

Fuel

Metagenomic analysis of the microbial community at the Riutort oil shale mine (NE Spain): Potential applications in bioremediation and enhanced oil recovery --Manuscript Draft--

Manuscript Number:	JFUE-D-22-11335R2
Article Type:	Research Paper
Keywords:	Riutort microbial consortium, hydrocarbon-degraders, 16S rRNA sequencing, bioremediation, MEOR
Corresponding Author:	Gonzalo Márquez Sevilla, Sevilla SPAIN
First Author:	Gonzalo Márquez, PhD
Order of Authors:	Gonzalo Márquez, PhD Elena GONZÁLEZ-TORIL, PhD Albert Permanyer, PhD José Luis R. Gallego, PhD Erica Lorenzo, PhD Angeles Aguilera, PhD
Abstract:	<p>Preservation of the environment of the Riutort oil shale mine for more than a century has favored the presence of a paradigmatic ecosystem of oil-degrading microorganisms. After extensive sampling and analysis by 16S rRNA sequencing, a marked prokaryotic community comprising diverse groups of bacteria (genus such as <i>Methylobacter</i>, <i>Thiothrix</i>, and <i>Desulfobacca</i>) and archaea (e.g., <i>Methanobrevibacter</i> genus) with hydrocarbon-degrading activity was found. Aerobic microorganisms were predominant in several samples but facultative microorganisms were also present, and there was an interesting transition to strict anaerobic conditions in some areas. One of the samples contained oil degrading aerobic bacteria such as <i>Pseudomonas</i> spp. and <i>Brevundimonas</i> spp. Of the microbes studied, we conducted a laboratory assessment of the capacity of this specific consortium for bioremediation of petroleum-polluted soil and microbial enhanced oil recovery processes. To this end, we used oily sludge-contaminated soil from La Libertad Refinery and cores from the Ancón Field, respectively, both sites in southwestern Ecuador. The Riutort consortium degraded 50.8% of total petroleum hydrocarbons, 64.2% of saturates, 41.3% of aromatics, and 37.4% of polar compounds after a 60-day incubation using oily sludge as the sole source of carbon. The performance of this consortium reflects its notable potential for bioremediation purposes. In turn, flooding with the natural Riutort consortium and its metabolites achieved a 7.2% (v/v) incremental recovery of crude oil through a sand-pack assay. These results are comparable to those reported using synthetic bacterial consortia, and thus reveal the great interest of the study seepages, not only for understanding microbial activities in oil degradation but also their use in biotechnological applications</p>
Suggested Reviewers:	<p>Michel C. Boufadel, PhD Full Professor, New Jersey Institute of Technology boufadel@njit.edu Expertise in Hydrocarbon Bioremediation and Petroleum Microbiology</p> <p>Ibrahim Banat, PhD Senior Researcher, Ulster University im.banat@ulster.ac.uk Expertise in Petroleum Microbiology</p> <p>Eliane Soares de Souza, PhD Senoir Professor, State University of Norte Fluminense eliane@lenep.uenf.br Expertise in Oil Geochemistry and Remediation</p>

Michael Kruge, PhD
Full Professor, Montclair State University
krugem@montclair.edu
Expertise in Oil Geochemistry

Ricardo Amils, PhD
Full Professor, Autonomous University of Madrid
ramils@cbm.csic.es
Expertise in Microbiology

Dear Kevin Van Geem:

I am submitting the last version of the manuscript entitled “**Metagenomic analysis of the microbial community at the Riutort oil shale mine (NE Spain): Potential applications in bioremediation and enhanced oil recovery**” for your considerations in order to be published in Fuel.

Attached you can find graphical abstract, highlights, manuscript, figures, tables and supplementary materials.

Yours sincerely,

Gonzalo Márquez

Dear Kevin Van Geem:

As shown below, the comments of the reviewer 3 have been addressed, whereas minor changes have been followed in the revised manuscript.

Comments	Changes
<i>Tables and figures are not displayed in the revised manuscript.</i>	There must have been a problem with the computer application, I added Figures 1 to 6 and tables 1 to 4 (plus Appendix) to the second version of the manuscript. Again, I have added the same Figures and Tables in the last version of the manuscript.
<i>Methodology flow chart, and mechanisms are not displayed as well.</i>	Among the six figures mentioned in the previous point, a flow chart (Fig. 3) was added. Key EOR mechanisms (viscosity reduction and decrease of interfacial tension) are displayed in the text.
<i>The below article is missing, which is similar to the electrical heating, and the difference is the strength of the generated magnetic field and heat effect: "New insights into the application of a magnetic field to enhance oil recovery from oil-wet carbonate reservoirs".</i>	A new reference has been added.

Note: Please, be aware of changes (**red color**) in the manuscript.

Enclosed you will find the text, figures and tables.

Yours sincerely, Gonzalo Marquez

Seepages containing ecosystems of oil-degrading microbes in the Riutort oil shale mine

Aerobic microbes were predominant in samples but facultative ones were also present

