Environmental Technology & Innovation 24 (2021) 102037

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Enhanced pilot bioremediation of oily sludge from petroleum refinery disposal under hot-summer Mediterranean climate

Olfa Ben Said [a,b], Cristiana Cravo-Laureau [b], Fabrice Armougom [c], Sabrina Cipullo [d], Meriem Ben Khelil [a], Marouen Ben Haj Yahiya [d], Abdeljabar Douihech [e], Hamouda Beyrem [a], Frédéric Coulon [d], Robert Duran [b,*]

[a] Environment Biomonitoring Laboratory LBE LR 01 ES 14, Bizerte Faculty of Sciences, 7021 Zarzouna, Bizerte, Tunisia
[b] Equipe Environnement et Microbiologie, MELODY group, Universite de Pau et des Pays de l'Adour, E2S-UPPA, IPREM UMR CNRS 5254, BP 1155, 64013 Pau Cedex, France
[c] Mediterranean Institute of Oceanography, MEB Team Bat OCEANOMED, Luminy, 13288 Marseille Cedex 9, France
[d] Cranfield University, School of Water, Energy and Environment, Cranfield, MK43 0AL, UK
[e] Centre Technique de la Chimie CTC Ministère de l'Industrie Tunis, Tunisia

ARTICLE INFO

Article history:
Received 19 July 2021
Received in revised form 13 October 2021
Accepted 16 October 2021
Available online 23 October 2021

Keywords:
Bacterial consortium
Illumina sequencing
Metal
Hydrocarbons
Ecotoxicity
Ecological succession

ABSTRACT

Large pilot scale bioremediation approaches were implemented for the treatments of oily sludge (OS) characterised by alkaline pH (pH > 9), high concentration of metals (3% dry weight) and high total petroleum hydrocarbons content (TPH) ranging between 22,000 and 67,300 mg kg^{-1} from a Tunisian petroleum refinery. The treatments included bioaugmentation and biostimulation approaches with autochthonous isolated bacterial strains and consortia. Chemical, microbial, and ecotoxicological analyses were performed over a period of 180 days incubation. The bioremediation treatments favoured the development of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* following an ecological succession of specialist bacterial groups, first associated to hydrocarbon degradation (e.g. *Marinobacter* and *Alcanivorax*) that resulted in a greater extent of TPH-degradation (up to 80%), and the selection of metal resistant bacteria including *Hyphomonas*, *Phaeobacter*, and *Desulfuromusa*. The best performances were obtained when bioaugmentation and biostimulation were combined. Over 90% of the TPH initial concentration was degraded over 180 days, which was accompanied with a 3-fold reduction of ecotoxicity. Our study demonstrates the efficacy of large pilot scale bioremediation of highly contaminated oily sludge, providing the evidence that the management of autochthonous microbial communities is of paramount importance for the success of the bioremediation process.
© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The production of large amounts of waste by oil refineries and petrochemical industries is a major environmental concern (Srinivasarao Naik et al., 2011). Oily sludge (OS), residue of the petroleum refining processes, represents the most substantial solid wastes produced by the petroleum industry (Hu et al., 2013). It corresponds up to 1/3 and 1/4 of the initial volume of the crude oil used during refining process. OS is constituted of water, sediment, aliphatic and aromatic hydrocarbons, resins and asphaltenes, and metals (Vdovenko et al., 2015), all of which are potentially toxic compounds

* Correspondence to: Universite de Pau et des Pays de l'Adour, E2S/UPPA, IPREM UMR CNRS 5254, BP 1155, 64013 Pau Cedex, France.
E-mail address: robert.duran@univ-pau.fr (R. Duran).

https://doi.org/10.1016/j.eti.2021.102037
2352-1864/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

(Jasmine and Mukherji, 2015). Among them, polyaromatic hydrocarbons (PAH) are of particular concern due to their toxicity, mutagenicity and carcinogenicity (Duran and Cravo-Laureau, 2016). They are highly hydrophobic, persistent in the environment because they tend to sorb in organic matter, their fate is dependent of biotic and abiotic factors (Duran and Cravo-Laureau, 2016). OS release in the environment is therefore a potential source of pollution for soil and water.

The spreading of untreated OS on agricultural land is hazardous for society and creates an economically and environmentally irreversible problem as OS significantly changes physical–chemical properties of the receiving soil affecting the soil microbial community (Aguelmous et al., 2019). Therefore, an adequate treatment of sludge before spreading or landfilling is thus required (Paton and Fletcher, 2008). The alternative biological treatment represents a reliable and cost-effective technology compared to the physical–chemical methods (Cravo-Laureau et al., 2017). In many cases, biological treatment has been preferred to clean moderate contaminated environments (Hu et al., 2013).

Sludge treatment is one of the most complicated pollution abatement processes because of the presence of multiple contaminants at the same time. Therefore, sludge characterisation is recommended before applying any treatment in order to adapt the treatment to the sludge characteristics, including physical–chemical parameters and the presence of specific pollutants (Jasmine and Mukherji, 2015). The capacity of microbial communities to thrive in highly polluted environments has been demonstrated in sites contaminated by either PAHs (Bordenave et al., 2004b; Stauffert et al., 2014) or metals (Bruneel et al., 2008; Giloteaux et al., 2010; Misson et al., 2016; Pringault et al., 2008), as well as in PAHs/metals co-contaminated sites (Ben Salem et al., 2019; Liu et al., 2019). Although the capacity to apply microorganisms for the degradation of organic compounds and the transformation of metals has been demonstrated in microcosm trials (Cravo-Laureau et al., 2011; Louati et al., 2013), only few studies have addressed the bio-treatment of OS at laboratory pilot scale (Zhou et al., 2019). Therefore, further studies following OS bio-treatment are required in order to optimise hydrocarbon degradation capacities and metal transformation, as well as characterise microbial communities at work during the process.

The main objective of the present study was to evaluate the efficacy of bioremediation treatments on the OS under hot-summer Mediterranean climate present in the waste storage at the Tunisian Company of Refining Industries (STIR) located at Bizerte, Tunisia. The refinery produces OS that are characterised by high moisture and alkalinity, containing high levels of hydrocarbon compounds and metals. Bio-treatment conditions were optimisedat laboratory scale and then evaluated at pilot scale. The degradation of hydrocarbon, the toxicity and the bacterial community dynamic were monitored during the bioremediation process.

2. Materials and methods

2.1. Site characterisation and oily sludge sampling

The Tunisian Company for Refining Industries STIR covers a surface of 180 ha, located at the Bizerte industrial-port area (Fig. 1A). It has the capacity to refine 1.5 million tons crude oil per year generating around 500 to 1000 m^3 of oily sludge (OS) per year, which were pilled over the years. Currently the OS storage is estimated at 4000 m^3 distributed in five saturated OS storage sites. Two OS types were identified: (i) the OS from the bottom of oil storage tanks, and (ii) the OS from the bottom API oil-water gravity separator (API, American Petroleum Institute) basins. Both are stored ashore in sloughs (OSS) within the refinery site (Fig. 1B).

For OS characterisation, oily sludge was collected from three storage sites OSS2, OSS3, OSS4 (Fig. 1B) in 2014 using a core drilling system (at 50 cm minimum depth). The OSS1 was not sampled due to maintenance for curettage operation. For each OSS five samples were collected (P1 to P5; Fig. 1C) following the standard procedures for soil quality and pre-treatment of samples for physical–chemical analysis (NF ISO 11464-1998) and the guidance of sampling techniques for soil quality sampling (NF ISO 10381-2 2003). Samples were transferred to 1 L sterile screw-capped glass amber bottles, immediately stored on ice and then transported to the laboratory.

2.2. Chemicals

All chemicals and vitamins used in this study for the culture media were analytical grade from Merck (Darmstadt, Germany). The deuterated analytical standards for petroleum hydrocarbons degradation were from Supelco Sigma Aldrich (Steinheim, Germany).

2.3. Physical-chemical characterisation of OS from OSS sites

Physical-chemical parameters including pH, temperature, conductivity, and salinity were directly measured on OS disposal site (Fig. 1B) using an Orion Star 140TM series metre (Thermo Electron Corporation, USA). Immediately after reaching laboratory, OS samples were kept at 4 °C, and aliquots were analysed for texture, humidity content (H), sulphate, ammonium (NH_4), nitrites (NO_2), nitrate (NO_3), orthophosphate (PO_4), silicon (Si), and total organic carbon (TOC) following standard spectrophotometric methods using Beckman Coulter DU370 UV/Vis spectrophotometer as described (Ben Salem et al., 2016).

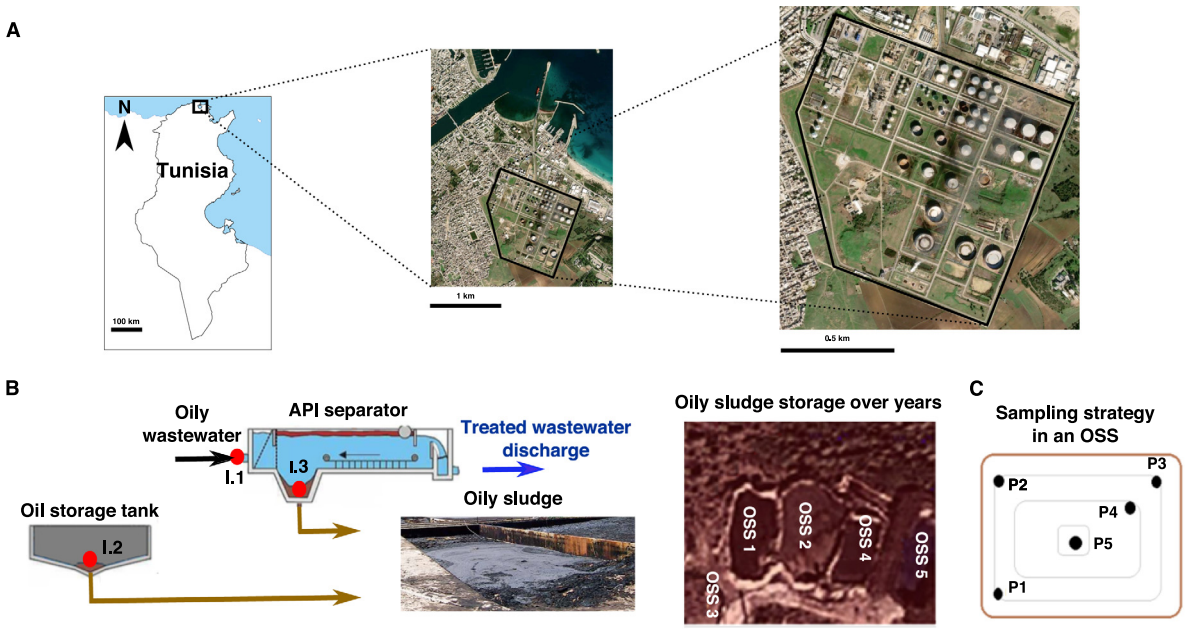


Fig. 1. Location of the Tunisian Company of Refining Industries STIR at Bizerte (A), schema of oily sludge (OS) generating process (B), and sampling strategy in oily sludge storage (C). The sites (I.1 to I.3) for sampling OS for bacterial isolation are indicated by red circles. OSS1 to OSS5: Oily Sludge Storage (OSS). P1 to P5: sampling sites in an OSS. Maps were generated using Plans (version 2.0).

