of DGGE patterns from the initial time shared a 100% similarity based on Dice coefficient, evidencing an adequate reproducibility of the method. The DGGE banding patterns from the original soil (0 days) and from the subsequent enrichment cultures with the assayed hydrocarbons shared different percentages of similarity (Fig. 2), evidencing that the structure of the initial community evolved divergently depending on both, culture time and the carbon source.

Band profile of the original soil (0 days) was the most different one, sharing only 20% similarity when compared with

Table 2 Main visual characteristics of the second (16 days, set 2) and third (24 days, set 3) subcultures with the different *n*-alkanes as carbon source. *n*C11: undecane, *n*C12: dodecane, *n*C13: tridecane, *n*C14: tetradecane, Mix: mixture 1:1:1:1 of each alkane, CL: control culture without carbon source)

Subculture	Carbon source	Turbidity	Color	Aggregate properties		
				Ammount	Shape	Size
2	*n*C11	+	Dark orange	++	Spherical	Small
2	*n*C12	+	Dark orange	1	Amorphous	Very large
2	*n*C13	+	Dark orange	++++	Oval	Large
2	*n*C14	+	Dark orange	++++	Oval	Medium
2	Mix	+	Dark orange	++++	Spherical	Medium
2	CL	–	Light pink	–	–	–
3	*n*C11	+	Yellow	++	Spherical	Small
3	*n*C12	+	Yellow	++++	Spherical	Medium
3	*n*C13	+	Yellow-white	++++	Spherical	Small, grouped
3	*n*C14	+	Dark orange	++++	Oval	Large
3	Mix	+	Yellow-white	++++	Oval	Large
3	CL	-	Colorless	–	–	–

1: one large aggregate; ++: 10 to 30 aggregates per flask, +++: 30 to 60 aggregates per flask; ++++: more than 60 aggregates per flask

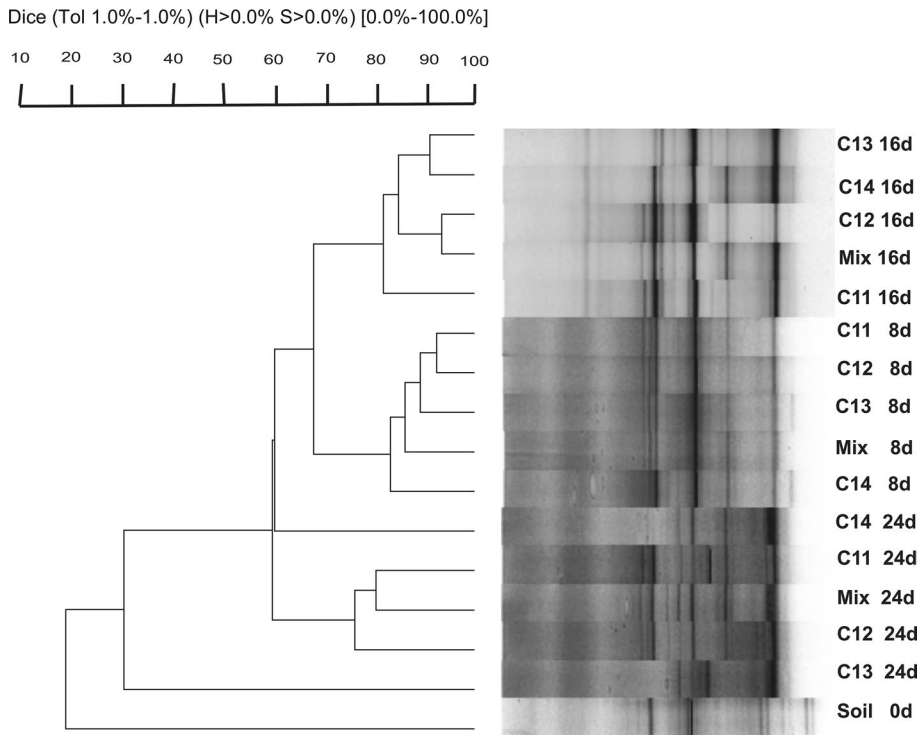

Fig. 2 Dendrogram (Dice) based on DGGE patterns from all subculture samples (0, 8, 16, and 24 days) of the cultures with different *n*-alkanes as sole carbon source (*n*C11, *n*C12, *n*C13, *n*C14, and Mixt)