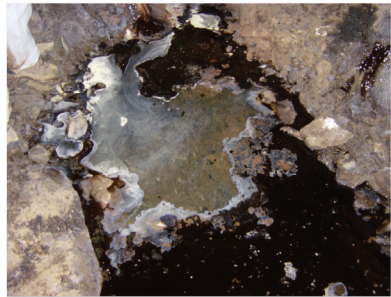
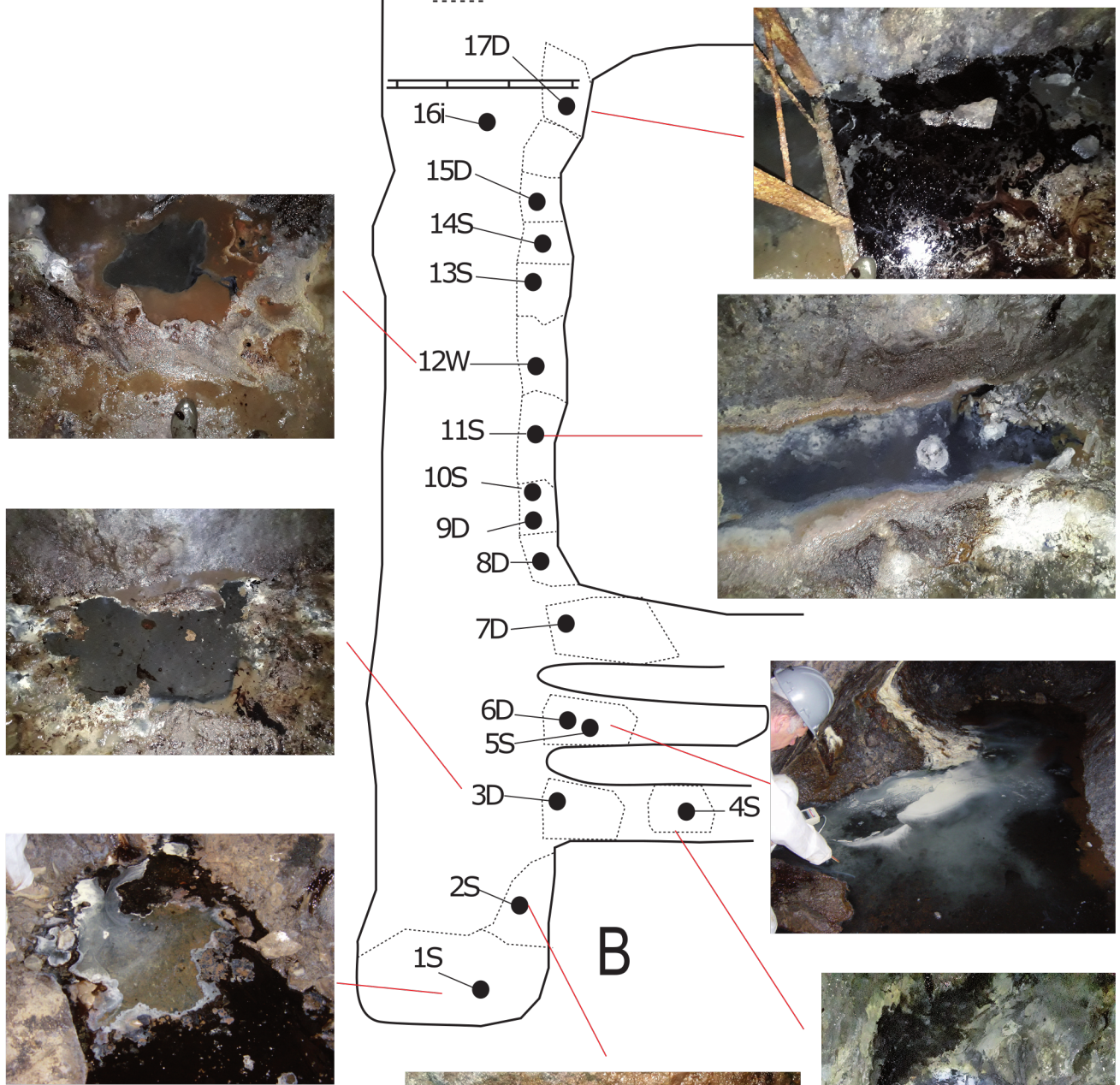
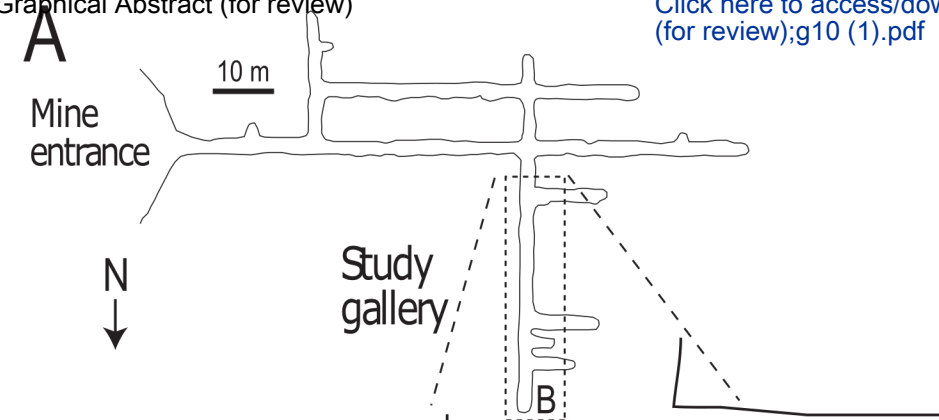
Bioremediation of soil and microbial enhanced oil recovery experiments were conducted

The Riutort consortium degraded 50.8% of TPH in oily sludge from La Libertad Refinery

This consortium and its metabolites achieved a 7.2% incremental recovery of Ancón oil



A



- Gallery door
- Oil-Water pools
- S: surface
- Samples: D: deep
- W: water

1 **Metagenomic analysis of the microbial community at the Riutort oil** 2 **shale mine (NE Spain): Potential applications in bioremediation and** 3 **enhanced oil recovery**

4
5 E. GONZÁLEZ-TORIL^a, A. PERMANYER^b, J.R. GALLEGO^c, G. MÁRQUEZ^{d,*}, E. LORENZO^e and A. AGUILERA^a
6

7 ^aCenter for Astrobiology (INTA-CSIC), Carretera de Ajalvir km 4, Torrejón de Ardoz, 28850 Madrid, Spain

8 ^bDepartment of Mineralogy, Petrology and Applied Geology, Universitat de Barcelona, 08028 Barcelona, Spain

9 ^c INDUROT and Environmental Biogeochemistry & Raw Materials Group, University of Oviedo, 33600 Mieres, Spain