Total petroleum hydrocarbons (TPH, C_{10} – C_{40}) were extracted and analysed according Risdon et al. (2008) with some modifications. Briefly, 5 g of OS were extracted in 20 mL dichloromethane:hexane (1:1, v/v) during 16 h at 150 rpm, then sonicated 30 min at 20 °C. Extracts, recovered by centrifugation, were cleaned on 6 mL SPE DSC-Si silica tubes (Florisil® columns) with hexane. Extracts were diluted (1:10) for GC–MS analysis using a deuterated alkane ($C_{10}d^{22}$, $C_{19}d^{40}$ and $C_{30}d^{62}$) and deuterated polycyclic aromatic hydrocarbons (PAH) (1,4-dichlorobenzene d4, naphthalene d^8, anthracene d^{10}, chrysene d^{12}, and perylene d^{12}) mix at 10 μg mL^{-1} each as standards. Analysis was performed using an Agilent 5973N GC–MS as previously described (Bourhane et al., 2022).

Total metals concentration was performed as described by Cipullo et al. (2018) following the extraction method ISO 11047 with aqua regia. Briefly, 0.5 g of air-dried OS was extracted with 8 mL hydrochloric/nitric acid mixture in a microwave digestion system (Multiwave 3000 microwave oven, Anton Paar/Perkin Elmer, UK). After filtration through 0.45 μm filters, the extracts were diluted to 50 mL with deionised water. All solutions were further diluted 2:8 ml in 1% HNO_3 before analysis using ICP-MS spectrometer NexION® 350D (Perkin Elmer, UK). The instrument was calibrated using major and trace elements mixtures, matching the sample matrix, ranging from 1, 5, 15, 20, 40 μg mL^{-1} and 0.01, 0.1, 0.5, 1, 2 μg mL^{-1} respectively. A mix of Sc, Ge, Rh, Bi was used as internal standard. Additionally, acid blanks (1% nitric acid), digestion blank, and guidance materials (BGS102) were analysed every 7 samples. Mean repeatability of BGS102 (expressed as relative standard deviation %) was lower than 8%.

2.4. Bacterial quantification and isolation of oil-degrading bacterial strains

2.4.1. Media preparation and bacteria enumeration

Marine broth medium was used for the quantification of total heterotroph in the sludges. Hydrocarbon degrading bacteria were quantified in mineral salt medium MSM (Álvarez-Barragán et al., 2021), supplemented with 2% crude oil under aerobic condition. Total heterotrophs and hydrocarbon degrading bacteria were quantified following the most probable number (MPN) method (Inglis et al., 2012). The MSM contained (gram per litre): KCl, 0.428; $CaCl_2.2H_2O$, 0.84; NH_4Cl, 0.015; $MgSO_4.7H_2O$, 3.794; $MgCl_2.6H_2O$, 3.017; NaCl, 15.14; Na_2CO_3, 0.15. After sterilisation, the medium was supplemented with 100 μL of 50 mM phosphate buffer solution, 1 mL of trace element stock solution composed of the following compounds (gram per litre): $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2; Na_2SO_3, 0.2; $CoCl_2.6H_2O$, 0.1; $MnSO_4.2H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; $AlCl_3.6H_2O$, 0.04; $NiCl_2.6H_2O$, 0.025; H_3BO_3, 0.01; $CuSO_4.5H_2O$, 0.01; and 1 mL of V7 vitamin stock solution that was composed of the following compounds (mg per 200 mL): vitamin B12, 10.0; p-aminobenzoic acid, 10.0; D-biotin, 2.0; acid nicotinic, 20.0; calcium pantothenate 5.0; pyridoxine.HCl, 50.0, thiamine.HCl.$2H_2O$, 10.0. Initial pH of the media was adjusted to 7.0–7.2 with sterile 10 M NaOH. Filter sterilised trace element and V7 solutions was added to the sterile MSM as described (Dias et al., 2008). Inoculated tubes were incubated at 30 °C and MPN were determined after 72 h using Mac Grady table.

2.4.2. Slurry enrichment

In order to obtain bacterial strains able to thrive in harsh conditions with hydrocarbon degradation capacity, sludge from different steps of the API separator processing were mixed. The mixture consisted of oily wastes from oily wastewater (I.1) and the crude oil Tank (I.2) at 25% each because they are the most toxic oily waste, and 50% of OS from the API separator basin (I.3), the end product of treatment corresponding to OSS. In this way, we assume that the I.3 will be enriched by microorganisms able to degrade hydrocarbon while reducing the content of toxic compounds. The mixture was sieved with a sterile sieve of 200 μm mesh. For slurry set up, the mixture (10% v/v) was used to inoculate 1 L of liquid MSM in 2 L Erlenmeyer glass. The slurry was incubated at 30 °C for 4 weeks at dark with shaking at 200 rpm in duplicate. An abiotic control of biodegradation was prepared by adding 300 mg L^{-1} of $HgCl_2$. During incubation 50 mL of slurry samples were collected every five days and analysed for TPH content and bacterial quantification. The subsamples were used for isolation of oil-degrading bacterial strains as described (Ben Said et al., 2008). In order to estimate the metabolic capacities of the bacterial isolates, the bacterial growth was determined in liquid MSM containing either a mixture of PAHs (fluoranthene, pyrene, and chrysene) or alkanes (octadecane and pristine) as carbon source. The bacterial growth was determined measuring the absorbance at λ 600 nm using a Beckman Coulter DU370 UV/Vis spectrophotometer as described (Ben Said et al., 2008).

2.4.3. Isolation and phylogenetic analysis of oil-degrading bacteria

Aliquot of 1 mL of slurry culture were diluted and spread on agar MSM added with 2% crude oil. After one-week incubation at 30 °C, morphologically different colonies were isolated and purified by the transplanting streaks method. Pure isolates (OSI1 to OSI22) were identified by microbiological tests including Gram staining, morphology, catalase and oxidase production as described (Ben Said et al., 2008). Identification of isolated cultivable bacterial strains was performed following Sanger sequencing procedure of PCR amplified 16S rRNA gene using 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′) primers as described (Ben Said et al., 2008). The PCR reactions were performed in the thermocycler Verity (Applied Biosystems, CA). The sequences determined in this study have been submitted to the EMBL database and assigned accession nos. from MW642208 to MW642229.

2.4.4. Mesocosm scale bioremediation treatments

In order to have better conditions for hydrocarbon degradation, OSS4 was selected for the bioremediation trials because it showed high cultivable oil-degrading bacterial abundance, and lower metal content (Supplementary Table S1).

Mesocosms consisted of 5 L PVC bottles containing 2 kg of wet OS from OSS4. Seven conditions were applied (Table S2) an experimental control with only addition of deionised water and intermittent manual aeration (Humidity Oxygen; HO_only), and six different treatments including biostimulation (NP), three bioaugmentation treatments (C1, C1x, C1_C1x), and two treatments combining biostimulation and bioaugmentation (NP_C1, NP_C1x). All treatments were performed in triplicates. All mesocosms were brought to 60% water holding capacity (WHC) by adding deionised water since maximum aerobic microbial activity in soil has been reported to occur at moisture between 50% and 70% of WHC (Franzluebbers, 1999). For the biostimulation treatments, C:N:P ratio was adjusted to 100:10:1 by adding $(NH_4)NO_3$ and KH_2PO_4.

The bioaugmentation treatments were performed with addition of 10^9 cells g^{-1} of bacterial consortia of isolated bacterial strains, each strain with same number of cells. Two different bacterial cocktails C1 (*Enterobacter* sp., *Bacillus Pseudofirmus*, *Pseudomonas xanthomarina*, *Rhizobium* sp. strains isolated in this study OSI2-3-4-11-14 respectively, Table 1) and C1x (*P. xanthomarina*, *P. stutzeri*, *Pseudomonas* sp., *Achromobacter spanius* strains isolated in this study OSI1-6-8-13-18 respectively, Table 1) were used, that resulted in three different bioaugmentation treatments: C1, C1x, C1_C1x. At 32 days of incubation, the isolated bacterial strain *Rhodococcus* sp. (OSI21, isolated in this study) capable of degrading complex alkanes such as octadecane and pristane was added. Finally, a C3 cocktail, comprising two strains of *Pseudomonas* sp. (OSI8 and OSI19, isolated in this study) degrading heavy PAHs such as fluoranthene, pyrene and chrysene were added after 120 days of incubation.

During the 180 days treatment, all mesocosms were manually aerated, nutrients added, and water content adjusted by the addition of sterile water every three days. The adjustment of the OS mesocosms moisture between 30% and 90% is often necessary for successful bioremediation (Alkorta et al., 2017). Metal and hydrocarbon contents, bacterial community abundance and diversity, and seed germination assays were analysed at 90, 120, 150 and 180 days of incubation. All analyses were performed in triplicate.

2.5. Bacterial diversity: DNA extraction, sequencing, and data analysis

Total DNA (metagenomic DNA) from untreated and treated OS (mesocosms) was extracted using the PowerSoil® DNA isolation Kit (MoBio Laboratories). Extractions were performed following manufacturer's instructions with slight modification in incubation times as described (Terrisse et al., 2017). The DNA quality and size were verified by electrophoresis on a 1% w/v agarose gel in Tris-Borate-EDTA buffer. The DNA extracts were aliquoted and stored at −80 °C until use.

The bacterial diversity was analysed by Illumina MiSeq 16S rRNA gene sequencing. MiSeq sequencing was performed at the PREMICE platform (Pau, France). The variable V1–V3 regions of the 16S rRNA gene were amplified with 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) primers as described (Jeanbille et al., 2016).

The 16S rRNA gene sequence reads were analysed using the QIIME package (Kuczynski et al., 2012) as previously described (Terrisse et al., 2017). Briefly, after removing low-quality and chimera sequences, the high-quality sequences were clustered into Operational Taxonomic Unit (OTU) using a 97% sequence identity threshold with UCLUST (Edgar, 2010). The OTU table was filtered for low abundance OTUs followed by a subsampling-based normalisation implemented in QIIME. The full taxonomic classification was performed by UCLUST querying the Greengenes database (McDonald et al., 2012), a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. The bioconductor Phyloseq package (McMurdie and Holmes, 2013) was used for alpha and beta diversity analysis, as well as graphic representation. The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database (SUB9082133) and it is available under the Bioproject ID PRJNA702548.

