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Bioremediation of oily hypersaline soil via autochthonous bioaugmentation with halophilic bacteria and archaea

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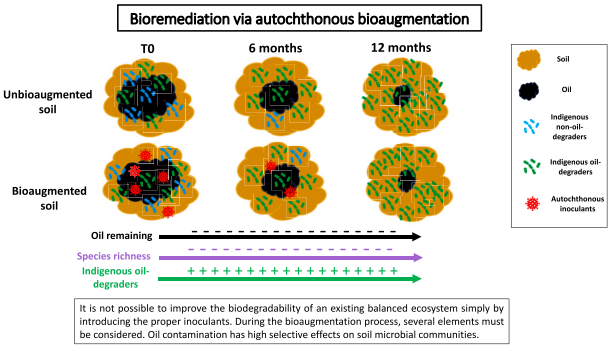
HIGHLIGHTS

- The microbial inoculants couldn't survive till the end of the bioremediation process.
- Oil hydrocarbons were attenuated in all samples including the unbioaugmented ones.
- Oil contamination has high selective effects on soil microbial communities.
- Autochthonous bioaugmentation does not significantly affect oil bioremediation.

GRAPHICAL ABSTRACT



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ABSTRACT

Kuwaiti hypersaline soil samples were contaminated with 5 % (*w*/w) weathered Kuwaiti light crude oil and bioaugmented with autochthonous halophilic hydrocarbonoclastic archaeal and bacterial strains, two each, individually and as consortia. Residual oil contents were determined, and microbial communities were analyzed by culture-dependent and culture-independent approaches initially and seasonally for one year. After one year of the bioremediation process, the mean oil degradation rate was similar across all treated soils including the controlled unbioaugmented one. Oil hydrocarbons were drastically reduced in all soil samples with values ranging from 82.7 % to 93 %. During the bioremediation process, the number of culturable oil-degrading bacteria increased to a range of 142 to 344 CFUx10^4 g^{-1} after 12 months of bioaugmentation. Although culture-independent analysis showed a high proportion of inoculants initially, none could be cultured throughout the bioremediation procedure. Within a year, microbial communities changed continually, and 33 species of halotolerant/halophilic hydrocarbonoclastic bacteria were isolated and identified belonged mainly to the three major bacterial phyla Actinobacteria, Proteobacteria, and Firmicutes. The archaeal phylum Halobacterota represented <1 % of the microbial community's relative abundance, which explains why none of its members were cultured. Improving the biodegradability of an already balanced environment by autochthonous bioaugmentation is more involved than just adding the proper oil degraders. This study emphasizes the possibility of

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a relatively large resistant population, a greater diversity of oil-degrading microorganisms, and the highly selective impacts of oil contamination on hypersaline soil bacterial communities.

1. Introduction

Oil pollution has become a significant global issue with negative implications, particularly in oil-producing countries such as Kuwait (Asif et al., 2022; Al-Dahanii et al., 2015). Industrial practices such as extraction, drilling, fracking, storing, and exporting cause pollution in such countries. During the Gulf War of 1991, Kuwait undoubtedly experienced the greatest oil slick in history, which exacerbated the pollution. Hydrocarbon pollution harmed terrestrial, aquatic, and atmospheric habitats, as well as hypersaline environments. This intensified the need to develop more effective remediation methods for cleaning contaminated areas.

Many physicochemical techniques, such as skimmers, dispersants, oil booms, sorbents, incineration, and landfilling, have been globally employed to remediate contaminated environments. However, these approaches have many drawbacks, including their high cost and ability to produce new pollution (Cordes et al., 2016; Ugwoha and Omenogor, 2017; Zamparas et al., 2020; Asif et al., 2022). In contrast, bioremediation is widely regarded as a more cost-effective, environmentally safe, and sustainable approach for removing spilled oil by utilizing naturally occurring hydrocarbonoclastic microorganisms in decomposing oil and hydrocarbon contaminants (Koshlaf and Ball, 2017; Zhang et al., 2019; Nwankwegu et al., 2022). Introducing exogenous hydrocarbonoclastic microorganisms into the polluted site in a process called bioaugmentation is considered a method of bioremediation, in which the newly introduced microorganisms must endure the stress of competition with the native microorganisms (Radwan, 1990; Nwankwegu et al., 2022). Thus, autochthonous bioaugmentation (ABA), in which only species native to the polluted location are used as inoculants, is an effective bioremediation strategy (Nikopoulou et al., 2013; DiGregorio et al., 2015). Autochthonous microorganisms are those who are ideally acclimated to their surroundings and hence contribute significantly to biochemical activity there.

In addition to being a country prone to oil pollution, Kuwait is geographically located in the semiarid region and has harsh climates. As a result, hypersaline areas with NaCl concentrations more than those of seawater are formed when coastal seawaters are trapped during tide movement and excessively evaporated throughout the long dry summer. When such areas are subjected to oil spills, like elsewhere (Dastgheib et al., 2012), only halophilic/halotolerant microorganisms that are adapted to extreme conditions will be effective in the bioremediating process (Madueno et al., 2014). Many studies on hydrocarbon biodegradation in high-salinity environments have been published over the last two decades and many indigenous hydrocarbonoclastic halophilic bacteria and archaea have been isolated (Zhao et al., 2009; Bonfa et al., 2011; Fathepure, 2014; Edbeib et al., 2016; Abou et al., 2021). During the past ten years, our research group has investigated the use of such microorganisms for bioremediation purposes. We brought experimental evidence that the addition of monovalent, divalent, and trivalent cations as osmoregulators (Al-Mailem et al., 2013, 2017), vitamins (Al-Mailem et al., 2014), ferric sulfate and proline (Al-Mailem et al., 2018) biostimulated oil removal in hypersaline soil and water samples. Within this context, our group reported on combined physical and microbiological approaches for removing oil by gelatinizing it in seawater (Radwan et al., 2017). Those latter studies were exclusively on biostimulation but not bioaugmentation. So far, no investigations on bioaugmentation as a technique for oil bioremediation in hypersaline environments have been conducted on a global or local scale, except the study of our group about cross-bioaugmentation among two Kuwaiti hypersaline soils, which was published recently (Kansour and Al-Mailem, 2023). Therefore, the goal of the current study was to help fill this information gap. Autochthonous hydrocarbonoclastic halophilic bacteria and archaea, which had been isolated from local hypersaline soil in our laboratory, were used for autochthonous bioaugmentation of oil-contaminated hypersaline soil samples. These microorganisms were studied individually and as consortia for their oil-degradation potential and their ability to colonize and propagate in the host soils during the bioremediation process.