the rest of the samples. At day 8 (set 1), all cultures shared more than 80% similarity, grouping in a unique cluster. After the first subculture, day 16 of the assay, all samples evolved in a way that they ended up being also 80% similar between them but keeping only 65% similarity with the group of samples at day 8 (Fig. 2). When the second 8-day-old subcultures were analysed, at day 24 of the assay, the community growing on *n*C14 alkane (C14 24 d) was the least divergent, exhibiting about 60% similarity with the communities in the previous stages of cultivation. On the contrary, the community growing on *n*C13 alkane was the most divergent from the previous state at day 16, sharing only 30% similarity with any other sample at any time of culture. On the other hand, communities developed after the second subculture, at day 24 of the assay, growing on *n*C11, *n*C12 and the mixture of alkanes clustered together, sharing 75–80% similarity between them and about 61% similarity with samples from the initial culture, the first subculture (set 1, day 8 and set 2, day 16) and with sample C14 24d.

In Fig. 3a, the number of bands for each subculture on the different hydrocarbons tested is shown. Assuming the number of bands in a single DGGE profile as indicative of the community richness, for *n*C11 and *n*C12 community richness reached a minimum after 8 days of culture and then rose gradually up to day 24 in both subcultures, but remained lower than the initial number of bands (12). On the contrary, bacterial community growing on *n*C13 showed a totally different dynamic, sharply decreasing in richness from 12 bands at day 0 to only 4 bands at day 24 of culture. When the initial bacterial community present in the soil was enriched on *n*C14 alkane, there was a fast reduction in richness at day 8, which remained stable until day 24, with the number of bands ranging from 8 to 6.

In cultures containing the hydrocarbons mixture as a substrate, richness decreased from 12 to 8 bands after the first 8 days of culture and remained stable until the end of the assay. However, although the number of bands at days 8, 16, and 24 could suggest some degree of stability of the bacterial community, the DGGE patterns from these three cultures shared only 5 bands, the other 3 bands being unique for each pattern. Figure 3b shows changes in the number of bands shared between all cultures (except for the Mix system). Out of the 12 bands present at day 0, only 5 were shared at the end of the initial culture (day 8) and the first subculture (day 16), and only 2 bands after the second subculture (24 days), also reflecting a gradual lack of similarity.

DGGE profiles of all cultures can be observed in Supplementary Material 1. There was one band at the bottom of the DGGE gel that, although not detected in the initial sample, was common to all subcultures profiles. Although it is known that reliable identification of one band cannot

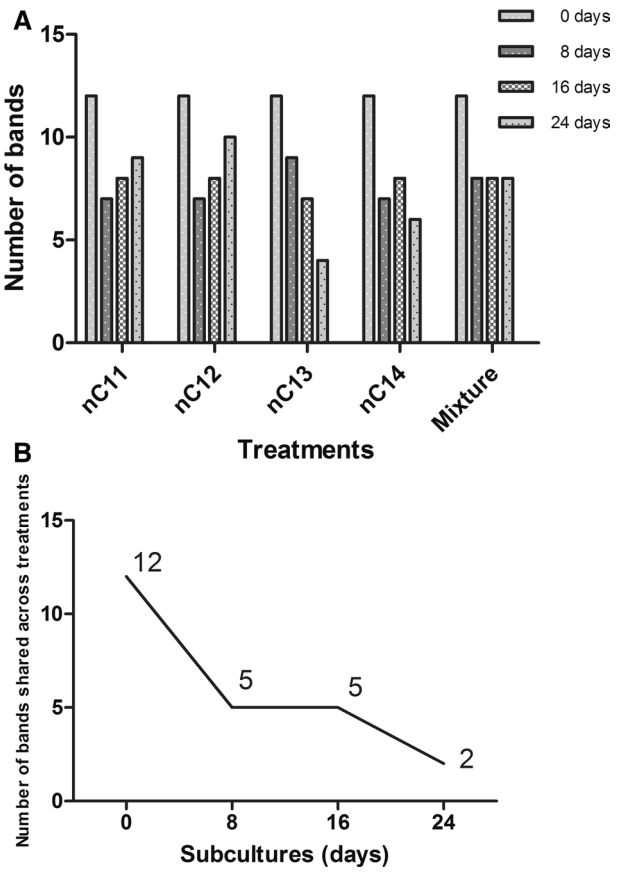

Fig. 3 DGGE profiles analysis **A** Number of bands exhibited by the subcultures with different *n*-alkanes as substrate for all subculture samples (0, 8, 16, and 24 days). **B** Number of shared bands in the DGGE patterns between subcultures corresponding to each time and bands present a t0