2.6. Toxicity bioassay

In order to assess the treatment performances in reducing the OS toxicity, wheat germination bioassay was performed, as it is well suited for the evaluation of soil quality for plant biomass production (Gariglio et al., 2002). For the assay, 10 wheat seeds were added separately in 3 replicates to 120 mL wide mouth glass jars containing 20 g of clean soil-OS mixture (1/3 v/v) and re-wetted to 75% WHC, mimicking the potential use of treated OS as amendment for soil in plant biomass production. The seeds were left to germinate at 25 °C, 80% humidity and dark for 7 days. After 4, 5, 6 and 7 days, germinated seeds were counted (G) and the radical length (L) was measured. The germination index (Gi) was calculated according to the formula $Gi = G/Gox\ L/Lo \times 100$ (Gariglio et al., 2002), where Go and Lo are respectively the germination percentage and radical growth of the 100% H_2O control, and G and L corresponds to the values obtained with the tested sample. A non-contaminated control soil, maintained at 44% WHC, was used as the baseline to obtain a 100% recovery in the assays.

2.7. Statistical analyses

Statistical analysis for comparing the effect of incubation time (90, 120, 150, and 180 days) and/or treatment (HO_only, C1, C1x, C1_C1x, NP, NP_C1, NP_C1x) on: (1) Aliphatic hydrocarbons alkanes, PAHs, TPH, metals and seeds germination was performed using PROC MIXED procedure in SAS (SAS version 9.4; Institute., 2014). Incubation time and treatment were modelled and along with the interactions were considered as fixed effects, and blocks (replications) along with the interactions were considered as random effects in the mixed model to fit this split split-plot design. The mean comparisons were done using Tukey's Honest Significant Difference (HSD, SAS Institute, 2014) with an alpha (α) of ≤ 0.05.

Multivariate analyses for comparing bacterial communities (clustering, CA, and CCA) were performed with MVSP v3.12d software (Kovach Computing Service, Wales). The two-ways repeated measures Analysis of Variance (ANOVA) was used to test the significance ($p \leq 0.05$) of incubation time (90, 120, 150, and 180 days) and treatment (HO_only, C1, C1x, C1_C1x, NP, NP_C1, NP_C1x) on: (1) Aliphatic hydrocarbons alkanes, PAHs, TPH and metals changes in concentration, (2) microbiological response (cultivable oil-degrading and non-cultivable detected bacteria), and (3) seeds germination. ANOVA was performed in R Studio using the "aov" function of the vegan library (Oksanen et al., 2013). Significance levels of each relationship were determined from the p value and recognised as significant where $p < 0.05$. Linear Spearman regression analysis was performed to determine significant ($r^2 > 0.75$) correlations between chemical data, and microbial or ecotoxicological response. Correlation was established with "cor" function of the Hmisc Library and plotted with "corrplot" (Harrell, 2019). All tests were computed with R Studio (Version 1.1.423 – © 2009–2018 RStudio, Inc.).

3. Results and discussion

3.1. Oily sludge characterisation

The physical–chemical properties and the cultivable microbial abundances of heterotrophic bacteria in marine broth medium and hydrocarbon degrading bacteria in MSM containing crude oil of the three OSS (OSS2 to 4, OSS1 being inaccessible for curettage) were determined in order to select the most appropriate OSS for bioremediation trials (Supplementary Table S1). All OS were alkaline (pH 9–10), with coarse grain structure (80%–97% of sand), which facilitate hydrocarbon bioavailability, and thus its biodegradation (Aguelmous et al., 2019). The OS showed high level of total N content (29 000, 25 000, and 33 000 mg kg^{-1} dry weight for OSS2, 3, and 4, respectively) while the total organic carbon (TOC) was above 184 mg kg^{-1} resulting in low C/N ratio (<0.05 mg kg^{-1} dry weight), which favour bacterial development (Touratier et al., 1999). In contrast, the total phosphorus content was low (0.8, 0.7, and 2.5 mg kg^{-1} dry weight for OSS2, 3, and 4, respectively) limiting microbial growth (Xu et al., 2015) while the total sulphur content (5.7, 9.9, and 6.3 mg kg^{-1} dry weight for OSS2, 3, and 4, respectively) was above the minimum requirements (Xu et al., 2015).

The TPH concentrations were largely above the critical limit of the Environment Quality Standards (EQS, 5000 mg kg^{-1}) for sandy soil recommended by the Tunisian government for industrial land use. Exceeding EQS leads to serious disruption of functional properties of soil or direct harm to human health and environment, requiring remediation treatment (Cabinet of Ministers Regulation No 804, 2005).

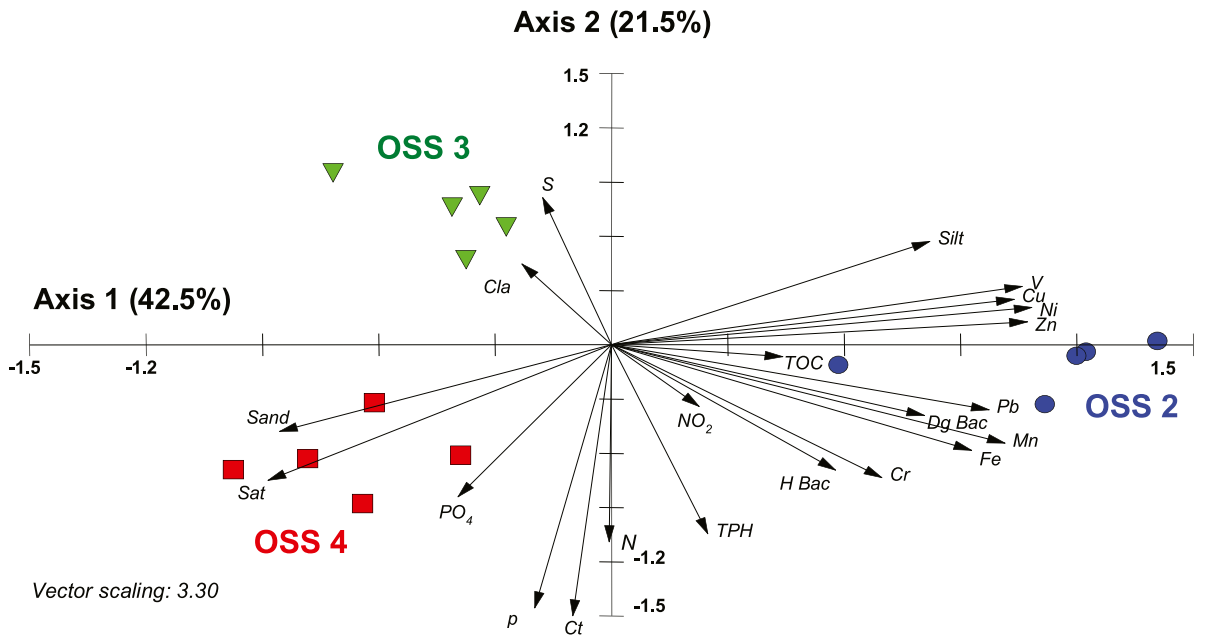


Fig. 2. Comparison of the oily sludge from the OSS2, OSS3 and OSS4 sites. The principal component analysis (PCA) was performed based on environmental variables including bacterial counts [mean profile of replicate samples (n = 3)]. The PCA was performed with 5 replicates for each OSS: OSS2 (blue circles), OSS3 (green triangles), and OSS4 (red squares). H Bac: cultivable heterotrophic bacterial abundance; Dg Bac: cultivable oil-degrading bacterial abundance; Sat: salinity; Ct: conductivity; S: sulphur; PO_4: inorganic phosphorus; NH_4: ammonia nitrogen; NO_2: nitrous oxide; H: humidity; NOT: total organic nitrogen; TOC: total organic carbon; MOT: total organic material. TPH: total petroleum hydrocarbon, Fe: iron; Zn: zinc; Cu: copper; Pb: lead; Ni: nickel; Mn: manganese; Cr: chromium; V: vanadium.

The principal component analysis (PCA) separated clearly the three OSSs indicating that OSS owns specific characteristics (Fig. 2). The PCA showed that OSS3 and OSS4 were close, separated along the axis 2 explaining 21.5% of the data distribution, while OSS2 was apart separated along the axis 1 (explaining 42.5%), forming a group associated mainly with metal content. Indeed, the metal content (including Fe) in OSS2 (30 305 mg kg^{-1}) was almost two times higher than OSS3 (15 708 mg kg^{-1}) and OSS4 (19 459 mg kg^{-1}). The lowest total hydrocarbon content was found in OSS3 (22 020 mg kg^{-1}), almost three times less than in OSS2 (52 800 mg kg^{-1}) and OSS4 (67 300 mg kg^{-1}) (Supplementary Table S1).

While the abundance of cultivable heterotrophic bacteria (5×10^4 bacteria g^{-1}) and oil degrading bacteria (2.7×10^3 bacteria g^{-1}) in OSS2 were about 2-fold higher than in OSS3 and OSS4 (Supplementary Table S1), overall the microbial abundance of the OSS samples indicates that bioremediation strategies are possible for the OS treatment.

3.2. Isolation and characterisation of oil-degrading bacteria

In order to isolate bacterial strains, a mixture of sludge collected at three steps (I.1, I.2, and I.3; 0.25/0.25/0.5 v/v) of the physical (separation/decantation) STIR process (Fig. 1B) was used as inoculate for enrichment cultures. The TPH degradation, monitored during the 30 days of slurry incubation, reached 90% at the end of incubation (Supplementary Figure 1). Noteworthy, the abiotic control showed 60% TPH degradation, indicating that both abiotic TPH degradation occurred and the $HgCl_2$ sterilisation was not efficient probably because the microbial communities are adapted to the presence of metals.

Following the isolation procedure 22 oil-degrading bacterial strains were obtained and identified by 16S rRNA gene sequences (Table 1). The majority of the isolated bacterial strains (63%) were affiliated to the Gammaproteobacteria, most of them (11) to the *Pseudomonas* genus (Table 1). The other bacteria were affiliated to Beta- and Alpha- proteobacteria (5 and 1 isolates, respectively), Firmicutes (1 isolate), and Actinobacteria (1 isolate).

3.3. Bioremediation in mesocosm

The OSS4 was selected for evaluating the performances of the bioremediation treatments in due to its high TPH content (67 300 mg kg^{-1}, with 38% of n-alkanes) and lower metal content in comparison to OSS2 (Supplementary Table S1). The physical–chemical characteristics of OSS4 are presented in Supplementary Table S3.

Different bioremediation treatments were applied (Supplementary Table S2), including bioaugmentation with the bacterial consortia C1 and C1x alone and in combination with inorganic biostimulation, for 180 days. Although OSS4

Table 1
Identification of bacterial strains isolated from STIR oily sludge.