2. Materials and methods

2.1. Soil sampling

Hypersaline soil samples were collected from the southern Kuwait coast of the Arabian Gulf in autumn in sterile plastic bags and transported to the laboratory to be processed on the same day. Some environmental parameters were measured in the field: pH using a pH meter (HANNA, USA), moisture, and temperature using a soil moisture kit (ML3 ThetaProbe, UK). Other chemical parameters were determined in the laboratory using an ion chromatograph model 850 Professional IC (Metrohm, Switzerland) for anions determination, and a CHNS-elemental analyzer (Elementar Unicube, Germany) for other elements.

2.2. Microbial cultures and experimental setup

Two hydrocarbonoclastic halophilic archaeal strains, *Halobacterium salinarum,* and *Haloferax elongans*; and two hydrocarbonoclastic halophilic bacterial strains *Halomonas axialensis* and *Marinobacter algicola* were used for the autochthonous bioaugmentation process. The strains were isolated earlier from the same hypersaline soil, identified by sequencing their 16S rRNA-coding genes, and deposited in our private culture collection.

The four microbial strains were chosen as inoculants according to their satisfactory oil-degradation capacity. Quantitative measurements of oil-degradation potential for the pure cultures revealed that the halophilic archaeal strains, *Halobacterium salinarum,* and *Haloferax elongans* could biodegrade crude oil up to 35 and 39 %, respectively and the halophilic bacterial strains *Halomonas axialensis* and *Marinobacter algicola* could biodegrade crude oil up to 43 and 44 %, respectively (Al-Mailem et al., 2010, 2017).

The pristine hypersaline soil samples were freshly collected and artificially polluted with 5 % (*w*/w) weathered Kuwaiti light crude oil (from the National Kuwait Company). The oil was thoroughly and evenly mixed with the soil before they were dispensed into 6 wooden boxes, 40 × 40 cm, to give 20 cm deep beds. One bed was not subjected to any further treatment to serve as a control. Four beds of the hypersaline soil were bioaugmented with the four individually halophilic microorganisms and the last bed was bioaugmented with a mixture of the four halophilic microorganisms.

For inoculants, the halophilic archaeal and bacterial strains were allowed first to propagate in Mevarech and Werzcberger (1985) liquid medium and halophilic broth (HIMEDIA, India), respectively. Archaeal and bacterial culture volumes containing 10^9 cells per Kg of soil (counted microscopically) were thoroughly and evenly mixed into each soil bed. The consortium soil core was receiving one-fourth of those volumes from the four individual cultures. The soil beds were incubated for a whole year in a protected area of the Botanical Garden, Faculty of Science, exposed to the open environment. They were irrigated with equal amounts of distilled water when needed depending on the season. Expectedly more frequent irrigation was needed during the long, hot summer. Residual oil contents were determined, and microbial communities were analyzed at the beginning of the experiment and

seasonally by harvesting three replicates from each bed.

2.3. Recovery and analysis of residual hydrocarbons

Triplicate amounts (5 g) of soil were harvested from each bed. The residual oil was recovered by extraction with three successive portions of 15 mL methylene chloride. Extracts were then combined, and the volume was raised to 50 mL with methylene chloride. The extracts were passed through a column of anhydrous sodium sulfate for dryness and evaporated by nitrogen gas to approximately 2 mL, with care not to evaporate to dryness. Subsequently, 1 μL was analyzed by Gas–Liquid Chromatography (GLC) using an Agilent 7890 A GLC (USA) system equipped with FID, a DB-5 capillary column (Agilent Technologies, USA), and He as a carrier gas. The oven temperature started at 50 °C for 3 min, then rose at 3 °C/min to 80 °C, then rose at 8 °C/min to 256 °C, then rose at 30 °C/min to 330 °C and held at this temperature for 11 min. The percentage of hydrocarbon consumption was calculated from the total peak-area reduction in the samples based on the peak areas of the controls (initial time in each beds). As a conserved internal standard, hopane was used to evaluate the consumption of hydrocarbons (Prince et al., 1994).

2.4. Culture-dependent analysis of hydrocarbonoclastic microorganisms

The dilution-plating method was used to count and isolate hydrocarbonoclastic microorganisms. For halophilic bacteria, mineral media described by Sorkhoh et al. (1990) was used, and for halophilic archaea, we used the medium suggested by Mevarech and Werzcberger (1985). The NaCl concentrations in those media were adjusted to 1.5 and 2.0 M, respectively. The sole carbon and energy source was oil vapor volatilizing from plate-lid-fixed filter papers infused with 2 mL of crude oil. Initially and at 3-month intervals, three replicate soil samples were taken from each soil bed to prepare a series of dilutions (10^{-1}–10^{-5}). Triplicate plates of both media were inoculated with 0.1 mL aliquots of common sample dilutions. The plates were sealed and incubated at 30 °C for bacteria, and 37 °C for archaea, for 12 d. The colony forming units (CFU) were counted, and the numbers per g of soil were calculated. Using the gram stain, colonies with identical appearance and cell morphology were enumerated, and representative colonies for each type were isolated and purified.