be assigned through direct comparison with those exhibited by an isolated strain, it is interesting to highlight that the mentioned band coincides with that obtained from an hydrocarbon-degrading Antarctic *Rhodococcus* strain previously isolated by our group (Ruberto et al. 2005) and that was included in the in-house marker. This fact would suggest the presence of a high G−C content bacterium, similar to *Rhodococcus*. The absence of this high-GC band in samples at day 0 and its appearance in the subculture samples would suggest the presence in the soil, as a minor component, of a ubiquitous bacterium with a G−C content compatible with *Rhodococcus.* This bacterium would be able to metabolize the four alkanes offered as a substrate or their metabolites. Although this component seems to be present in a very low proportion in the original soil community and therefore was not detected by the DGGE, it becomes a dominant member after the enrichment cultures. Further studies will allow its clear identification.

Isolation and identification of culturable hydrocarbon-tolerant bacteria

Petri dishes containing well-isolated colonies corresponding to the end of the second subculture (day 24) were used to isolate hydrocarbon-tolerant bacteria, assuming that bacteria able to grow at this stage should be highly adapted to tolerate and grow on the corresponding *n*-alkane used as substrate. Twenty-six colonies were picked and transferred to plates containing fresh media. The taxonomic assignment of the isolates obtained (12 for *n*C11, 5 for *n*C12, 5 for *n*C13, and 4 for *n*C14) showed that members of the genus *Pseudomonas* were present in cultures with all the carbon sources, evidencing the adaptation of *Pseudomonas* species to the presence of alkanes as substrate. In this sense, a putative *Pseudomonas yamanorum* was present in enrichment cultures containing *n*C12, *n*C13, and *n*C14 (Table 3). On the other hand, a *Rhodococcus* sp. was the only high-GC content bacteria recovered and it was found in *n*C13 and *n*C14 cultures. These two genera and also the others identified among the isolates are already described as hydrocarbon tolerant/degrading bacteria (Ruberto et al. 2005; Ma et al. 2012).

Discussion

The results presented in this work suggest that, when an Antarctic hydrocarbon-contaminated soil is used as inoculum for enrichment cultures, its bacterial community evolves divergently depending on the *n*-alkane (C11 to C14 in this case) used as sole carbon and energy source as well as with the incubation time. Similarity percentage among cultures differed either with the number of subcultures but also with the carbon source. This fact suggests that the use of different substrates leads to different microbial community structures, even when *n*-alkanes differed only in one carbon atom. These results are surprising considering that the catabolic machinery needed for alkane degradation is simple and relatively conserved among the Bacteria domain. For instance, alkane hydroxylases proved to have a wide range of substrates (Beilen and Funhoff 2007).

These results were obtained by incubation at 15 °C, a temperature which is in the range (5 to 20 °C) commonly used for the study of hydrocarbon-degrading bacteria in cold soils (Eriksson et al. 2003). As temperature is a key factor affecting bacterial growth (Ratkowsky et al. 1982), it is reasonable to presume that incubation at a different temperature could result in a different community structure than those obtained at 15 °C. In any case, beyond the temperature dependence of the phenomenon, the results suggest that the divergent evolution that leads to the different structures of the community observed at 15 °C is a phenomenon that also depends on the length of the hydrocarbon chain and that it could happen at any other temperature at which the phenomenon was studied. According to Rojo (2009) and Austin et al. (2011), bacterial enzymes involved in C7–C17 degradation are soluble P450 cytochrome oxidases and *alkB* monooxygenases. Hydrocarbons with longer chain lengths (≥ 18C) are catabolized through other *alkB* non-related hydroxylases (Wang and Shao 2013). For this reason, it is possible to assume, in a first instance, that all the studied *n*-alkanes (*n*C11, *n*C12, *n*C13, and *n*C14), and hence most of the Antarctic gasoil components, would be catabolized by the same enzymatic system. It is important to consider that *n*-alkane-degrading pathways present a strong regulation, only being active in the presence of the adequate

Table 3 Closest relative type strain (according to 16S rRNA gene sequence) for each isolate obtained from the 24-day subcultures using C11, C12, C13, and C14 *n*-alkanes as carbon source