Isolate[a]	Closest relatives	Accession n° of closest relative	Similarity (%)	Growth on Flu + Pyr + Chry[b]	Growth on Oct + Pris[b]
OSI1	*Pseudomonas stutzeri*	CP000304.1	99.67	+	–
OSI2	*Enterobacter* sp.	KF411349.1	99.29	–	–
OSI3	*Bacillus pseudofirmus*	MK100781.1	99.83	–	-
OSI4	*Enterobacter* sp.	KY673185.1	91.57	–	–
OSI5	*Achromobacter deleyi*	MT415976.1	99.91	+	-
OSI6	*Achromobacter* sp.	MN647627.1	99.73	+	-
OSI7	*Enterobacter cloacae*	MN904978	99.49	–	–
OSI8	*Pseudomonas* sp.	KY020317.1	99.67	+++	–
OSI9	*Pseudomonas stutzeri*	EU275359	99.76	+	+
OSI10	*Achromobacter* sp.	MN647627.1	99.59	+	+
OSI11	*Pseudomonas stutzeri*	EU275359.1	99.92	+	+
OSI12	*Pseudomonas* sp.	KY020317.1	99.84	+	+
OSI13	*Pseudomonas stutzeri*	EU275359.1	99	–	–
OSI14	*Rhizobium* sp.	KU904509.1	99.73	–	-
OSI15	*Pseudomonas* sp.	KY020317.1	99.67	+	+
OSI16	*Pseudomonas songnenensis*	MN176506.1	99.58	+	–
OSI17	*Pseudomonas stutzeri*	KM874452.1	99.76	+	–
OSI18	*Achromobacter spanius*	MK000623.1	99.75	+	-
OSI19	*Pseudomonas* sp.	MG547951.1	94.68	+++	-
OSI20	*Achromobacter* sp.	MN647606	99.35	–	+
OSI21	*Rhodococcus* sp.	MT012184.1	100	–	+++
OSI22	*Pseudomonas stutzeri*	MN252070	100	–	–

[a]OSI: oily sludge isolate.
[b]Growth on liquid MSM medium containing a mixture or either PAHs (Flu: fluoranthene, Pyr: pyrene, Chry: chrysene) or alkanes (Oct: octadecane, Pris: pristine) was estimated measuring absorbance at λ 600 nm.

showed alkaline conditions (pH 10.36, Supplementary Table S3) effective hydrocarbons degradation was observed in all treatments (Table 2), suggesting the presence of alkaliphilic and alkali-tolerant degraders (McGenity et al., 2010). The TPH removal was significantly higher (Tukey–Kramer HSD test, $p < 0.05$) when the bioaugmentation with C1x consortium was combined with biostimulation (NP_C1x), which was already observable after 90 days and until the end of the experiment (Table 2). Noteworthy, the treatment by bioaugmentation with the consortium C1x alone showed the lowest TPH removal. These observations highlight the importance of providing suitable nutrient content for stimulating the bacterial communities. After 180 days, the TPH removal was > 65% for all treatments. Interestingly, the TPH removal with bioaugmentation by the C1 or C1x consortium was similar with or without biostimulation from day 120 onwards (Table 2). It is likely that the biostimulation accelerate the TPH removal at the beginning reducing the lag phase for TPH removal. Several studies on bioremediation treatment of hydrocarbon contaminated soils reported contradicting results. For example, Louati et al. (2014) found biostimulation strategies were more successful than bioaugmentation while Aguelmous et al. (2019) found the opposite. It is likely that the success of bioremediation treatments is dependent on both the intrinsic properties of the solid matrix to be treated and the ability of hydrocarbon degrader isolates to cope with the existing environmental conditions.

The cultivable oil-degrading bacteria increased during the bioremediation in all treatments (Table 2). As expected, the number of oil-degrading bacteria was higher in the bioaugmentation treatments and further increased with biostimulation. Notably, the cultivable oil-degrading bacteria increased significantly according to incubation time (Tukey–Kramer HSD test, $p < 0.0001$). By comparing the increase of oil-degrading bacteria in the treatments using C1 bacterial consortium (*Enterobacter* sp., *B. pseudofirmus*, *P. xanthomarina*, *Rhizobium* sp.) with that using C1x bacterial consortium (*P. xanthomarina*, *P. stutzeri*, *Pseudomonas* sp., *A. spanius*), it is likely that the C1x bacterial consortium contains bacterial strains showing a better ability to colonise the mesocosms. Such observation was in accordance with previous reports showing that microorganisms preferentially colonise microenvironments according to their metabolic capacity and adaptation strategies (Terrisse et al., 2017). Future studies should focus on establishing protocols for the selection of the most appropriate strains based on the environmental conditions at the contaminated site.

The total metal (Fe, Cu, Pb, Mn, Ni, Zn, Cr) content was stable during the bioremediation treatments (Table 2), except Fe for which the content decreased between 9%–35% (Table 2). The combination of bioaugmentation and biostimulation showed the most important Fe content decrease (Tukey–Kramer HSD test, $p < 0.0001$) during the treatment (Table 2). Such results were not surprising since metals are biodegraded but transformed or transferred into less bioavailable forms or fractions, which was in accordance with previous reports showing that the bioavailability of metals was reduced during bioremediation treatment by maintaining metals trapped in insoluble fractions (Cipullo et al., 2018).

The performance of the bioremediation treatments was assessed by determining the toxicity during the treatment period with the wheat germination assay. Although different trends were observed depending on the treatment and incubation period, all treatments showed high germination index (Gi, less toxicity) at the end of incubation, except for bioaugmentation treatment with C1_C1x consortia (Table 2). All biostimulated mesocosms (NP, NP_C1 and NP_C1 and NP_C1X) showed less toxicity, the optima for decreasing toxicity being obtained with the treatment using the combination

Table 2
Total Petroleum hydrocarbon TPH (Alk + PAH) degradation, Fe removal, cultivable oil-degrading bacteria abundance, and toxicity (wheat germination assay) during the bioremediation treatments. Mean value ± SD (n = 3) are presented. In each column, the same small letter indicates no significant difference of means compared by Tukey–Kramer HSD test, $p < 0.05$.

	Bioremediation treatment	TPH degradation (%)	Fe removal (%)	Oil-degrading bacteria abundance*	Wheat germination (%)#	Germination index Gi
C1	C1_090	23.11 ± 17de	11.39 ± 2.97^{bcd}	1.67 ± 2^{b}	4.7 ± 8^{a}	5.48 ± 1.26^{b}
	C1_120	67.98 ± 7^{abc}	13.69 ± 1.22^{abcd}	2.67 ± 1^{b}	4.7 ± 8^{a}	7.88 ± 4.79^{ab}
	C1_150	64.56 ± 12^{abc}	31.59 ± 1.93^{abcd}	2.98 ± 2^{b}	4.7 ± 8^{a}	7.83 ± 3.5^{ab}
	C1_180	81.71 ± 6^{ab}	19.54 ± 2.09^{abcd}	3.31 ± 3^{b}	4.7 ± 8^{a}	4.35 ± 2.99^{b}
C1_C1x	C1_C1x_090	48.63 ± 17^{abcd}	8.03 ± 2.82^{cd}	13.44 ± 0.61^{abcd}	4.7 ± 8^{a}	5.55 ± 2.8^{b}
	C1_C1x_120	62.46 ± 16^{abc}	10.74 ± 0.19^{cd}	7.31 ± 0.69^{cd}	4.7 ± 8^{a}	6.75 ± 4.42^{ab}
	C1_C1x_150	69.67 ± 8^{abc}	32.05 ± 1.42^{bcd}	32.08 ± 1.83^{abc}	0 ± 0^{a}	10 ± 5.25^{ab}
	C1_C1x_180	78.83 ± 5^{abc}	18.28 ± 0.48^{abcd}	18.48 ± 0.47^{abcd}	19 ± 21^{a}	7.43 ± 3.54^{ab}
C1x	C1x_090	9.61 ± 9^{e}	13.44 ± 0.61^{abcd}	3.72 ± 4^{b}	4.7 ± 8^{a}	6.98 ± 3.13^{ab}
	C1x_120	66.99 ± 9^{abc}	7.31 ± 0.69^{cd}	2.45 ± 1^{b}	0 ± 0^{a}	8.89 ± 3.23^{ab}
	C1x_150	61.63 ± 8^{abc}	32.08 ± 1.83^{abc}	4.57 ± 4^{b}	4.7 ± 8^{a}	6.98 ± 5.42^{ab}
	C1x_180	81.13 ± 1^{ab}	18.48 ± 0.47^{abcd}	16.1 ± 7^{ab}	4.7 ± 8^{a}	10 ± 5.58^{ab}
HO_only	HO_only_090	41.45 ± 35^{cde}	8.56 ± 1.79^{cd}	8.24 ± 13^{ab}	0 ± 0^{a}	5.29 ± 2.32^{b}
	HO_only_120	74.90 ± 12^{abc}	11.51 ± 1.28^{cd}	1.65 ± 1^{b}	0 ± 0^{a}	6.11 ± 1.51^{b}
	HO_only_150	76.13 ± 6^{abc}	26.92 ± 2.05^{abcd}	1.39 ± 0.9^{b}	4.7 ± 8^{a}	6.94 ± 0.83^{ab}
	HO_only_180	76.75 ± 3^{abc}	14.18 ± 2.72^{abcd}	5.3 ± 3^{b}	19 ± 16^{a}	16.51 ± 7.21^{ab}
NP	NP_090	45.05 ± 13^{bcde}	8.90 ± 1.84^{cd}	1.19 ± 0.9^{b}	4.7 ± 8^{a}	4.91 ± 3.8^{b}
	NP_120	78.45 ± 11^{abc}	15.72 ± 4.73^{abcd}	6.55 ± 7^{ab}	4.7 ± 8^{a}	7.46 ± 2.39^{ab}
	NP_150	65.75 ± 9^{abc}	35.36 ± 4.20a^{b}	5.37 ± 1^{b}	0 ± 0^{a}	7.92 ± 3^{ab}
	NP_180	69.58 ± 9^{abc}	19.28 ± 1.52^{abcd}	10.87 ± 1^{ab}	14 ± 14^{a}	13.14 ± 3^{ab}
NP_C1	NP_C1_090	51.22 ± 11^{abcd}	12.33 ± 0.39^{bcd}	2.98 ± 2^{b}	9.3 ± 8^{a}	12.5 ± 4.5^{ab}
	NP_C1_120	65.59 ± 8^{abc}	10.71 ± 1.83^{abcd}	4.3 ± 0^{b}	0 ± 0^{a}	8.22 ± 4^{ab}
	NP_C1_150	61.85 ± 6^{abc}	37.35 ± 0.82^{a}	9.27 ± 12^{ab}	0 ± 0^{a}	8.78 ± 2^{ab}
	NP_C1_180	65.25 ± 1^{abcd}	11.84 ± 0.73^{abcd}	15.5 ± 8^{ab}	28.3 ± 24^{a}	19.78 ± 2^{a}
NP_C1x	NP_C1x_090	69.72 ± 6^{abc}	12.14 ± 2.22^{cd}	1.12 ± 1^{b}	14 ± 0^{a}	7.92 ± 4.13^{ab}
	NP_C1x_120	75.99 ± 6^{abc}	4.51 ± 1.06^{d}	3.46 ± 4^{b}	0 ± 0^{a}	5.63 ± 5.3^{b}
	NP_C1x_150	72.58 ± 11^{abc}	32.70 ± 2.19^{abc}	8.62 ± 13^{ab}	9.3 ± 8^{a}	12.8 ± 6^{ab}
	NP_C1x_180	83.84 ± 6^{a}	9.97 ± 3.45^{abcd}	24.1 ± 21^{a}	19 ± 8^{a}	11.33 ± 2.88^{ab}

*Abundance of cultivable oil-degrading bacteria ($\times 10^3$ bacteria per g) counted on media containing oil as unique carbon source.
#A non-contaminated control served as 100% baseline.

of biostimulation involving the C1 consortium and "NP_C1" addition (Table 2). However, the differences in decreasing toxicity between bioremediation treatments were significant considering the time of incubation rather than the type of treatment (Tukey–Kramer HSD test, $p < 0.0001$).