For isolate identification, their 16S rRNA genes were sequenced, and the sequences were compared with those of type strains in GenBank. The total genomic DNA of each isolate was extracted by homogenizing 300 mg of the fresh 48-h bacterial biomass in 100 μL of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA) and 200 μL molecular water (Sigma, UK). The mixture was then incubated in a water bath for 10 min at 100 °C, cooled for 2 min, and then centrifuged at 14,000 ×*g* for 3 min to collect the DNA-containing supernatant. The polymerase chain reaction (PCR) was used to amplify the 16S rRNA genes. For halophilic bacteria, the reaction mixture contained puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK), 1 μL (25 ng) of DNA template, and 1 μL each of the universal primer combinations of the forward primer, GM5F (5′-CCTACGGGAGGCAGCAG-3′) and the reverse primer, 907 R (5′-CCGTCAATTCMTTTGAGTTT-3′) (Santegoeds et al., 1998). For halophilic archaea, archaeal primer combinations 0018F (5′-ATTCCGGTTGAGCC TGCC-3′) and 1518R (5′-AGGAGGTGAGC CAGCCGC-3′) (Cui et al., 2009) were used. The reaction volume was made up to 25 μL with molecular water. Amplification was done in a Veriti Thermal Cycler (Applied Biosystems, USA) by touch-down PCR in which the initial denaturation was at 95 °C for 5 min, and the annealing temperature started at 65 °C and decreased by 1 °C every cycle to 55 °C; 15 additional cycles were carried out at this temperature. The PCR products were purified using a QIA quick PCR purification kit (Qiagen, USA) to remove the Taq polymerase, primers, and dNTPs. Partial sequencing of the 16S rRNA-gene was done using a BigDye version Terminator Kit (Applied Biosystems, USA); 20 ng of the DNA template was added to 2 μL of a Big Dye v 3.1 terminator, and 2 μL of Big Dye Terminator v 1.1, v 3.1 5× sequencing buffer; l μL of either 907 R or GM5F was added to the mixture in case of halophilic bacteria and l μL of either 0018F or 1518R in case of halophilic archaea, and the final volume was brought up to 10 μL with molecular water. Labeling was completed in a Veriti Thermal Cycler (Applied Biosystems, USA) using one cycle of 96 °C for l min, then 25 cycles of l min at 96 °C, 5 s at 50 °C and 4 min at 60 °C. The pure template DNA samples were processed in a 3130xl genetic analyzer (Applied Biosystems, USA). Sequencing analysis (SeqA 7 software) was used to analyze the results. Sequences were subjected to basic local alignment search tool analysis with the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) GenBank database (Altschul et al., 1997).

2.5. Culture-independent analysis of total microbial populations and bioinformatics

Three replicate soil samples were taken from each soil bed initially and every 3 months to molecularly study the bacterial and archaeal communities inhabiting soil samples during one year of bioaugmentation. Following the manufacturer's instructions, FastDNA SPIN Kit for Soil (MP, USA) was used to extract the total genomic DNA of the soil samples. DNA extracts of each site were pooled, and the purity of the DNA was checked using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific) and for quantification, a Qubit 2.0 fluorometer (Thermo Fisher Scientific) was used. DNA samples were analyzed by Next-Generation Sequencing (NGS) using Illumina MiSeq chemistry, following the manufacturer's protocols at Mr. DNA (MRDNA, Molecular Research LP, Shallowater TX, USA) and 16S primers and data processing protocols as previously described (Archer et al., 2019, 2023). Briefly, libraries were processed with cutadapt v2.7 (Martin, 2011), ASVs were generated with dada2 v1.14 (Callahan et al., 2016), and taxonomic classification was done with the SILVA v138 database (Quast et al., 2013). ASV data statistics and visualization were conducted using the R package phyloseq and ggplot2 respectively (McMurdie and Holmes, 2013; Wickham, 2016).

3. Results and discussion

3.1. Hydrocarbon consumption during bioremediation

The data shown in Table 1 imply that halophilic archaea and bacteria have the capacity to bioremediate oil in situ given appropriate environmental circumstances, including pH, salinity, moisture, and nitrogen levels. During the bioremediation period, the oil hydrocarbons were significantly attenuated in all soil samples (ANOVA, $n = 3$, $P < 0.05$), including the unbioaugmented (control) sample (Fig. 1). After three months of bioremediation, the proportions of the removed oil ranged between 37.2 %, in the soil, which was bioaugmented with a mixture of 4 strains, and 48.7 % in the soil which was not bioaugmented (control). A period of 3 months could be enough time for normal (not hypersaline) soil to be bioremediated, but it is considered a short time to

Table 1
Environmental parameters of the soil samples.

pH	8.2
Temperature	34.7 °C
Moisture	0.4 %
C%	1.38 % ±0.46
H%	0.14 % ±0.010
N%	0.53 % ±0.03
S%	0.06 % ±0.005
Fluoride (mg/kg)	0.7 ± 0.06
Chloride (mg/kg)	132.8 ± 1.3
Bromide (mg/kg)	4.5 ± 0.01
Nitrate (mg/kg)	5.1 ± 0.02
Sulphate (mg/kg)	70.5 ± 0.06