10 ^dCenter for Research in Sustainable Chemistry (CIQSO), University of Huelva, 21006 Huelva, Spain

11 ^eSchool of Engineering Sciences, State University Santa Elena Peninsula, 240204 La Libertad, Ecuador
12

13 **Abstract**

14 Preservation of the environment of the Riutort oil shale mine for more than a century has favored the presence of a
15 paradigmatic ecosystem of oil-degrading microorganisms. After extensive sampling and analysis by 16S rRNA
16 sequencing, a marked prokaryotic community comprising diverse groups of bacteria (genus such as *Methylobacter*,
17 *Thiothrix*, and *Desulfobacca*) and archaea (e.g., *Methanobrevibacter* genus) with hydrocarbon-degrading activity was
18 found. Aerobic microorganisms were predominant in several samples but facultative microorganisms were also present,
19 and there was an interesting transition to strict anaerobic conditions in some areas. One of the samples contained oil
20 degrading aerobic bacteria such as *Pseudomonas* spp. and *Brevundimonas* spp. Of the microbes studied, we conducted a
21 laboratory assessment of the capacity of this specific consortium for bioremediation of petroleum-polluted soil and
22 microbial enhanced oil recovery processes. To this end, we used oily sludge-contaminated soil from La Libertad
23 Refinery and cores from the Ancón Field, respectively, both sites in southwestern Ecuador. The Riutort consortium
24 degraded 50.8% of total petroleum hydrocarbons, 64.2% of saturates, 41.3% of aromatics, and 37.4% of polar
25 compounds after a 60-day incubation using oily sludge as the sole source of carbon. The performance of this consortium
26 reflects its notable potential for bioremediation purposes. In turn, flooding with the natural Riutort consortium and its
27 metabolites achieved a 7.2% (v/v) incremental recovery of crude oil through a sand-pack assay. These results are
28 comparable to those reported using synthetic bacterial consortia, and thus reveal the great interest of the study seepages,
29 not only for understanding microbial activities in oil degradation but also their use in biotechnological applications.
30

31 **Keywords:** Riutort microbial consortium, hydrocarbon-degraders, 16S rRNA sequencing, bioremediation, MEOR.
32

33 **1. Introduction**

34 The biodegradation of hydrocarbons alters the properties of these compounds and can bring about
35 significant economic losses for the petroleum industry [1]. Microbial oxidation of hydrocarbons

Corresponding author: G. Márquez (gonzalo.marquez@diq.uhu.es)

36 (petroleum biodegradation) generally occurs at temperatures below 75–80°C; i.e. above this range
37 there is scarce microbial life and only hyperthermophilic bacteria and archaea grow [2-5]. Meteoric
38 water influx can cause aerobic biodegradation processes and also remove more water-soluble
39 compounds [6]. However, anaerobic hydrocarbon breakdown is the common process of
40 biodegradation in most petroleum reservoirs [7-8]. Prior studies have verified the predominance of
41 bacteria and archaea in anaerobic processes controlling subsurface hydrocarbon degradation in
42 reservoirs. These processes are linked mostly to methanogenesis and iron reduction [9-11] or, when
43 free sulfate is abundant, to sulfate reduction [12]. Conversely, aerobic microbes are the main agents
44 of surface biodegradation processes after oil spills [13], but also in oil reservoirs whenever they are
45 shallow and contain oxygenated waters. However, anaerobic bacteria may initiate biodegradation
46 under certain conditions [14]. In addition to free or combined oxygen, microbes in shallow
47 environments need water with salinity levels < 100-150‰ and enough nutrients to metabolize
48 hydrocarbons [5] as the degradation of these compounds is frequently limited by phosphorus or
49 nitrogen [9]. Irrespective of whether the process is aerobic or anaerobic, the impact of microbial
50 degradation on crude oil can be notable [3,15]. As biodegradation progresses, it leads to a decrease
51 in oil quality, diminishing its economic value as API gravity decreases, while increasing viscosity
52 and density [5]. Interestingly, the compositional changes caused by biodegradation of petroleum in
53 reservoirs are similar to those observed in surface oil seeps and oil spills [9,16]. They result in a
54 lowering of saturated hydrocarbons and, to a lesser extent, aromatic compounds, thereby
55 concentrating resins and asphaltenes in the residual oil, while also enriching metal and sulfur
56 content [17]. In addition, biodegradation causes an increase in the acidity of the oil [18].

57

58 Hydrocarbons are magnificent growth media for microorganisms [19], and one of the challenges in
59 petroleum microbiology is to identify the microbial diversity in oilfields and hydrocarbon-
60 containing habitats. Although a major research focus has been placed on anaerobes, microbial

61 populations in shallow oil reservoirs also include aerobic microbes introduced through aqueous
62 fluid injection, drilling muds, and natural flows of surface water and groundwater [20]. Aerobic
63 hydrocarbon-degrading and biosurfactant-producing microbial populations are, for the most part,
64 advantageous for bioremediation processes, both in marine and terrestrial spills [21-22], and for
65 microbial enhanced oil recovery (MEOR) operations [23]. In both technologies, microbial consortia
66 can give yields that are greater than those achieved by a single species, as consortia benefit from
67 interrelations between distinct types of microbes [24-25].