3.4. Bacterial community dynamics during OS bioremediation treatment

The dynamic of bacterial community was followed in the different bioremediation treatments by 16S rRNA gene sequencing at 90, 120, 150 and 180 days for all biological triplicates representing a total of 84 samples (7 treatments × 4 times × 3 replicates). The 16S rRNA gene sequencing generated 14,226 high-quality sequences per sample after trimming and normalisation (Supplementary Table S5). The rarefaction curves reached a plateau (Supplementary Figure S2) indicating that the sequencing effort was adequate to recover almost complete bacterial diversity. Although the OTU number decreased during the bioremediation treatments, the diversity indices indicated that the bacterial diversity was maintained during the treatments period (Supplementary Table S5). Overall, the different treatments showed similar alpha diversity indices, no significant differences being identified (Supplementary Table S5). Nevertheless, the "NP_C1x" treatment, showing the highest bacterial richness (observed OTUs) and Shannon diversity (Fig. 3A and B), exhibited significantly higher (Tukey–Kramer HSD test, $p < 0.05$) TPH removal (Table 2). Such observation was consistent with previous studies showing that higher diversity is observed in bioaugmentation and biostimulation treatments (Wu et al., 2017), the nutrient addition maintaining high level of microbial diversity (Shahi et al., 2016). It is likely that combining biostimulation and bioaugmentation treatments helps the bacterial community to tolerate the toxic metabolites generated during hydrocarbons degradation as previously proposed (Xu et al., 2018), which relies on co-metabolism of hydrocarbons (Zhang et al., 2019).

The bacterial community composition included 13 phyla with a major dominance of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* accounting for 93 to 97% of the sequences (Fig. 3C), which have been recognised as essential phyla in bioremediation process (Shahi et al., 2016). The bacterial communities were largely dominated by *Proteobacteria*

with relative abundance remaining stable (60%–70%) during the treatments (Fig. 3C). Several studies have shown that *Proteobacteria* are increasing during bioremediation treatments, especially those involving biostimulation and/or bioaugmentation (Shahi et al., 2016; Wu et al., 2017) while other studies reported a decrease of *Proteobacteria* (Deng et al., 2020). *Marinobacter* genus dominated the *Proteobacteria* (Supplementary material Figure S3). Such observation was not surprising since members of the *Marinobacter* genus, known as obligate hydrocarbon bacteria (Grimaud et al., 2012), have been found to play a critical role in petroleum hydrocarbon degradation (Bordenave et al., 2008; Chronopoulou et al., 2013; Guermouche M'rassi et al., 2015).

In contrast the relative abundance of *Firmicutes* and *Bacteroidetes* shifted during the treatments, the relative abundance of *Firmicutes* (from 23%–31% at day 90 to 17%–20% at day 180) decreasing while that of *Bacteroidetes* increasing (from 5%–8% at day 90 to 12%–15% at day 180) (Fig. 3C). Such observation was in contradiction with a previous study showing simultaneous increase of the *Bacteroidetes* and *Firmicutes* relative abundance during the bioremediation of oil-contaminated soil (Shahi et al., 2016). The decrease of the relative abundance of *Firmicutes* corresponded to the decrease of *Dethiobacter*, *Tindallia* genera and two uncultured bacterial genera belonging to the *Syntrophomonadaceae* family (Supplementary Figure S4) suggesting that these genera play a limited role in the bioremediation process. The increase of the relative abundance of *Bacteroidetes* corresponds to the increase of the *Brumimicrobium* genus and an uncultured bacterium (Supplementary Figure S5). *Brumimicrobium* members have been isolated in hydrocarbon-contaminated marine coastal sediments and hypersaline environments (Yang et al., 2013) suggesting that they own metabolic capabilities to thrive in extreme ecosystems.

3.5. Identification of bacteria specifically associated with pollutants

In order to identify the associations between bacterial OTUs and pollutants a clustered correlation (ClusCor) analysis was performed (Fig. 4). The ClusCor analysis revealed four main groups. The Group G1 gathers bacteria, belonging to *Halomonas*, *Salinarimonas*, *Marinicella*, *Methylonatrum*, *Anoxynatronum*, *Erysipelothrix* genera, which abundance showed strong positive correlations (Pearson correlation > 0.5) with chromium and iron (Fig. 4). Most of these genera own several species detected in contaminated alkaline hypersaline extreme environments (Sorokin et al., 2007; Yin et al., 2019). Interestingly, *Halomonas* species have been isolated for their capacity to reduce Cr(VI) (Piñón-Castillo et al., 2010), while *Marinicella* have been shown synergist with annamox bacteria in presence of high Fe content (Yin et al., 2019). It is likely that the bacteria related to the Group G1 bring important metabolic functions allowing to the microbial community to cope with the harsh conditions prevailing in the OS during the treatment.

The abundance of the genera constituting the Group G2 was strongly correlated (Pearson correlation > 0.5) with hydrocarbons (alkane and PAH), chromium and iron content (Fig. 4). The Group G2 contains several bacterial genera found in hydrocarbon-contaminated sites such as *Marinobacter* and *Alcanivorax* known as marine hydrocarbon-degraders (Terrisse et al., 2017), *Aliidiomarina*, *Bacteroidetes*, *Halomonas,* and *Dethiobacter* often detected in hydrocarbon-containing waste (Potts et al., 2019), *Pelagibacterium* in oil reservoir (Chai et al., 2018), and *Fastidiosipila*, *Alkalibacterium* in solid wastes (Kiesel et al., 2007). Thus, the Group G2 represents the hydrocarbon degradation potential of the bacterial community by either direct hydrocarbon degradation as *Marinobacter* and *Alcanivorax* (Terrisse et al., 2017) or indirectly providing surfactant as demonstrated for *Pelagibacterium* (Chai et al., 2018).

The abundance of the OTUs belonging to the Group G3 showed low correlations positive and negative ($-0.5 <$ Pearson correlation < 0.4) with most pollutant contents (Fig. 4). Among them, *Parvibaculum* and *Azoarcus* have also been detected in hydrocarbon-contaminated sites associated with *Marinobater* and other hydrocarbon-degrading genera (Igun et al., 2019), suggesting that the Group G3 play a key role in hydrocarbon degradation.

The Group G4 assembles genera which abundance was strongly positively correlated (Pearson correlation > 0.5) with metal content and strongly negatively correlated (Pearson correlation < -0.5) with hydrocarbon compounds (alkane and PAH) content (Fig. 4). Most of the genera of Group G4 have been detected in alkaline (hyper)-saline environments such as *Roseinatronobacter*, *Oceanibaculum*, *Brumimicrobium*, *Geoalkalibacter*, *Thioalkalispira*, *Glycocaulis*, *Nitriliruptor*, *Thioalkalivibrio*, *Cyclobacterium*, and *Methylonatrum* (Chakraborty et al., 2020; Sorokin et al., 2007). These genera, although not reported to be detected in metal contaminated sites, own probably metabolic capacities to cope with the extreme conditions of the OS. The role of metal transformation/resistance needs to be elucidated. Some genera from the Group G4 have been described in contaminated environments such as *Hyphomonas* and *Phaeobacter* in metal contaminated site (Zhang et al., 2019), and *Desulfuromusa* in petroleum and metal waste (Holmes et al., 2004).

Noteworthy, several genera show OTUs in various Groups such as *Alcanivorax* (G2 and G4), *Marinobater* (G2, G3, and G4), and *Dethiobacter* (G2 and G4), indicating the versatility and adaptability of members of these genera to survive in environments contaminated with various contaminants as previously reported for *Alcanivorax* and other genera (Terrisse et al., 2017). Also, it is important to note that the ClusCor analysis revealed genera known for their anaerobic metabolism, which abundances were correlated with pollutants, suggesting that anaerobic metabolism play a critical role in the remediation process. Particularly, genera involved in the sulphur and nitrogen cycles have been detected, such as *Anoxynatronum* (Ryzhmanova et al., 2017), *Dethiobacter* (Sorokin et al., 2008), and *Geoalkalibacter* (Greene et al., 2009). Importantly, among the bacteria included in the bacterial consortium used for the bioaugmentation treatment, only two OTUs related to *Pseudomonas* were present in the 100 more abundant OTUs included in the ClusCor analysis, indicating that the added bacteria were unable to colonise the mesocosm. Such result was in accordance with previous reports