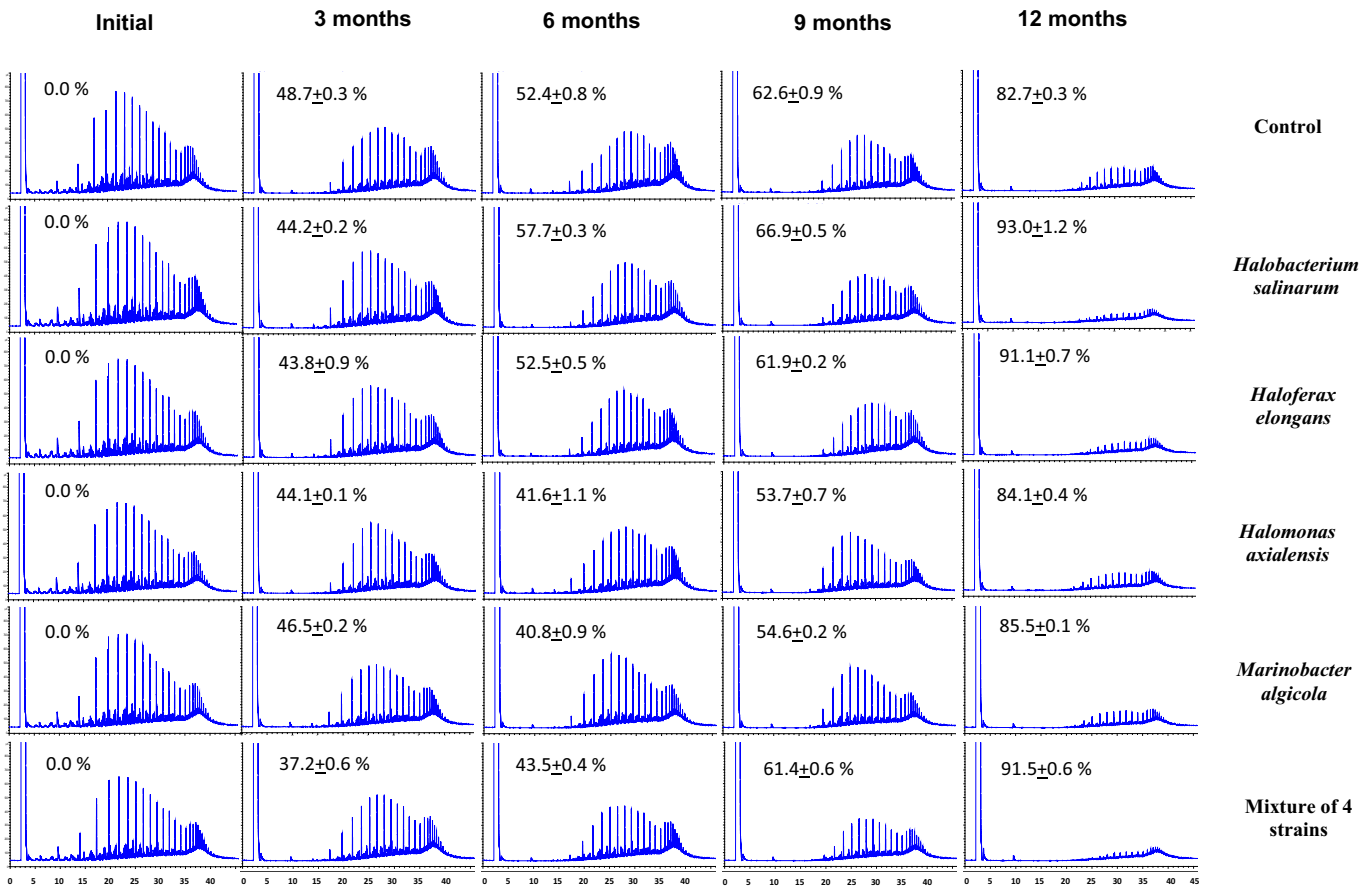

Fig. 1. Typical GLC profiles of residual oil in the bioremediated hypersaline soil. Smaller peaks indicate more oil consumption. Values on the individual profiles are those of the oil-consumption values; they were means of 3 replicates, ± standard deviation values.

bioremediate a contaminated hypersaline soil since high salinity slowed hydrocarbon biodegradation by decreasing oxygen dissolution and hydrocarbon solubility (Ward and Brock, 1978). Chemically, crude oil is a highly complex mixture of organic materials comprising thousands of chemical compounds. Saturated hydrocarbons, including normal alkanes with chains of up to 44 and more carbon atoms, branched alkanes, and cycloalkanes, are the major constituents of the crude, making up between 40 and 60 % of the total weight (Leahy and Colwell, 1990). The majority of hydrocarbonoclastic microorganisms are more capable of degrading hydrocarbon fractions with low molecular weight such as n-alkanes than higher molecular weight hydrocarbons (Agarwal and Liu, 2015; Nwankwegu et al., 2016), which explains the high rate of oil degradation within the first three months of the bioremediation process.

Oil degradation persisted in the sixth and ninth months, albeit slowly, due to the decline in bacterial numbers, as will be detailed later, and lasted until the conclusion of the bioremediation period, at which point the proportions of the consumed oil ranged between 82.7 % in the control. soil and 93 % in the soil which was bioaugmented with *Halobacterium salinarum*. Statistically, from the ANOVA test, after one year of the bioremediation process, the mean oil degradation rate was similar across the six different treatments ($P = 0.9963$) (StataIC 16), indicating that there is no statistically significant difference in oil-degradation rates between unbioaugmented and bioaugmented soils (StataIC 16), although the bacterial strains used as inoculants were isolated from the same tested soil previously. It seems that autochthonous halotolerant/halophilic bacteria that already exist in the soil, rather than inoculants, are playing an important role in the oil degradation process, although the latter have satisfactory oil-degradation capacity, and the already balanced ecosystem is not able to be improved via further additions. These results support the importance of natural attenuation, a biological mechanism that reduces the hazardous percentage of contaminants as quickly as feasible after a fresh hydrocarbon spill (Nwankwegu et al., 2022).

There is still much debate over the usefulness of bioaugmentation in addressing environmental oil spills. Some authors reported that inoculating proper microorganisms into a site is not a guarantee of successful pollutant removal (El Fantroussi and Agathos, 2005), or is associated with the inconsistency of the bioremediation results (Al-Mailem et al., 2019; Kansour and Al-Mailem, 2023). On the other hand, it was reported that bioaugmentation over the years has been proven effective, fast, and affordable greener cleanup option (Chen et al., 2015; Zabbey et al., 2017). Several aspects must be considered during the bioaugmentation process, some of which are biotic, such as competition between inoculates and indigenous microorganisms, and others that are abiotic, such as pH and temperature.

3.2. Culture dependent analysis

3.2.1. Numbers of culturable hydrocarbonoclastic bacteria during bioremediation

The number of culturable oil-degrading bacteria increased during the bioremediation process (Fig. 2). As the hydrocarbon degradation mechanism is complex, allochthonous diversity often requires a long period of adaptation before utilizing the contaminants as the sole source of energy and carbon when a pristine ecosystem is freshly contaminated with oil (Nwankwegu et al., 2022). This explains the low number of halotolerant/halophilic hydrocarbonoclastic bacteria in all treated soil samples at initial, which ranged between 21 and 43 CFUx10^4 g^{-1}. The number of bacteria continued to rise in the third and sixth months of bioremediation to reach a range of 52 to 610 CFUx10^4 g^{-1} before

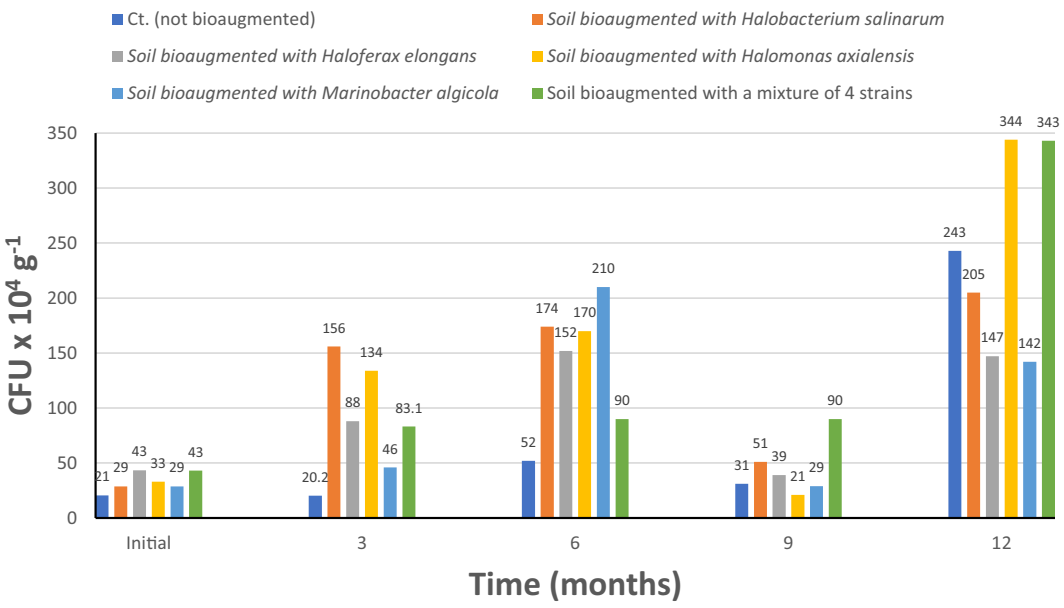

Fig. 2. Numbers of cultivable oil-utilizing bacteria in the unbioaugmented and bioaugmented hypersaline soil samples during one year of the bioremediation process.