68

69 In the SE Pyrenees (Cadí thrust sheet, NE Spain), the organic-rich materials of the Armàncies unit
70 (Eocene) are the most important oil source rock, cropping out for more than 100 km from near the
71 Terrades sector in the east to beyond the village of Bagá in the west (Fig. 1). Part of the Armàncies
72 Formation is characterized by a significant period of anoxia, which induced sedimentation and
73 conservation of organic matter [26]. The abundance of oil shows in outcropping materials from the
74 Cadí thrust sheet generated interest in this area for oil exploration. In this regard, the Riutort oil
75 shale mine was opened in 1912 and operated for around 10-15 years, producing approximately 3400
76 tons of rock during this time [27]. The main underground gallery (74 m long, still accessible) cuts
77 into the Armàncies Formation that has a thickness of 58 m, of which 50 m are oil shales. The slow
78 percolation of groundwater through the mine galleries has resulted in pools of water of various
79 sizes. Oil is present in some of these pools and is accompanied by the growth of microbial mats [28-
80 30]. The biodegradation of seepage oil at level 3-4 on the PM scale [15] can be observed in situ, as
81 attested by the presence of hydrocarbon-degrading bacteria [31]. Increasing levels of CO₂ in the air
82 towards the bottom of the galleries account for predominantly aerobic biodegradation processes,
83 while darkness has excluded the growth of photosynthetic microbes. The environment of the Riutort
84 oil shale mine has allowed oil biodegradation processes to take place for more than a century, and
85 thus the microbial ecology of this site is of great interest [31]. In this regard, the characterization of

86 microbial communities in oil-containing ecosystems can be used to elucidate the role of microbes in
87 relevant biogeochemical processes [32] and may also be useful to enhance bioremediation and
88 MEOR technologies [33-34].

89 *Figure 1*

90 Given the preceding considerations, here we sought to: (i) study in detail the microbial communities
91 involved in the biodegradation processes at the Riutort oil shale mine; ii) examine the impact of
92 using the Riutort microbes for hydrocarbon bioremediation; and (iii) evaluate the potential of the
93 natural Riutort microbial consortium for MEOR in shallow reservoirs. The use of non-subsurface
94 microbial populations such as the Riutort consortia can provide a novel and eco-friendly option for
95 improving hydrocarbon production from mature oilfields during the energy transition [35-36].

96

97 **2. Materials and methods**

98 **2.1. Sample collection and initial characterization**

99 Several underground galleries were identified in the Riutort oil shale mine (Fig. 2a). In particular,
100 the “study” gallery (Fig. 2b), located around 70 m from the mine entrance with 12 °C of constant
101 temperature, was selected as a sampling site due to the preservation of oil seeps (in oily-water
102 pools). To analyze microbial diversity within the pools on the gallery floor, we collected 17 water
103 samples belonging to four types (see Fig. 2b): 8 were surface pool samples (S), 7 deeper pool
104 samples (D), 1 water sample (W), and 1 isolated sample (I) from a pool located away from the
105 gallery walls and characterized by zero seepage flow rate and a lower proportion of water. In all
106 cases, 50 ml of sample was taken in sterile tubes and kept at -20°C until processing.

107

Figure 2

108 For bioremediation trials, oily sludge samples were taken from La Libertad Oil Refinery (longitude
109 80°53’56” W, latitude 2°13’32” S) in southwestern Ecuador, where the average temperature is
110 around 25 °C. Oily sludge is a typical waste of oil refineries, and it consists of a mixture of

111 hydrocarbons, water, and sediments [37]. The amount of total petroleum hydrocarbons (TPH)
112 recovered was determined gravimetrically as described by Mishra and co-workers [38] and
113 accounted for approximately 60% of total TPH, 34% water, and 6% sediments. TPH were
114 fractionated into saturates, aromatics, asphaltenes, and resins (SARA fractioning) by liquid
115 chromatography in a column containing silica gel (activated in an oven at 105 °C for 12 h). In brief,
116 asphaltenes were separated as the insoluble fraction by dissolving 1 g of TPH in *n*-pentane and
117 using 0.45 µm filters. The saturated, aromatic, and resin fractions were then eluted with *n*-hexane,
118 dichloromethane:hexane (4:1, v/v), and dichloromethane:methanol (1:1, v/v), respectively [13].
119 Solvents were evaporated at room temperature under a nitrogen stream in a fume hood, and the
120 SARA fractions were determined gravimetrically (45%, 29%, 5%, and 21% respectively). All
121 measurements were conducted in triplicate. The soil used in the bioremediation trials described in
122 section 2.3.3 was collected from the surface top layer (15 cm depth) in the surroundings of the
123 refinery mentioned above. Samples were homogenized through a 1-mm sieve to remove roots and
124 larger particles [39]. Organic carbon (0.97%), total nitrogen (0.03%), and bulk density (1.6 g/ml)
125 were then determined using standard methods [40-41]. A pH value of 8.0 was also measured in a
126 mixture of soil and water (1:1).

127

128 In turn, for the MEOR tests, rock cores, formation water, and crude oil samples were collected at a
129 depth of 343 m from the ANC-0008 well (80°51'55" W, 2°19'08" S) of the Ancón oilfield in
130 southwestern Ecuador. The physico-chemical properties of the formation fluids were examined
131 using 200-ml samples, which were first filtered. Organic compounds were then extracted using Sep-
132 Pak C18 solid phase extraction cartridges (Waters Chromatography). Following the procedure
133 described above, the initial SARA composition of the crude oil (32°API and viscosity of 3.15 mPa·s
134 at 37°C) from the ANC-0008 well was 69% saturates (SAT), 22% aromatic hydrocarbons (ARO),
135 and polar (POL) compounds (3% resins and 6% asphaltenes). Crude oil viscosity was measured

136 using an Oswald viscometer maintained at a constant temperature (37°C) in a water bath. Three
137 repeated measurements were performed at a 100 s⁻¹ shear rate. Finally, salinity, pH, and electrical
138 conductivity of the formation water were measured with a TPH 02154 device, a BASIC 20 pH
139 meter, and an EC Meter BASIC 30+, and the main ions were determined in an ion chromatograph
140 883 Basic IC Plus (Metrohm). As shown in Table 1, our data indicated low salinity and slightly
141 alkaline pH for these formation waters (Socorro reservoir in the Ancón field).