Hydrocarbon	Closest relative type strain according to 16S rRNA gene sequence	Number of isolates
*n*C11	*Pseudomonas mandelii* CIP 105273 (T)	3
	Pseudomonas extremaustralis 14-3 (T)	4
	Sphingobacterium anhuiense CW 186 (T)	4
	Microbacterium oxydans DSM 20,578 (T)	1
*n*C12	*Janthinobacterium lividum* DSM 1522 (T)	1
	Stenotrophomonas rhizophila DSM 14405 (T)	2
	Pseudomonas yamanorum 8H1 (T)	2
*n*C13	*Janthinobacteriumlividum* DSM 1522 (T)	1
	Pseudomonas yamanorum 8H1 (T)	3
	Rhodococcus qingshengii JCM 15477(T)	1
*n*C14	*Rhodococcus erytropolis* NBRC 15,567 (T)	1
	Microbacterium oxydans DSM 20,578(T)	1
	Pseudochrobactrum kiredjianiae CCUG 49584(T)	1
	Pseudomonas yamanorum 8H1 (T)	1

hydrocarbon and in the absence of any preferred substrate (Rojo 2009). Under the culture conditions imposed on the soil bacterial community in the present work, both conditions were accomplished.

The divergent changes observed in bacterial communities from the different cultures, open several questions: how does microbial community change when a complex hydrocarbon mixture, such as gasoil, is spilled on a soil? How does it evolve when hydrocarbons are chronically spilled on a soil? Is this dynamic predictable? Does this community reach a stable structure at some point or changes continuously built on the previous states? If enzymes involved in alkane degradation are ubiquitous and similar among hydrocarbon degrading bacteria, differences observed in the community's structure could not be attributed to catabolic machinery. At this point, it is relevant to consider hydrocarbons uptake. Different alkanes could require a variety of uptake mechanisms involving different efficiencies which could favour one strain over others and, therefore, different community structures. In this sense, Hua & Wang (2014) reviewed evidence showing that different microorganisms have different affinities to hydrocarbons and bacterial associations could even modify the way in which each component of the association interacts with the hydrocarbon, in a process called functional complementation. These differences could be one of the driving forces of the divergent behaviour observed in the present work, especially considering that, although similar in structure, the evaluated alkanes show a low but different range of solubility in water (Table 1). Future studies, studying changes in these bacterial communities in the presence of surface-active compounds (surfactants), could help to understand if this biophysical factor contributes to the observed differences between cultures.

DGGE is considered a powerful tool for analysing changes in community structure. It has been proposed that band profiles reflect the number and relative abundances of the bacterial 16S rDNA amplicons present in the analysed sample after PCR. Each band could be linked to a single amplicon that represents a different bacterial population present in the original sample (Watanabe 2001). On the other hand, artefacts associated mainly with the presence of multiple bands for a unique strain call in question its utility as tools for the estimation of α-diversity based on band number or OTU abundance based on band intensity (Neilson et al. 2013). The fingerprint provided by this methodology allows the description of differences and similarities between communities. In this work, interesting observations emerged from the analysis of the number of bands obtained for each treatment. If the number of bands is related to the richness of the microbial community, it is possible to say that richness decreased significantly in all treatments at day 8, 16, and 24 compared to the original community (T0). This observation is in accordance with the idea pointed out by Erkus et al. (2013) who stated that strongly selective conditions reduce the microbial community diversity. In this case, the use of *n*-alkanes and a hydrocarbon mixture imposed selective (and slightly different) conditions.

On the other hand, isothermal incubation (15 °C) constitutes another selection factor. Bare surface soils in Antarctica are exposed, during summer, to a wide temperature range, extended from 0 °C or less to 15–20 °C during sunny days. This range involves drastic changes in availability of water and substrates. As was pointed by Pettersson and Baath (2003) temperature is a physical factor showing a significant influence in microbial communities. Such a range could be associated with the presence of microbial actors able to flourish at different temperatures. When a piece of this soil is taken and incubated isothermally, the selected temperature (15 °C in this case) exert an important selection pressure, allowing an increase in the number of microorganisms showing optimal growth temperature close to this incubation temperature and limiting the growth of others adapted to a different one.