dropping dramatically in the ninth month. As was previously noted, the experiment began in the autumn early October, therefore the ninth month of the study was in the summer (July), when the temperature in Kuwait may exceed 55 °C. At high temperatures (over 40 °C), oxygen solubility declines, as does aerobic bacteria's metabolic activity; the membrane becomes more sensitive to hydrocarbon toxicity (Sihag et al., 2014; Ferreira et al., 2012) and consequently reduces bacterial numbers. When the temperature began to drop near the end of the bioremediation process in October, bacterial counts increased again, ranging from 142 to 344 CFUx10^4 g^{-1}.

3.2.2. *Dynamics of culturable bacterial communities during bioremediation*

Although it is known that the culture-dependent strategy using oil vapor as the sole carbon source captures just a small portion of the total microbial community, it has the valuable advantage of capturing exclusively hydrocarbonoclastic microorganisms. As a result, the isolates described in this experiment should be regarded as the most prominent representatives of the culturable hydrocarbonoclastic bacterial communities in the treated soils. Even though the bacterial and archaeal strains employed as inoculants were autochthonous and isolated from the same soil previously, none of them could be cultivated from the bioaugmented soil during the whole year of the bioremediation process (Fig. 3). This was related to these microorganisms' inability to

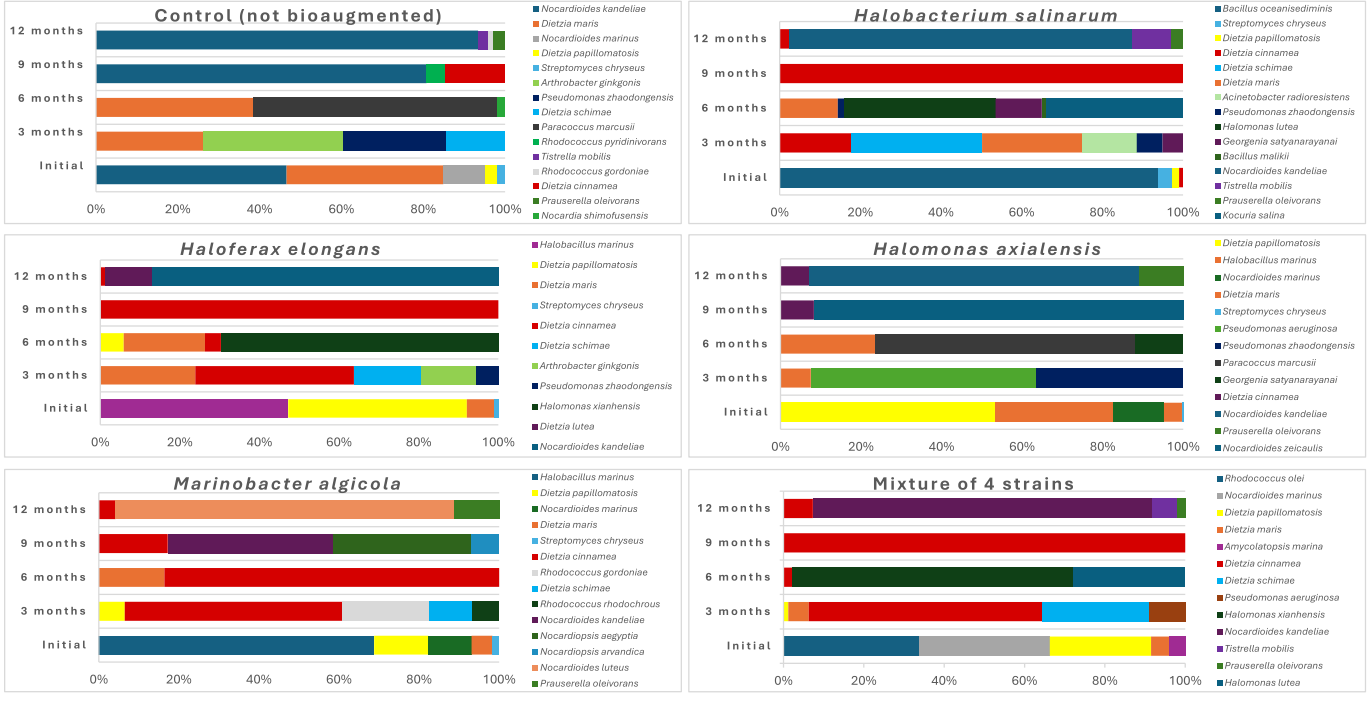

Fig. 3. Dynamics of hydrocarbonoclastic bacterial communities in the treated hypersaline soil samples during 1 year of bioremediation.

compete with already existing ones consequently, their limited number, which makes them undetectable by culture-dependent methods. The bacterial communities are continuously changing, as this text will describe later, which explains why autochthonous inoculants compete to survive with new members who did not previously exist. In addition, many other factors could affect the growth and propagation of the introduced microorganisms e.g., hydrocarbon and soil characteristics, nutrient availability, temperature, moisture availability, pH, and salinity (Kebede et al., 2021).

In total, 33 species of halotolerant/halophilic hydrocarbonoclastic bacteria were isolated from the unbioaugmented and bioaugmented soil samples. They belonged mainly to the following genera arranged in decreasing order of species numbers: five *Dietzia*, four each of *Nocardioides* and *Rhodococcus*, three *Pseudomonas*, two each of *Bacillus*, *Halomonas*, and *Nocardiopsis* spp. Other genera were presented by one species, each (Table 2). Different ratios of the three major bacterial phyla Actinobacteria, Proteobacteria, and Firmicutes represented the cultured soil communities.