142 *Table 1*

143 **2.2 Assessment of microbial communities**

144 *2.2.1 DNA extraction, amplification and sequencing, and OTU detection*

145 Genomic DNA was extracted from 1 ml of representative homogenized subsamples using the
146 FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC) following the manufacturer's instructions
147 and it was quantified by PICOGREEN®. The extracted DNA was used in a first PCR of 27 cycles
148 with Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs) in the presence of 100
149 nM primers for 16S rRNA amplification. The primers used amplify the V3-V4 region of 16S rRNA
150 Archaea or Bacteria domain [42]. Each sample was amplified and, after the first PCR, a second
151 PCR of 14 cycles was performed with Q5® Hot Start High-Fidelity DNA Polymerase (New
152 England Biolabs) in the presence of 400 nM of the primers (5' AATGATACGGCGACCACCGAG
153 ATCTACACTGACGACA TGGTTCTACA-3' and 5'-CAAGCAGAAGACGGCATACGAG AT-
154 [barcode]-TACGGTAGCA GAGACTTGGT CT-3') of the Access Array Barcode Library for
155 Illumina Sequencers (Fluidigm). Finally, the pool of amplicons was denatured before being seeded
156 on a flow cell at a density of 10 pM, where clusters were formed and sequenced using a "MiSeq
157 Reagent Kit v3", in a 2x300 pair-end sequencing run on a MiSeq sequencer.

158

159 For the detection and classification of OTUs (operational taxonomic units), sequence processing,
160 alignment and analysis were carried out with Mothur v.1.38.1 [43-44] and The Basic Local

161 Alignment Search Tool (BLAST) [45]. The databases used were SILVA-SEED (v138) [46] and the
162 Genome Taxonomy Database [47]. Chao 1 estimator and Shannon index were calculated to predict
163 community richness using PAST software 50 [48].

164

165 *2.2.2. 16S rRNA gene amplification and library construction*

166 The microbial species of the Riutort-16I sample were further studied by 16SrRNA gene
167 amplification and cloning. Amplification of nearly full-length bacterial and archaeal 16S rRNA
168 genes was carried out with the primer sets 8F/1492R [49] and 25F/1492R [50]. Each 50- μ L reaction
169 tube contained 20–30 ng of template DNA, 1 \times PCR reaction buffer (Promega Biotech Iberica,
170 Spain), 2.5 μ M of each dNTP (Amersham Biosciences, UK), 2.5 mM of MgCl₂, 1 mg mL⁻¹ of
171 bovine serum albumin, 500 mM of the forward and reverse primers, and 0.025 U μ L⁻¹ of DNA Taq
172 polymerase (Promega Biotech). PCR reactions were performed in a Perkin Elmer Thermocycler
173 with the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of
174 denaturation at 95°C for 1 min, annealing at 49°C for the bacterial primer set and 52°C for the
175 archaeal primer set, and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was
176 performed. Amplified 16S rRNA gene products (> 1400 bp) were cloned using the TOPO TA
177 Cloning Kit (Invitrogen, CA) and sequenced using a Big-Dye kit (Applied Biosystems), following
178 the manufacturer's instructions. Taxonomic assignation was performed by BLAST
179 (<https://blast.ncbi.nlm.nih.gov/>).

180

181 *2.2.3. Statistical analyses*

182 Principal Component Analyses (PCA) on the relative proportion of OTUs (at a distance of 0.03)
183 among the samples were performed. Using the three components as new variables, hierarchical
184 dendrograms by Euclidean squared distance method were also calculated (IBM SPSS Statistics 24
185 package). Using relative percentages, new PCA analyses were performed by CANOCO 4.5

186 (Microcomputer Power, Ithaca, NY, USA) [51]. The program CANODRAW 4.0 (in the Canoco
187 package) was used for graphical presentation of ordination results. Distribution analyses of OTU
188 abundance were done in Tableau Software (www.tableau.com).

189

190 **2.3 Biotechnological applications**

191 *2.3.1. Preparation of inoculums*

192 The microbial populations present in the Riutort-16I water pool sample (see results) were picked up
193 from oil agar plates and added to 50 ml basal medium [(grams per liter of brine): glucose (10),
194 sodium nitrate (1)]; and [(mg per liter of brine): manganese sulfate (0.25), magnesium sulfate (2.5),
195 zinc sulfate (0.25), copper sulfate (0.025), ferrous sulfate (0.25), aluminum potassium sulfate
196 (0.025), sodium molybdate (0.025), sodium selenate (0.013), nickel chloride (0.075) and boric acid
197 (0.025)]. They were incubated under aerobic conditions in a shaker at 37°C and 150 rpm [52]. Cell
198 growth was monitored by measuring the optical density at 600 nm through a UV-2550 Shimadzu
199 spectrophotometer [53]. When the culture reached the late exponential phase of growth, the cells
200 were resuspended in sterile basal medium, which was incubated at 37°C (rotatory shaker, 150 rpm)
201 for 24 h. Agitation and aeration were maintained using glass gas dispersion tubes with fritted
202 cylinders (Fisher Scientific). Microbial populations were counted using the most probable number
203 (MPN) method [54]. Each dilution was placed on a nutrient agar plate at 25°C. The number of
204 CFUs (Colony-Forming Units) at each dilution rate was counted after incubation and the average
205 CFU/ml or g of soil was determined. Figure 3 shows the overall experimental workflow.

206

Figure 3

207 *2.3.2. Consortium properties*

208 To determine surface tension (ST) reduction caused by the selected consortium, 1% (v/v) of
209 Riutort-16I consortium was added to a 250 ml Erlenmeyer flask containing 100 ml of sterile basal
210 medium supplemented with 1% (w/v) of La Libertad Refinery's oily sludge and placed in a shaker

211 incubator at 25°C and 150 rpm. Aliquots of this culture broth were sampled at different intervals
212 over 16 days. Cells were harvested by centrifugation at 9000 rpm and 4 °C for 15 min and then
213 discarded. Finally, the surface tensions of the supernatants were measured by a Gibertini
214 tensiometer (TSD 132389), following the method described by Abouseoud and co-workers [55].
215 Interfacial tension (IFT) determinations were performed against 1% (v/v) of ANC-0008 crude oil in
216 the same way at 37 °C (the well's bottom-hole temperature). Measurements were carried out in
217 triplicate.