The observed decrease in the numbers of DGGE bands (from 12 to 8) mentioned for the flasks containing the hydrocarbons-mixture could be interpreted as a kind of stability. However, deeper analysis showed that communities in the three cultures only shared 5 out of 8 bands, supporting the idea about progressive changes in this kind of cultures.

Appearances of bands in some profiles that were not detected at T0 could be explained mainly by the increase in the size of the populations of these strains, which were below the detection limit of the method at T0 but they grow enough to reach detectable levels in further enrichment cultures. A similar statement was made by Coats et al. (2007) working with sequencing batch bioreactors inoculated with different wastewaters. These authors also attributed the appearance of new bands in their DGGE analysis to the enrichment in populations previously undetected in the systems and argue as possible additional factors the bacterial contamination and/or provision of cells with the feedstock used. However, in this work, no feeding was used, and all the culture medium components were previously sterilized, the enrichment of selected populations being the most plausible cause of the observation.

Dendrogram from DGGE gels showed that the experimental systems were mainly differentiated by set, each one comprising the three successive subcultures, suggesting that changes were mainly driven by the elapsed time of culture. However, within each set, the differences between cultures with different substrates, although slower, were also evident. Thus, the similarity between samples in Set 1 and Set 2 was comparable (approximately 80%), but in Set 3 differences increased significantly, with the C13 and C14 cultures showing the most differentiable band patterns, whereas C11, C12, and Mix were grouped with 75% similarity. These results

seem to suggest that the use of different carbon sources leads to differences in microbial communities. In a similar work reported by Jiao et al. (2016), the authors clearly showed that microbial communities from a single soil sample used as inoculum in batch culture, gradually changed during successive subcultures depending on the carbon source, evidencing the influence of contaminants on microbial succession. Although the mentioned work used hydrocarbons having great differences in its chemical structure (C18, phenanthrene and the mixture of both, also combining them with the presence of a heavy metal such as Cd) as carbon source, it was clearly established that the differences in the substrates result in a strong selection pressure for the microorganisms of the community. In our work, the studied substrates were *n*-alkanes that differ only in one carbon atom. As far as we know, there are no previous works exploring the differences in the bacterial communities induced by this set of *n*-alkanes. However, it is important to note that in a study where the degradation capacity of *n*-alkanes was studied using a bacterial strain isolated from contaminated soil (*Pseudomonas aeruginosa* MGP-1), Salgado-Brito et al. (2007) reported that the growth rate and degradation capacity of this strain were optimal with the *n*-alkane of C20, and decreased when the length of the carbon chain was larger o shorter. These authors clearly show that growth rate of the MGP-1 strain was notoriously low with C11 and gradually increases with C12, C13 and C14. Although attempting to make a strict comparison between the two trials is not our aim, if some of the members of the soil community of Carlini station present a behaviour like that shown byMGP-1 strain, these differences in the ability to degrade the *n*-alkanes of C11 to C-14 could clearly lead to the observed differentiation of the cultures. As in the different systems those components exhibiting a higher growth rate with the available substrate would predominate, it could cause the divergence of bacterial community's structure developing in each culture, even though substrates differed only in a C atom of their chain.

In this study, the existence of a mixture of populations instead of a single bacterial strain could constitute an advantage for biotechnological processes. For example, undefined cultures composed of a mixture of strains used in dairy food processes, such as yogurt and cheese elaboration, are more resilient and display a more robust performance when compared with defined low-strain-diversity cultures (de Vos 2011). For bioremediation, the use of microbial consortia is also considered advantageous, especially when contaminants are complex mixtures of compounds, such as crude oil and gasoil. For the soil used in this study, the use of bioaugmentation as a second stage after a previous biostimulation strategy would be useful for the removal of the remnant hydrocarbon fraction. Such strategy should involve the addition of a significant number of microorganisms targeting specifically the C11–C14 alkanes. Other approaches, such as surfactant addition or the introduction of hydrocarbon-tolerant plants to increase pollutant removal, could be effective and deserve specific experimentation.

In conclusion, we found that the structure of the bacterial communities from hydrocarbon contaminated Antarctic soils previously treated by biostimulation follows a divergent evolution when C11 to C14 *n*-alkanes are provided as sole C and energy source. In our opinion, this work opens the way to deeper studies dealing with the influence of the hydrocarbon chemical structure on the microbial community composition and hence, on the performance of bioremediation processes applied to these extremely cold soils.

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Compliance with ethical standards

Conflict of interest The authors declare that they have conflict of interest.

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