Scanning the pie charts in Fig. 3 shows several interesting observations. At the beginning of the bioremediation process (T0), Firmicutes members *Bacillus oceanisediminis* and *Halobacillus marinus* were predominant in most of the treated soil samples. Proteobacteria first appeared in bacterial communities between the third and sixth months. *Acinetobacter radioresistens*, *Pseudomonas aeruginosa,* and *Pseudomonas zhaodongensis* which belonged to Gammaproteobacteria were isolated after three months of bioremediation. More members of Gammaproteobacteria were cultured in the sixth month e.g., *Halomonas lutea* and *Halomonas xianhensis* in addition to *Paracoccus marcusii* which belongs to Alphaproteobacteria.

Another important point to be addressed is that Actinobacteria members were predominant in the second half of the bioremediation process. In the ninth month, only Actinobacteria, primarily *Dietzia cinnamea* and *Nocardioides* spp., could be cultured. Actinobacteria predominancy continued till the end of the bioremediation period with a sweep of *Nocardioides* spp. and a modest presence of *Tistrella mobilis* which is one of Alphaproteobacteria members. It has been reported that various genes encoding many aromatic compound degradations have been identified in the genera, Nocardioides (Lu et al., 2011). This explains why this group of bacteria predominated at the end of the bioremediation process where, as well known, high molecular weight hydrocarbons usually remain.

The data in Fig. 3 show continuous changes in the microbial communities over time. According to research, when hydrocarbon pollutants are introduced into the soil environment, bacterial communities fluctuate and certain bacterial species experience immediate and significant declines, while others become more abundant (Omrani et al., 2018). Thus, the diversity, richness, and evenness of the microbial community change, which affects the metabolic activity of hydrocarbon-degrading bacteria (Wu et al., 2017). These changes are continuous and strongly tied to the types and nature of hydrocarbon pollutants, as well as environmental conditions (Xu et al., 2018). The presence of hydrocarbon contaminants in the soil will result in the formation of appropriate consortia composed of several bacterial strains capable of metabolizing a wide spectrum of pollutants. This is due to the fact that bacterial consortiums have broad degradative enzymes, degrade different hydrocarbon components, have a higher tolerance to physicochemical parameters (temperature, pH, and salinity), and have a higher rate of biosurfactants synthesis (Hamzah et al., 2017; Moliterni et al., 2012).

During the whole year of bioremediation, no archaeal strains, including those used as inoculants, could be cultivated. The reason for this could be that, as previously indicated, the culture-dependent technique can only identify a tiny fraction of the microbial community, and there is a paucity of these extreme microorganisms.

The 33 halotolerant/halophilic hydrocarbonoclastic bacterial strains that had been isolated in this study are listed in Table 2 which includes data related to the sequencing of their 16S rDNA and their accession

Table 2
Information related to 16S rRNA gene sequencing of the halotolerant/halophilic bacterial isolates from treated hypersaline soil samples.

Isolate no.	Total bases	Phyla	Nearest GenBank match	Similarity %	Bases compared	GenBank accession no.
1	546	Gammaproteobacteria	*Acinetobacter radioresistens*	100	546/546	OR674004
2	532	Actinobacteria	*Amycolatopsis marina*	99	534/535	OR674005
3	536	Actinobacteria	*Arthrobacter ginkgonis*	100	536/536	OR674006
4	551	Bacilli	*Bacillus malikii*	100	551/551	OR674007
5	548	Bacilli	*Bacillus oceanisediminis*	100	548/548	OR674008
6	534	Actinobacteria	*Dietzia cinnamea*	100	534/534	OR674009
7	529	Actinobacteria	*Dietzia lutea*	100	529/529	OR674010
8	534	Actinobacteria	*Dietzia maris*	100	534/534	OR674011
9	535	Actinobacteria	*Dietzia papillomatosis*	100	535/535	OR674012
10	532	Actinobacteria	*Dietzia schimae*	100	532/532	OR674013
11	545	Actinobacteria	*Georgenia satyanarayanai*	100	545/545	OR674014
12	546	Bacilli	*Halobacillus marinus*	100	546/546	OR674015
13	552	Gammaproteobacteria	*Halomonas lutea*	100	552/552	OR674016
14	547	Gammaproteobacteria	*Halomonas xianhensis*	100	547/547	OR674017
15	528	Actinobacteria	*Kocuria salina*	100	528/528	OR674018
16	537	Actinobacteria	*Nocardia shimofusensis*	100	537/537	OR674019
17	535	Actinobacteria	*Nocardioides kandeliae*	100	535/535	OR674020
18	523	Actinobacteria	*Nocardioides luteus*	100	523/523	OR674021
19	532	Actinobacteria	*Nocardioides marinus*	100	532/532	OR674022
20	516	Actinobacteria	*Nocardioides zeicaulis*	99	528/534	OR674023
21	537	Actinobacteria	*Nocardiopsis aegyptia*	100	537/537	OR674024
22	537	Actinobacteria	*Nocardiopsis arvandica*	100	537/537	OR674025
23	522	Alphaproteobacteria	*Paracoccus marcusii*	100	522/522	OR674026
24	533	Actinobacteria	*Prauserella oleivorans*	100	533/533	OR674027
25	547	Gammaproteobacteria	*Pseudomonas aeruginosa*	100	547/547	OR674028
26	545	Gammaproteobacteria	*Pseudomonas stutzeri*	100	545/545	OR674029
27	559	Gammaproteobacteria	*Pseudomonas zhaodongensis*	100	559/559	OR674030
28	532	Actinobacteria	*Rhodococcus gordoniae*	100	532/532	OR674031
29	529	Actinobacteria	*Rhodococcus olei*	99	531/532	OR674032
30	537	Actinobacteria	*Rhodococcus pyridinivorans*	100	537/537	OR674033
31	534	Actinobacteria	*Rhodococcus rhodochrous*	100	534/534	OR674034
32	535	Actinobacteria	*Streptomyces chryseus*	100	535/535	OR674035
33	449	Alphaproteobacteria	*Tistrella mobilis*	99	451/452	OR674036

numbers in GenBank. The table also shows that the sequence similarities of all strains to those of the type strains were between 99 and 100 %.