218

219 The hydrophobicity of hydrocarbonoclastic microbial cells from the Riutort-16I sample was
220 measured through the Bacterial adhesion to hydrocarbons (BATH) assay, following the method
221 previously reported [56]. Phosphate urea magnesium sulfate (PUM) buffer [pH equals 7.1 and
222 composition (g per liter): $K_2HPO_4 \cdot 3H_2O$ (22.2), KH_2PO_4 (7.26), urea (1.8) and $MgSO_4 \cdot 7H_2O$ (0.2)]
223 was used to wash the cells twice and suspend them to obtain an optical density (OD) value of 0.6 at
224 600 nm. Thus, 2 ml of the cell suspension was mixed with 3 ml of *n*-hexadecane, and then the
225 mixture was vortexed for 2 min. Finally, *n*-hexadecane and the aqueous phase were held at room
226 temperature for 30 min to separate the phases. OD at 600 nm was quantified in the latter phase.
227 Hydrophobicity is expressed as the percentage of adherence to hexadecane, and it was calculated as
228 follows: $100 \cdot [(1 - OD_{600} \text{ of the aqueous phase}) / (OD_{600} \text{ of the initial cell suspension})]$. This analysis
229 was done in triplicate.

230

231 Emulsification activity was also used as an indicator of biosurfactant production in the culture broth
232 to determine a relationship between ANC-0008 crude oil biodegradation and biosurfactant
233 production [23]. The emulsion stability of the culture samples was determined by adding 3 ml of *n*-
234 hexadecane to 2 ml of culture supernatant in glass test tubes, shaking vigorously using a vortex for

235 2 min, and later incubating at 37 °C for 24 h. The emulsification index (E24) was calculated as the
236 percentage of the emulsified layer height divided by the total height of the liquid column [57].

237

238 2.3.3. *Bioremediation trials*

239 The biodegradation of the oily sludge from La Libertad Refinery was examined using three
240 approaches: natural attenuation, biostimulation, and combined biostimulation-bioaugmentation.
241 These trials were carried out in 250-ml sterile Erlenmeyer flasks containing 100 ml of soil spiked
242 with 1% (w/v) oily sludge and mixed thoroughly for 72 h. The natural attenuation trial was done
243 without any amendment, whereas biostimulation was carried out by adding 5% (v/v) sterile basal
244 medium, and biostimulation+bioaugmentation with 5% (v/v) basal medium including the microbial
245 inoculum from Riutort-16I. The experiments were done at 25°C (Santa Elena Peninsula's annual
246 mean temperature). As soil moisture content influences the biodegradation of petroleum compounds
247 [11,58], it was maintained around 60% throughout the experiment. TPH extraction and SARA
248 fractionation were done at 30 and 60 days. All tests were performed three times.

249

250 2.3.4. *Oil recovery tests*

251 Core-flooding experiments were performed to study the efficacy of the Riutort-16I microbial
252 consortium to enhance oil recovery when compared to conventional water flooding. Lab-scale
253 simulations for oil recovery were conducted following the procedure described by Gao and co-
254 workers [59]. Two core tubes, 50 cm long and 2.5 cm inner diameter, were filled with sandstone of
255 the Socorro Formation of the Ancón Field by mechanical loading. After packing with sand,
256 cleaning, and drying [60], each core tube was saturated with formation water from the ANC-0008
257 well, at a flow rate of $0.25 \text{ cm}^3 \cdot \text{s}^{-1}$, then a 0.7 pore volume (PV) was determined using the dry-
258 weight and wet-weight of the cores. Formation water was previously passed through a 0.45- μm
259 Millipore Filtration Unit [61]. Each core was then flooded with dehydrated ANC-0008 crude oil to

260 irreducible water saturation, as observed in petroleum reservoirs; the total volume of injected crude
261 oil was recorded. Both core tubes were allowed to soak at 37 °C for a week. One of the cores was
262 then injected with several pore volumes of the ANC-0008 formation water to simulate the
263 secondary oil recovery stage until reaching a water-cut level of 98%. The volume of oil recovered
264 by the formation water flooding was then determined. Finally, to simulate the tertiary recovery
265 process, microbial flooding was carried out by injecting 0.3 PV of the natural Riutort-16I
266 consortium (10^7 CFU/ml) mixed with nutrient basal medium (1.0%) in water into the second core
267 tube, which was then closed for 4 days. After that, the core was injected with water until no more
268 oil was released. The additional oil recovered by the microbial flooding was recorded. The
269 secondary and tertiary recoveries were calculated as a percentage of the amount of oil recovered in
270 each case in relation to the total mass of crude oil injected. The tests were carried out at 37 °C to
271 simulate the bottom-hole temperature of the Ancón-0008 well, with a displacement pressure of
272 about 4 MPa. All tests were done in triplicate.

273

274 **3. Results**

275 **3.1. Microbial diversity**

276 *3.1.1. Sequencing analysis and estimated richness*

277 The collections of amplicons were sequenced, and the number of reads is summarized in Appendix.
278 Using bacterial-specific primers, a total of $\sim 2 \times 10^6$ reads were obtained (about 120,000 per sample
279 on average), whereas using archaeal-specific primers a total of $\sim 4 \times 10^6$ reads were achieved
280 ($\sim 250,000$ per sample). Analysis of the read length distributions indicated a modal distribution
281 between 442 and 444 nucleotides. After quality control checks, chimera sequence removal, and the
282 subtraction of control sequences, a total of $\sim 1.5 \times 10^6$ sequences remained, about 86,000 per sample
283 on average in the case of the Bacteria domain, and 700,000 with about 40,000 per sample on
284 average for archaeal-specific primers. The number of bacterial OTUs detected at a distance of 0.03

285 ranged from 255 to 477 per sample and from 4 to 16 per sample in the case of archaeal OTUs.
286 Nevertheless, Chao1 estimations suggest that we were able to identify between 52 and 79% of the
287 total bacterial OTUs and between 53 and 97% of the total archaeal OTUs.