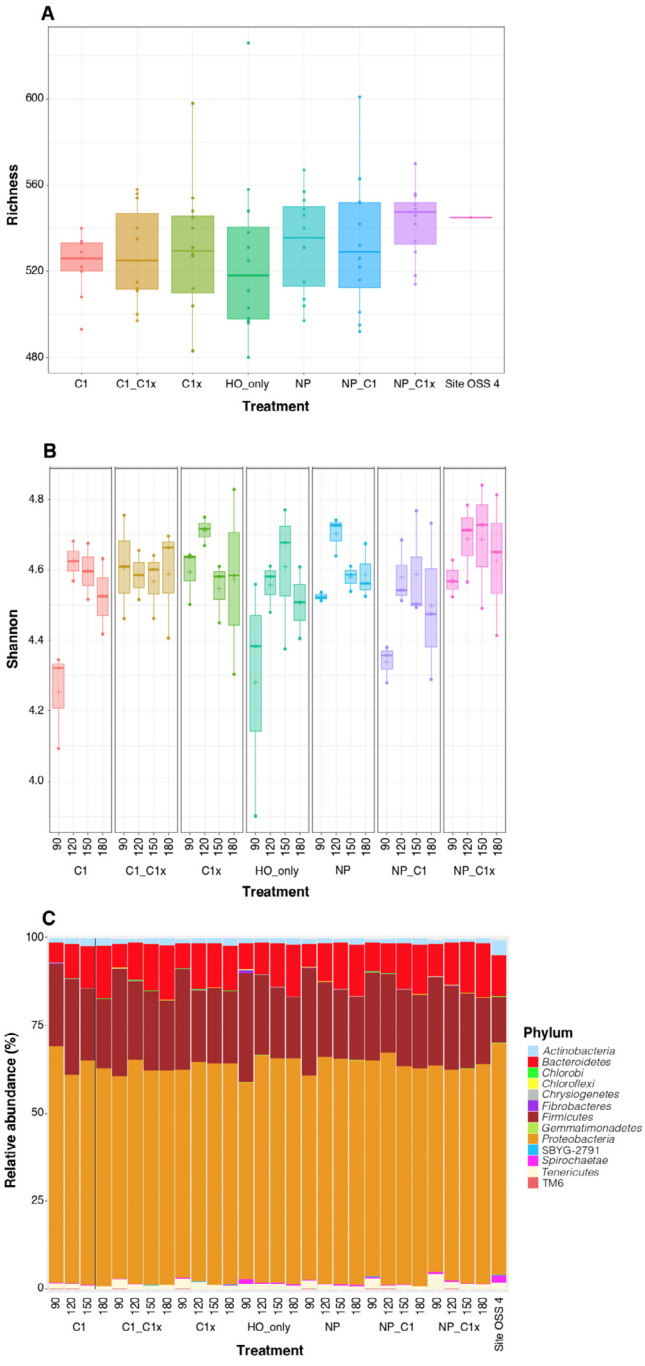


Fig. 3. Comparison of bacterial communities during OSS4 treatment: Richness (A), Shannon (B), and bacterial composition at the phylum level (C). The mean of all times is present for each treatment (n = 12) for the Richness (A). For Shannon (B) and bacterial composition (C) the mean (n = 3) is present for each time (90, 120, 150, and 180 days) for the different treatments. Bioaugmentation with the bacterial consortia C1 and C1x alone (C1 and C1x), and with both (C1_C1x); Biostimulation alone (NP) and combined with bioaugmentation with the consortia C1 (NP_C1) or C1x (NP_C1x); The control corresponds to treatment with only addition of deionised water and intermittent manually aeration (Humidity Oxygen; HO_only); Témoin is the initial OSS4 sample corresponding to the bacterial communities from the site.

showing that added bacteria do not predominate in bioaugmentation treatments (Ben Said et al., 2015). Moreover, the ClusCor analysis revealed specific bacterial groups harbouring the metabolic capacities to colonise contaminated extreme environments, which is consistent with previous reports revealing specific bacterial assemblages according to pollutants

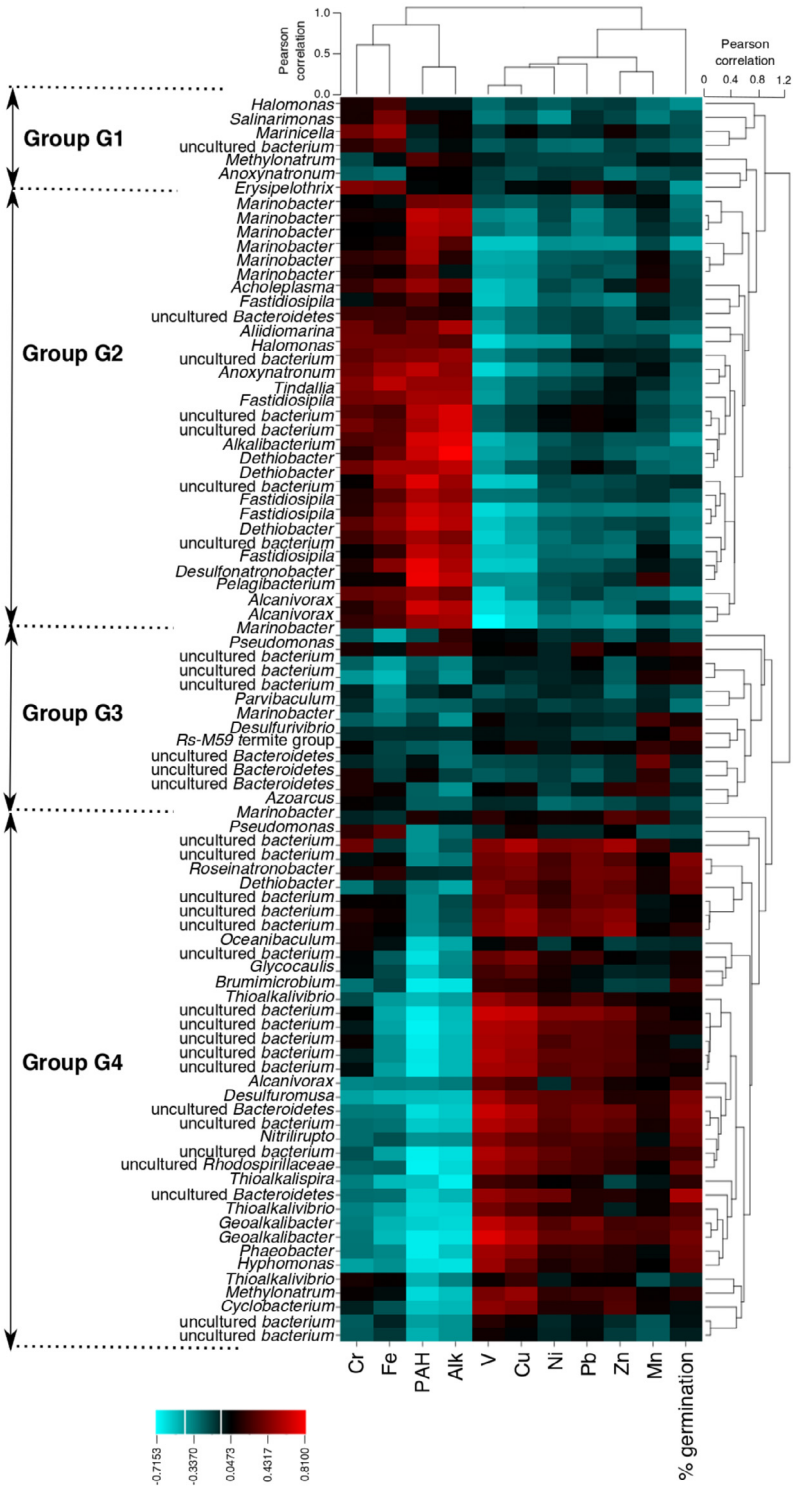


Fig. 4. Clustered correlation (ClusCor) between bacterial OTU abundances and pollutant (metals and hydrocarbons) contents, and wheat germination. Clustering was performed applying Pearson correlations and Ward algorithm, red indicating high positive correlation and blue high negative correlation (p value < 0.01). The analysis was performed at the OTU level, with 100 most abundant OTUs, applying a threshold similarity of 97% for OTU identification. Biological replicates (n = 3) were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

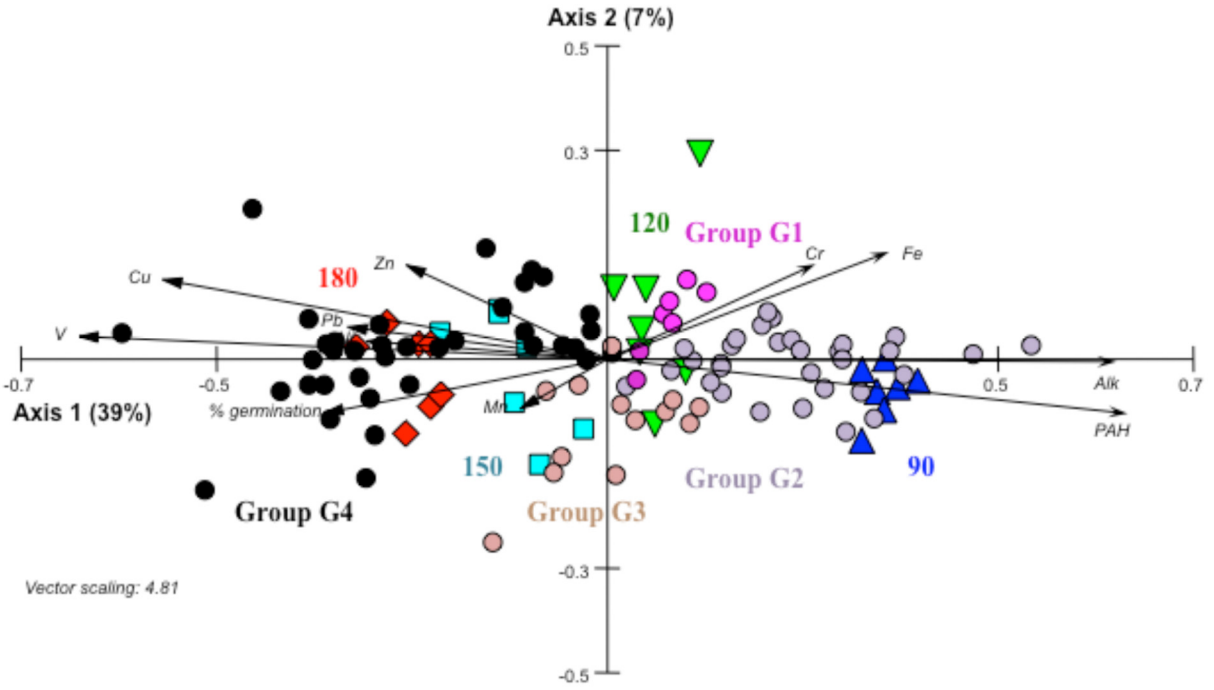


Fig. 5. Comparison of bacterial communities during bioremediation treatments. The canonical correspondence analysis (CCA) was performed to correlate bacterial communities during the treatments (based on OTUs) with pollutants parameters (metals and hydrocarbons), and wheat germination. The CCA analysis includes the different treatments at 90 (blue triangles), 120 (green inversed triangles), 150 (light blue squares), and 180 (red diamonds) days. The OTUs (circles) are indicated according to the groups identified in the ClusCor analysis (Fig. 5): Group G1 (pink), Group G2 (violet), Group G3 (brown), and Group G4 (black). PAH, polycyclic aromatic hydrocarbon; AlK, alkane; Metals (Fe, Cu, Pb, Mn, Ni, Zn, Cr, V). Biological replicates (n = 3) were used.

(Duran et al., 2015; Jeanbille et al., 2016). However, further studies are necessary to decipher the factors driving the microbial community organisation, particularly for ensuring pollutants degradation and transformation.

The comparison of bacterial communities of the treatments (HO_only, C1, C1x, C1_C1x, NP, NP_C1, NP_C1x) at different times by canonical correspondence analysis (CCA) showed clusters according to time rather than treatment (Fig. 5). The clusters were separated along the axis 1 explaining 39% of the data distribution. The cluster gathering the bacterial communities at day 90 was correlated with hydrocarbon compounds (alkane and PAH) and associated to the bacterial Group G2 defined by the ClusCor analysis. The bacterial communities shifted then to a mixture containing OTUs from Group G1, Group G2, and Group G3 at day 120. At day 150, the bacterial communities consisted to mixture of OTUs from Group G3 and Group G4, to finally be composed by OTUs from Group G4 at day 180, which were correlated with metals (Cu, V, Pb, Ni), as shown by the ClusCo analysis (Fig. 4). Such observations revealed thus the ecological succession during the treatment, modification of bacterial communities according to time concomitantly with changes of the conditions corresponding particularly to the hydrocarbon degradation as shown in Table 2. The ecological succession describes the bacterial colonisation of an ecosystem that has been reported in many environments, including volcano (Medrano-Santillana et al., 2017), mine impacted soils (Liu et al., 2019), and petroleum hydrocarbons contaminated sites (Bordenave et al., 2004a). Here, the ecological succession relates the bacterial community plasticity adapting the metabolic activity during the bio-treatments. It is also important to notice that the treatments were significantly separated (ANOVA, $p = 0.02$) along the axis 2 (explaining 7% of the data distribution) showing a slight effect of the treatments on bacterial communities.