3.3. Culture-independent analysis

The relative abundance of inoculant strains represented a high proportion of the corresponding experimental soils at T0, however, decreased to very low relative abundances by the 12-month mark (Fig. 4). Given that no culturable inoculants were identified throughout the experiment (Fig. 3), this would indicate that the inoculants quickly perished and were degraded by resident microorganisms in the soil. Despite the selection of autochthonous isolates for this purpose, the challenges of applying lab-grown inoculants to environmental conditions are well-known (El Fantroussi and Agathos, 2005).

Interestingly, the amplicon-based Chao1 estimates generally decreased with time, contrasting the increasing CFU identified (Fig. 5). This would indicate that a small number of hydrocarbonoclastic taxa increased on the substrate, outcompeting much of the resident microbial diversity and an ongoing negative effect of oil toxicity. However, Shannon index values of the communities showed that the diversities at 6 and 12 months were roughly the same (or even increased at 12 months). The difference between richness and diversity trends suggests while the number of ASVs present decreased throughout the experiment period ($0 > 6 > 12$ months), the communities at 12 months are more even perhaps reaching an equilibrium and thus influencing the diversity values ($0 > 6 \mid 12$ months).

Further investigation of the major phylum identified in the amplicon results shows a dominance of Proteobacteria (Fig. 6). This varies from the cultivation effort which selected for hydrocarbonoclastic taxa, while the amplicon results represent the total resident community indicating that numerous Proteobacteria taxa are at least oil resilient in the community, even if they do not survive directly off the substrate. Some Pseudomonas taxa, genera of Proteobacteria, were identified in cultivation efforts as proportionately minor, however, due to their known polycyclic aromatic hydrocarbon-degrading capacity they may have represented a larger proportion than previously thought. This aligns with previous work that has shown a dominance of Proteobacteria in oil-contaminated and controlled desert soils. The presence of the Archaeal phylum Halobacterota in bioaugmented soils where this was included but lacking in other samples indicates they naturally represent <1 % relative abundance of the microbial community. The sudden increase in Chloroflexi at 12 months may be a result of the community's recovery as the oil concentration has dropped below toxic levels. Taken together, the culture-independent results confirm the highly selective effects of oil contamination on soil bacterial communities and highlight the potential for a relatively extensive resistant population or a larger diversity of oil-degrading microorganisms than first thought.

4. Conclusions

Autochthonous bioaugmentation does not significantly affect oil bioremediation of contaminated hypersaline soils. It has been experimentally proven by both culture-dependent and culture-independent approaches that autochthonous inoculants could not survive and propagate in the inoculated soil samples. It is not possible to improve the biodegradability of an already balanced environment by simply introducing the appropriate microorganisms. Several biotic and abiotic factors must be considered during the bioaugmentation process.

CRediT authorship contribution statement

Kevin C. Lee: Software, Formal analysis, Data curation. **Stephen D. J. Archer:** Writing – review & editing, Formal analysis. **Mayada K. Kansour:** Visualization, Resources, Investigation, Data curation. **Dina M. Al-Mailem:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

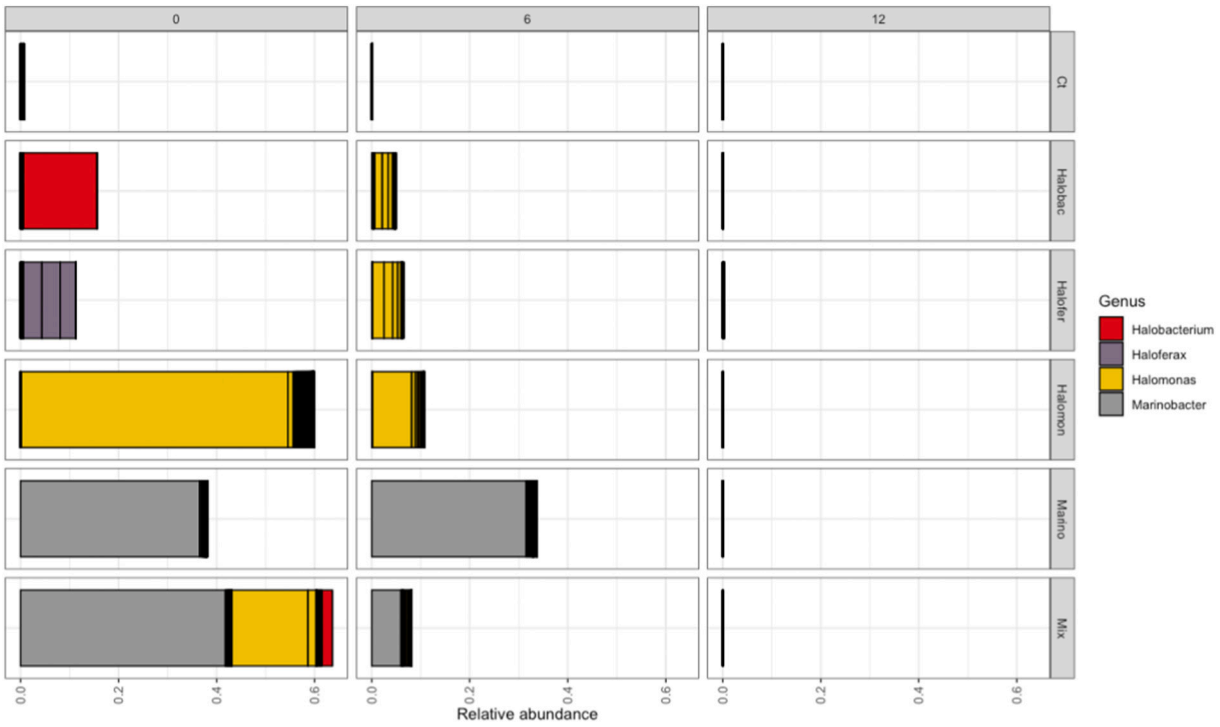

Fig. 4. Proportion of bacterial diversity belonging to the same genera as the four introduced species. The bars are arranged to represent treatment (y) and incubation time (x). Relative abundance here represents the fraction of each taxon in relation to the total counts of the sample (adds up to 1).