288

289 The bacterial community along the gallery showed a high diversity (Shannon index > 3; Appendix)
290 and multiple phenotypes. Shannon indices higher than 4 were detected in every pool sampled
291 through the gallery. Sample 16I showed the lowest value (4.42) and 11S the highest (5.04), with a
292 low deviation (0.16). With respect to *Archaea*, the Shannon indices showed a lower diversity. The
293 lowest value was detected in 5S (1.21) and the highest in 6D (2.47), with a deviation of 0.3.
294 Correlation networks of microbial populations and metabolic role of *Bacteria* and *Archaea* related
295 with oily-water pools in the Riutort oil shale mine were also studied (see Supplementary Material).

296

297 3.1.2. Bacterial community profiles

298 Taxonomic analyses of bacterial sequences were analyzed using the SILVA classifier algorithm
299 against SILVA.SEED V138 16S rRNA database. Reads were allotted to OTUs with an identity
300 cutoff of $\geq 97\%$, which were further assigned to higher taxa: 1 domain (*Bacteria*), 41 phyla, 119
301 classes, 287 orders, 460 families, and 746 genera. Most of the *Bacteria* were distributed among the
302 phyla *Proteobacteria*, *Planctomycetota*, *Bacteroidota*, *Verrucomicrobiota*, *Desulfobacterota*,
303 *Nitrospirota*, *Patescibacteria*, *Chloroflexi*, *Acidobacteriota*, *Firmicutes*, *Actinobacteriota*,
304 *Myxococcota* and *Dependentiae*, which accounted for $\geq 80\%$ of the total *Bacteria* within each
305 sampling site (Fig. 2). *Proteobacteria* were the most relative abundant group in all pools, except for
306 6D, where *Desulfobacterota* was predominant. The *Gammaproteobacteria* class dominated among
307 the *Proteobacteria* phylum and, to a lesser extent, *Alphaproteobacteria*. More precisely,
308 *Gammaproteobacteria* were predominant in 1S, 2S, 4S, 5S, 8D, 9D, 10S, 11S, 13S, 15D, and 16I
309 (between 14 and 37%). *Verrucomicrobiota* was the most relatively abundant phylum in 7D (15%),

310 14S (13%), and 17D (15%). *Planctomycetota* was the most abundant in 3D (15%) and 12W (23%).
311 Finally, only in 6D (25%) was *Desulfobacterota* the phylum with the most relative abundance.
312 Further analysis of the *Proteobacteria* community revealed a high proportion of unidentified
313 *Gammaproteobacteria* (between 3 and 17% in the different pools) and *Alphaproteobacteria* (1-
314 9%). With regard to *Alphaproteobacteria*, it is interesting to note the presence of *Brevundimonas* in
315 16I. Within the *Gammaproteobacteria* that had been identified, there were members of the
316 *Burkholderiales* order, the *Comamonadaceae* and *Methylomonadaceae* families, and the genera
317 *Methylobacter*, *Pseudomonas* and *Thiothrix*. The *Methylobacter* genus includes aerobic
318 methanotrophic bacteria and was detected (1-3%) mostly at the beginning of the gallery (8D, 9D,
319 10S, 11S, 12W, 13S, 15D, 16I, and 17D). Up to 7% of the *Pseudomonas* genus was detected in pool
320 16I, but was negligible in the other pools. The OTUs of *Thiothrix*, an aerobic sulfur-oxidizing
321 bacterium, were detected in only two pools, namely 5S (6%) and 11S (1%). A large number of
322 *Planctomycetota* resulted unclassified (1-5%), and between 1 and 3% of OTUs of the *Pirellulaceae*
323 family were also detected. With respect to *Desulfobacterota*, the third most abundant phylum, again
324 a good part of the sequences remained unclassified (between 1 and 6%). A high percentage (1-3%)
325 of unidentified microorganisms was also found at the *Desulfosarcinaceae* family level. Finally,
326 *Desulfobacca*, *Desulfocapsa*, and *Smithella* were the dominant genera of *Desulfobacterota*. The
327 sulfate-reducing bacteria (SRB) *Desulfobacca* was detected at between 1 to 7% in 1S, 2S, 3D, 5S,
328 6D, 7D, 8D, 9D, 10S, 11S, 14S, 15D, and 17D. *Desulfocapsa* was detected in high numbers (1-3%)
329 of sequences in 1S, 2S, 5S, 6F, 7F, 11S, and 13S, and the anaerobic propionate-degrading
330 syntrophic *Smithella* was found in important numbers (between 1-3%) in only 5 pools: 1S, 3D, 5S,
331 6D and 7D. This genus has been frequently detected in oil environments [62]. All sequences from
332 the *Nitrospirota* group were related to unclassified *Thermodesulfovibrionia*, which are mainly
333 anaerobic, moderately thermophilic, and SRB; they were detected mostly (1-4%) in 3D, 6D, 8D,
334 9D, 14S and 17D. In summary, 4S was the only sample in which no SRB were detected, and in

335 12W and 16I these bacteria were scarce, with only 1% *Desulfobacterota* and 1%
336 *Desulfosarcinaceae*, respectively. The *Verrucomicrobiota* group included sequences belonging to
337 the *Candidatus* “Omnitrophus” (1-6%) and *Candidatus* “Protochlamydia” (1-6%), and also part of
338 the sequences remained as unclassified *Chlamydiales* (1-4%). For a better understanding, Figure 4
339 summarizes the relative proportions of the most representative bacterial genera (those with a
340 relative abundance >3% in at least one sample).