4. Conclusion

Industrial oily sludge (OS) landfills are threatening the environment and human health. In order to set up bioremediation treatments for mitigating the effect of OS in the environment, hydrocarbon-degrading bacteria were isolated from OS. The bioaugmentation adding mixtures of the isolated bacteria combined with biostimulation was the most effective strategy to remove hydrocarbon compounds (alkane and PAH). During the treatment the characterisation of the bacterial communities revealed the ecological succession of specialised bacterial groups associated to a pollutant type. Obtaining such bacterial groups in culture will allow to better understand their metabolic potential and, moreover, improve the bioremediation along the different steps of the treatment.

CRediT authorship contribution statement

Olfa Ben Said: Conceptualisation, Investigation, Resources, Formal analysis, Writing – original draft, Funding acquisition. **Cristiana Cravo-Laureau:** Conceptualisation, Validation, Writing. **Fabrice Armougom:** Formal analysis, Validation. **Sabrina Cipullo:** Formal analysis, Validation, Writing. **Meriem Ben Khelil:** Validation. **Marouen Ben Haj Yahiya:** Validation. **Abdeljabar Douihech:** Validation. **Hamouda Beyrem:** Validation. **Frédéric Coulon:** Conceptualisation, Resources, Chemical analysis, Investigation, Validation, Writing – original draft. **Robert Duran:** Conceptualisation, Investigation, Resources, Formal analysis, Writing – original draft, Supervision, Funding acquisition.

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.

Acknowledgements

We would like to thank MELODY group for the useful discussions. We acknowledge the Regional Platform for Environmental Microbiology PREMICE supported by the Aquitaine Regional Government Council (France) and the urban community of Pau-Pyrénées (France).

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2021.102037.

References

Aguelmous, A., El Fels, L., Souabi, S., Zamama, M., Hafidi, M., 2019. The fate of total petroleum hydrocarbons during oily sludge composting: a critical review. Rev. Environ. Sci. Bio/Technol. 18 (3), 473–493.
Alkorta, I., Epelde, L., Garbisu, C., 2017. Environmental parameters altered by climate change affect the activity of soil microorganisms involved in bioremediation. FEMS Microbiol. Lett. 364 (19).
Álvarez-Barragán, J., Cravo-Laureau, C., Wick, L.Y., Duran, R., 2021. Fungi in PAH-contaminated marine sediments: Cultivable diversity and tolerance capacity towards PAH. Mar. Pollut. Bull. 164, 112082.
Ben Said, O., Goñi-Urriza, M.S., El Bour, M., Dellali, M., Aissa, P., Duran, R., 2008. Characterization of aerobic polycyclic aromatic hydrocarbon-degrading bacteria from Bizerte lagoon sediments, Tunisia. J. Appl. Microbiol. 104 (4), 987–997.
Ben Said, O., Louati, H., Soltani, A., Preud'homme, H., Cravo-Laureau, C., Got, P., Pringault, O., Aissa, P., Duran, R., 2015. Changes of benthic bacteria and meiofauna assemblages during bio-treatments of anthracene-contaminated sediments from Bizerta lagoon (Tunisia). Environ. Sci. Pollut. Res. 22 (20), 15319–15331.
Ben Salem, F., Ben Said, O., Aissa, P., Mahmoudi, E., Monperrus, M., Grunberger, O., Duran, R., 2016. Pesticides in Ichkeul Lake–Bizerta Lagoon Watershed in Tunisia: use, occurrence, and effects on bacteria and free-living marine nematodes. Environ. Sci. Pollut. Res. 23 (1), 36–48.
Ben Salem, F., Ben Said, O., Cravo-Laureau, C., Mahmoudi, E., Bru, N., Monperrus, M., Duran, R., 2019. Bacterial community assemblages in sediments under high anthropogenic pressure at Ichkeul Lake/Bizerte Lagoon hydrological system, Tunisia. Environ. Pollut. 252, 644–656.
Bordenave, S., Fourçans, A., Blanchard, S., Goñi, M.S., Caumette, P., Duran, R., 2004a. Structure and functional analyses of bacterial communities changes in microbial mats following petroleum exposure. Ophelia 58 (3), 195–203.
Bordenave, S., Goñi-Urriza, M., Vilette, C., Blanchard, S., Caumette, P., Duran, R., 2008. Diversity of ring-hydroxylating dioxygenases in pristine and oil contaminated microbial mats at genomic and transcriptomic levels. Environ. Microbiol. 10 (12), 3201–3211.
Bordenave, S., Jézéquel, R., Fourçans, A., Budzinski, H., Merlin, F.X., Fourel, T., Goñi-Urriza, M., Guyoneaud, R., Grimaud, R., Caumette, P., Duran, R., 2004b. Degradation of the Erika oil. Aquat. Living Resour. 17 (3), 261–267.
Bourhane, Z., Lanzén, A., Cagnon, C., Ben Said, O., Mahmoudi, E., Coulon, F., Atai, E., Borja, A., Cravo-Laureau, C., Duran, R., 2022. Microbial diversity alteration reveals biomarkers of contamination in soil-river-lake continuum. J. Hard Mater. 421, 126789.
Bruneel, O., Pascault, N., Egal, M., Bancon-Montigny, C., Goñi-Urriza, M.S., Elbaz-Poulichet, F., Personné, J.C., Duran, R., 2008. Archaeal diversity in a Fe-As rich acid mine drainage at Carnoulès (France). Extremophiles 12 (4), 563–571.
Chai, L., Li, X., She, Y., Banat, I., 2018. Characterization of bacterial communities in high-temperature heavy oil reservoir. J. Adv. Oxid. Technol. 21 (2), 201706651.
Chakraborty, J., Sapkale, V., Rajput, V., Shah, M., Kamble, S., Dharne, M., 2020. Shotgun metagenome guided exploration of anthropogenically driven resistomic hotspots within lonar soda lake of India. Ecotoxicol. Environ. Saf. 194, 110443.
Chronopoulou, P.M., Fahy, A., Coulon, F., Païssé, S., Goñi-Urriza, M., Peperzak, L., Acuña Alvarez, L., McKew, B.A., Lawson, T., Timmis, K.N., 2013. Impact of a simulated oil spill on benthic phototrophs and nitrogen-fixing bacteria in mudflat mesocosms. Environ. Microbiol. 15 (1), 242–252.
Cipullo, S., Snapir, B., Tardif, S., Campo, P., Prpich, G., Coulon, F., 2018. Insights into mixed contaminants interactions and its implication for heavy metals and metalloids mobility, bioavailability and risk assessment. Sci. Total Environ. 645, 662–673.
Cravo-Laureau, C., Cagnon, C., Lauga, B., Duran, R., 2017. Microbial Ecotoxicology. Springer International Publishing.
Cravo-Laureau, C., Hernandez-Raquet, G., Vitte, I., Jézéquel, R., Bellet, V., Godon, J.-J., Caumette, P., Balaguer, P., Duran, R., 2011. Role of environmental fluctuations and microbial diversity in degradation of hydrocarbons in contaminated sludge. Res. Microbiol. 162 (9), 888–895.
Deng, Z., Jiang, Y., Chen, K., Gao, F., Liu, X., 2020. Petroleum depletion property and microbial community shift after bioremediation using Bacillus halotolerans T-04 and Bacillus cereus 1-1. Front. Microbiol. 11, 353.
Dias, M., Salvado, J.C., Monperrus, M., Caumette, P., Amouroux, D., Duran, R., Guyoneaud, R., 2008. Characterization of Desulfomicrobium salsuginis sp. nov. and Desulfomicrobium aestuarii sp. nov. two new sulfate-reducing bacteria isolated from the Adour estuary (French Atlantic coast) with specific mercury methylation potentials. Syst. Appl. Microbiol. 31 (1), 30–37.
Duran, R., Bielen, A., Paradžik, T., Gassie, C., Pustijanac, E., Cagnon, C., Hamer, B., Vujaklija, D., 2015. Exploring actinobacteria assemblages in coastal marine sediments under contrasted Human influences in the West Istria Sea, Croatia. Environ. Sci. Pollut. Res. 22 (20), 15215–15229.