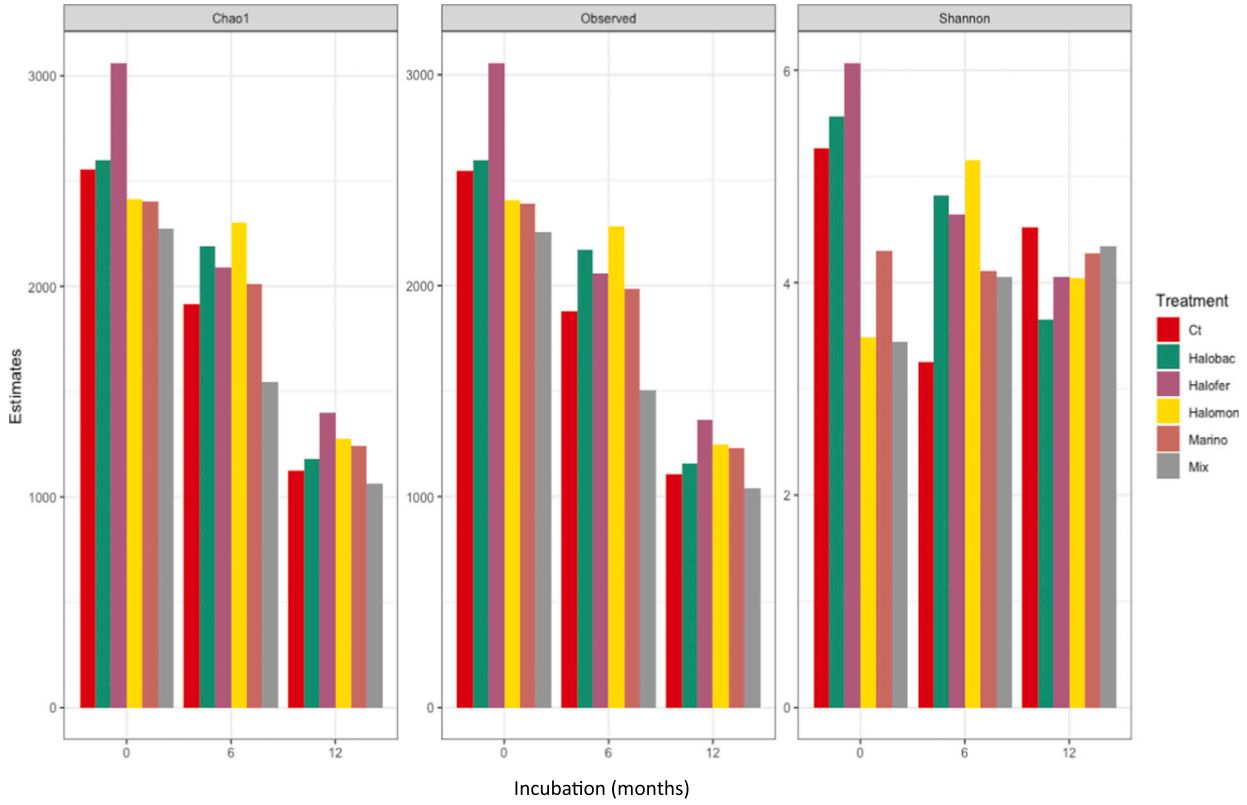

Fig. 5. Species richness and diversity in relation to incubation time and treatments.

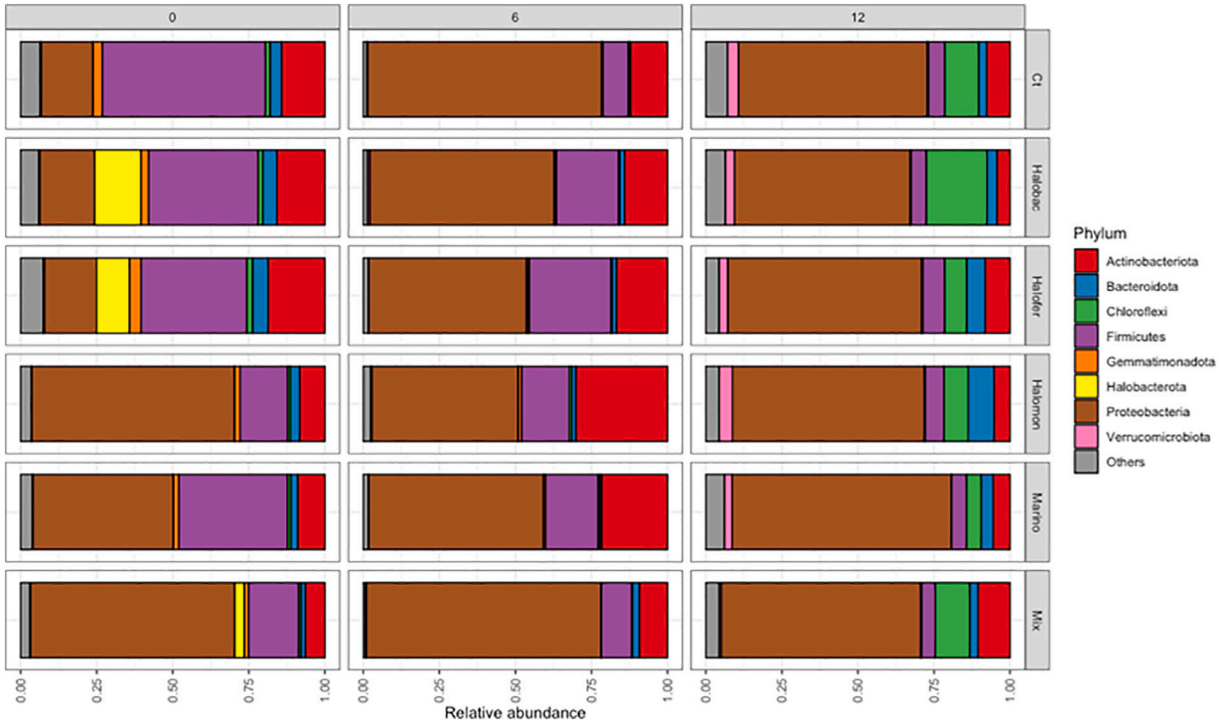

Fig. 6. Proportion of bacterial and archaeal phyla observed. The bars are arranged to represent treatment (y) and incubation time (x). Minor phyla with less than <1 % mean relative abundance are grouped into "Others".

Data availability

The datasets generated or analyzed during the current study (the isolates accession numbers - OR674004 to OR674036 - under which their sequences have been deposited) are available in the [GenBank] repository, [https://blast.ncbi.nlm.nih.gov/Blast]. Sequence data for all

samples and controls are accessible in the European Nucleotide Archive at EMBL-EBI under project accession number PRJEB72245 (https://www.ebi.ac.uk/ena/browser/view/ PRJEB72245).

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