Duran, R., Cravo-Laureau, C., 2016. Role of environmental factors and microorganisms in determining the fate of polycyclic aromatic hydrocarbons in the marine environment. FEMS Microbiol. Rev. 40 (6), 814–830.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26 (19), 2460–2461.
Franzluebbers, A., 1999. Microbial activity in response to water-filled pore space of variably eroded southern Piedmont soils. Appl. Soil Ecol. 11 (1), 91–101.
Gariglio, N., Buyatti, M., Pilatti, R., Russia, D.G., Acosta, M., 2002. Use of a germination bioassay to test compost maturity of willow (Salix sp.) sawdust. N. Z. J. Crop Hortic. Sci. 30 (2), 135–139.
Giloteaux, L., Goñi-Urriza, M., Duran, R., 2010. Nested PCR and new primers for analysis of sulfate-reducing bacteria in low-cell-biomass environments. Appl. Environ. Microbiol. 76 (9), 2856–2865.
Greene, A.C., Patel, B.K., Yacob, S., 2009. Geoalkalibacter subterraneus sp. nov. an anaerobic Fe (III)-and Mn (IV)-reducing bacterium from a petroleum reservoir, and emended descriptions of the family desulfuromonadaceae and the genus Geoalkalibacter. Int. J. Syst. Evol. Microbiol. 59 (4), 781–785.
Grimaud, R., Ghiglione, J.-F., Cagnon, C., Lauga, B., Vaysse, P.-J., Rodriguez-Blanco, A., Mangenot, S., Cruveiller, S., Barbe, V., Duran, R., 2012. Genome sequence of the marine bacterium Marinobacter hydrocarbonoclasticus SP17, which forms biofilms on hydrophobic organic compounds. Am. Soc. Microbiol..
Guermouche M'rassi, A., Bensalah, F., Gury, J., Duran, R., 2015. Isolation and characterization of different bacterial strains for bioremediation of n-alkanes and polycyclic aromatic hydrocarbons. Environ. Sci. Pollut. Res. 22 (20), 15332–15346.
Harrell, F.E., 2019. Package 'hmisc'. In: CRAN2018 2019. pp. 235–236.
Holmes, D.E., Nevin, K.P., Lovley, D.R., 2004. Comparison of 16s rRNA, nifD, recA, gyrB, rpoB and fusA genes within the family Geobacteraceae fam. nov. Int. J. Syst. Evol. Microbiol. 54 (5), 1591–1599.
Hu, G., Li, J., Zeng, G., 2013. Recent development in the treatment of oily sludge from petroleum industry: A review. J. Hard Mater. 261, 470–490.
Igun, O.T., Meynet, P., Davenport, R.J., Werner, D., 2019. Impacts of activated carbon amendments, added from the start or after five months, on the microbiology and outcomes of crude oil bioremediation in soil. Int. Biodeterior. Biodegrad. 142, 1–10.
Inglis, G.D., Enkerli, J., Goettel, M.S., 2012. Chapter VII - Laboratory techniques used for entomopathogenic fungi: Hypocreales. In: Lacey, L.A. (Ed.), Manual of Techniques in Invertebrate Pathology (Second Edition). Academic Press, San Diego, pp. 189–253.
Jasmine, J., Mukherji, S., 2015. Characterization of oily sludge from a refinery and biodegradability assessment using various hydrocarbon degrading strains and reconstituted consortia. J. Environ. Manag. 149, 118–125.
Jeanbille, M., Gury, J., Duran, R., Tronczynski, J., Ghiglione, J.F., Agogué, H., Saïd, O.B., Taïb, N., Debroas, D., Garnier, C., Auguet, J.C., 2016. Chronic polyaromatic hydrocarbon (PAH) contamination is a marginal driver for community diversity and prokaryotic predicted functioning in coastal sediments. Front. Microbiol. 7 (AUG).
Kiesel, B., Müller, R., Kleinsteuber, R., 2007. Adaptative potential of alkaliphilic bacteria towards chloroaromatic substrates assessed by a gfp-tagged 2, 4-D degradation plasmid. Eng. Life Sci. 7 (4), 361–372.
Kuczynski, J., Stombaugh, J., Walters, W.A., González, A., Caporaso, J.G., Knight, R., 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. Curr. Protoc. Microbiol. 27 (1), 1E. 5.1–1E. 5.20.
Liu, J., Yao, J., Sunahara, G., Wang, F., Li, Z., Duran, R., 2019. Nonferrous metal (loid) s mediate bacterial diversity in an abandoned mine tailing impoundment. Environ. Sci. Pollut. Res. 26 (24), 24806–24818.
Louati, H., Said, O.B., Got, P., Soltani, A., Mahmoudi, E., Cravo-Laureau, C., Duran, R., Aissa, P., Pringault, O., 2013. Microbial community responses to bioremediation treatments for the mitigation of low-dose anthracene in marine coastal sediments of Bizerte lagoon (Tunisia). Environ. Sci. Pollut. Res. 20 (1), 300–310.
Louati, H., Said, O.B., Soltani, A., Got, P., Cravo-Laureau, C., Duran, R., Aissa, P., Pringault, O., Mahmoudi, E., 2014. Biostimulation as an attractive technique to reduce phenanthrene toxicity for meiofauna and bacteria in lagoon sediment. Environ. Sci. Pollut. Res. 21 (5), 3670–3679.
McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6 (3), 610–618.
McGenity, T.J., Whitby, C., Fahy, A., 2010. Alkaliphilic hydrocarbon degraders. In: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1931–1937.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE 8 (4), e61217.
Medrano-Santillana, M., Souza-Brito, E.M., Duran, R., Gutierrez-Corona, F., Reyna-López, G.E., 2017. Bacterial diversity in fumarole environments of the Paricutín volcano, Michoacán (Mexico). Extremophiles 21 (3), 499–511.
Misson, B., Garnier, C., Lauga, B., Dang, D.H., Ghiglione, J.F., Mullot, J.U., Duran, R., Pringault, O., 2016. Chemical multi-contamination drives benthic prokaryotic diversity in the anthropized Toulon Bay. Sci. Total Environ. 556, 319–329.
NF ISO 10381-2 2003, 2003. The guidance of sampling techniques for soil quality sampling. https://m.boutique.afnor.org/fr-fr/norme/nf-iso-103811/qualite-du-sol-echantillonnage-partie-1-lignes-directrices-pour-letablissem/fa041879/21375.
NF ISO 11464-1998, 1998. The standard procedures for soil quality and pre-treatment of samples for physical–chemical analysis. https://www.boutique.afnor.org/fr-fr/norme/nf-iso-11464/qualite-du-sol-pretraitement-des-echantillons-pour-analyses-physicochimique/fa134263/28341.
Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Package 'vegan'. Community Ecol. Package Version 2 (9), 1–295.
Paton, W., Fletcher, P., 2008. Challenges of waste management in environmentally developing countries. In: SPE International Conference on Health, Safety, and Environment in Oil and Gas Exploration and Production. OnePetro.
Piñón-Castillo, H., Brito, E., Goñi-Urriza, M., Guyoneaud, R., Duran, R., Nevarez-Moorillon, G., Gutiérrez-Corona, J., Caretta, C., Reyna-López, G., 2010. Hexavalent chromium reduction by bacterial consortia and pure strains from an alkaline industrial effluent. J. Appl. Microbiol. 109 (6), 2173–2182.
Potts, L.D., Calderon, L.J.P., Gubry-Rangin, C., Witte, U., Anderson, J.A., 2019. Characterisation of microbial communities of drill cuttings piles from offshore oil and gas installations. Mar. Pollut. Bull. 142, 169–177.
Pringault, O., Duran, R., Jacquet, S., Torréton, J.P., 2008. Temporal variations of microbial activity and diversity in marine tropical sediments (New Caledonia Lagoon). Microb. Ecol. 55 (2), 247–258.
Risdon, G.C., Pollard, S.J., Brassington, K.J., McEwan, J.N., Paton, G.I., Semple, K.T., Coulon, F., 2008. Development of an analytical procedure for weathered hydrocarbon contaminated soils within a UK risk-based framework. Anal. Chem. 80 (18), 7090–7096.
Ryzhmanova, Y., Oshurkova, V., Troshina, O., Abashina, T., Ariskina, E., Avtukh, A., Shcherbakova, V., 2017. Anoxynatronum buryatiense sp. nov., an anaerobic alkaliphilic bacterium from a low mineralization soda lake in Buryatia, Russia. Int. J. Syst. Evol. Microbiol. 67 (11), 4704–4709.
Shahi, A., Aydin, S., Ince, B., Ince, O., 2016. Reconstruction of bacterial community structure and variation for enhanced petroleum hydrocarbons degradation through biostimulation of oil contaminated soil. Chem. Eng. J. 306, 60–66.
Sorokin, D.Y., Tourova, T., Muzmann, M., Muyzer, G., 2008. Dethiobacter alkaliphilus gen. nov. sp. nov., and desulfurivibrio alkaliphilus gen. nov. sp. nov.: two novel representatives of reductive sulfur cycle from soda lakes. Extremophiles 12 (3), 431–439.
Sorokin, D.Y., Trotsenko, Y.A., Doronina, N.V., Tourova, T.P., Galinski, E.A., Kolganova, T.V., Muyzer, G., 2007. Methylohalomonas lacus gen. nov. sp. nov. and methylonatrum kenyense gen. nov. sp. nov. methylotrophic gammaproteobacteria from hypersaline lakes. Int. J. Syst. Evol. Microbiol. 57 (12), 2762–2769.

Srinivasarao Naik, B., Mishra, I.M., Bhattacharya, S.D., 2011. Biodegradation of total petroleum hydrocarbons from oily sludge. Bioremed. J. 15 (3), 140–147.
Stauffert, M., Duran, R., Gassie, C., Cravo-Laureau, C., 2014. Response of archaeal communities to oil spill in bioturbated mudflat sediments. Microb. Ecol. 67 (1), 108–119.
Terrisse, F., Cravo-Laureau, C., Noël, C., Cagnon, C., Dumbrell, A.J., McGenity, T.J., Duran, R., 2017. Variation of oxygenation conditions on a hydrocarbonoclastic microbial community reveals Alcanivorax and Cycloclasticus ecotypes. Front. Microbiol. 8, 1549.
Touratier, F., Legendre, L., Vezina, A., 1999. Model of bacterial growth influenced by substrate C: N ratio and concentration. Aquat. Microb. Ecol. 19 (2), 105–118.
Vdovenko, S., Boichenko, S., Kochubei, V., 2015. Composition and properties of petroleum sludge produced at the refineries.
Wu, M., Ye, X., Chen, K., Li, W., Yuan, J., Jiang, X., 2017. Bacterial community shift and hydrocarbon transformation during bioremediation of short-term petroleum-contaminated soil. Environ. Pollut. 223, 657–664.
Xu, X., Hui, D., King, A.W., Song, X., Thornton, P.E., Zhang, L., 2015. Convergence of microbial assimilations of soil carbon, nitrogen, phosphorus and sulfur in terrestrial ecosystems. Sci. Rep. 5 (1), 1–8.
Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., Gao, X., Li, F., Li, H., Yu, H., 2018. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. Front. Microbiol. 9, 2885.
Yang, S.-H., Seo, H.-S., Oh, H.-M., Kim, S.-J., Lee, J.-H., Kwon, K.K., 2013. Brumimicrobium mesophilum sp. nov. isolated from a tidal flat sediment, and emended descriptions of the genus Brumimicrobium and Brumimicrobium glaciale. Int. J. Syst. Evol. Microbiol. 63 (3), 1105–1110.
Yin, S., Li, J., Dong, H., Qiang, Z., 2019. Enhanced nitrogen removal through marine anammox bacteria (MAB) treating nitrogen-rich saline wastewater with Fe (III) addition: nitrogen shock loading and community structure. Bioresour. Technol. 287, 121405.
Zhang, S., Hu, Z., Wang, H., 2019. Metagenomic analysis exhibited the co-metabolism of polycyclic aromatic hydrocarbons by bacterial community from estuarine sediment. Environ. Int. 129, 308–319.
Zhou, H., Huang, X., Bu, K., Wen, F., Zhang, D., Zhang, C., 2019. Fungal proliferation and hydrocarbon removal during biostimulation of oily sludge with high total petroleum hydrocarbon. Environ. Sci. Pollut. Res. 26 (32), 33192–33201.



https://dspace.org/

Enhanced pilot bioremediation of oily sludge from petroleum refinery disposal under hot-summer Mediterranean climate

Said, Olfa Ben; Cravo-Laureau, Cristiana; Armougom, Fabrice; Cipullo, Sabrina; Khelil, Meriem Ben ...

2021-10-23
Elsevier
https://doi.org/10.1016/j.eti.2021.102037
Said OB, Cravo-Laureau C, Armougom F, et al., (2021) Enhanced pilot bioremediation of oily sludge from petroleum refinery disposal under hot-summer Mediterranean climate. Environmental Technology and Innovation, Volume 24, November 2021, Article number 102037

Downloaded from a DSpace repository. https://dspace.org/