341 *Figure 4*

342 The PCA for bacterial phyla and *Proteobacteria*-class level was conducted and based on number of
343 genus-level OTUs; three components accounted for 89% of the variance. The point cloud in Figure
344 5a shows how the D points (deep samples) tend to cluster together. In contrast, the S (surface)
345 points are more dispersed, except 14S, which joins 3D and 8D robustly. Various clusters are marked
346 in the dendrogram in Figure 5b.

347 *Figure 5*

348 3.1.3. Archaeal community profiles

349 Archaeal sequences were also taxonomically classified by SILVA.SEED V138 16S rRNA database.
350 Again, reads were allotted to OTUs with an identity cutoff of $\geq 97\%$, and these were further
351 assigned to higher taxa: 1 domain (*Archaea*), 5 phyla, 12 classes, 15 orders, 18 families, and 23
352 genera. Samples 1S (44 OTUs), 2S (51), 4S (13), 5S (17), and 7D (42) showed the lowest data and
353 15D and 6D the highest. In general, deep pools (D) showed the largest number of OTUs. The
354 majority of the archaeal sequences resulted unclassified (53-89%). Overall, a very low number of
355 sequences were identified. Sample 14S showed the most OTUs (314), and 2S, 5S and 4S the fewest
356 (8, 8, and 6 respectively). With the exception of 14S, the deep samples were the ones in which most
357 archaeal OTUs were identified. The OTUs detected belonged to only 5 phyla according to SILVA's
358 taxonomic classification v138 [46], namely *Halobacterota* (377 OTUs in total), *Thermoplasmata*
359 (377), *Crenarchaeota* (186), *Asgardarchaeota* (53) and *Euryarchaeota* (10). Note that although

360 *Halobacterota* and *Thermoplasmatota* showed the same relative abundance, only *Halobacterota*
361 was identified at all the sampling sites. A large proportion of the *Halobacterota* sequences were
362 phylogenetically affiliated to methanogenic archaea. Especially abundant were those affiliated to
363 the genus *Methanoregula*, which occurred in all sampling sites except 5S and 12W. This genus was
364 especially abundant in 2S (25%). The rest of the methanogens of this phylum were related to an
365 unclassified *Methanosarcinales* order (up to 53% in 1S). The rest of the *Halobacterota* sequences
366 were affiliated to the ANME-1 group (genus level). This anaerobic and methanotrophic genus was
367 detected at all the sampling sites except 1S, and it was especially abundant in 14S, where it showed
368 the highest relative abundance (64%). A large number of *Thermoplasmata* resulted unclassified (1-
369 39%). This clade was detected in all samples except 5S. In addition, a significant number of
370 sequences were taxonomically affiliated to *Candidatus Proteinoplasmatales* archaeon SG8-5 (1-
371 38%), an uncultured benthic archaeon, probably methanogenic [63]. Finally, 20% of the archaeal
372 sequences detected in 6D and 6% in 3D were affiliated to *Methanomethylophilaceae*, a
373 methanogenic anaerobic archaeal *Thermoplasmatota*. *Crenarchaeota* sequences were grouped in
374 three clades: *Bathyarchaeia*, *Nitrosopumilales*, and unclassified *Crenarchaeota*. In 1S, 2S and 10S,
375 *Crenarchaeota* were not identified. Representatives of *Bathyarchaeia* were found up to 36% in 7P,
376 while representatives of *Nitrosopumilales* accounted for 80% of the sequences obtained in 12W.
377 Most of the *Asgardarchaeota* sequences were related to *Lokiarchaeia*; this group was detected in 11
378 pools. In the case of 5S, 50% of the sequences were affiliated to *Lokiarchaeia*, although it should be
379 noted that very few archaeal OTUs (17) were obtained in this sample. In addition to *Lokiarchaeia*, a
380 significant number of *Asgardarchaeota* remained unclassified (up to 25%, again in 5S).
381 Furthermore, 2% of the sequences obtained in 6D were classified as *Odinarchaeia*. Members of
382 phyla *Euryarchaeota* were found only in 1S, 2S, 6D, 8D, 9D, 13S, and 15D. All sequences were
383 phylogenetically affiliated to the methanogenic genus, most of them to *Methanobrevibacter* and a
384 minority to *Methanobacterium* and *Methanofollis* (only 1% in 8D). Figure 6 summarizes the

385 relative proportions of the most representative archaeal genera (those with a relative abundance
386 >3% in at least one sample).

387 *Figure 6*

388 *3.1.4. 16S rRNA gene amplification and library construction*

389 Upon completing the study of the microbial communities, we focused on the selection of an
390 appropriate consortium for biotechnological applications. In this regard, the high percentage of
391 *Pseudomonas* (7%) detected in sample 16I from the NGS results was outstanding, as the potential
392 of these bacteria in oil biodegradation is well known [64-65]. We therefore chose sample 16I.

393
394 To precisely identify species of Riutort-16I, bacterial 16S rRNA gene amplification and cloning
395 was carried out. A total of 96 clones were sequenced and classified using BLAST. 31% of the
396 OTUs showed similarity to *Pseudomonas marincola* strain 002-Na3, 19% to an uncultured
397 bacterium clone (*Pseudomonas* sp.), 13% to *Brevundimonas mediterranea* strain N7, 12% to
398 uncultured *Desulfobacula* sp. clone LU2-210 (sulfate-reducing bacteria), 12% to the uncultured
399 *Gammaproteobacteria* clone 91-13 (probably sulfur-oxidizing bacteria), 6% to *Microbacterium* sp.
400 BA45(2011), and finally 6% to *Mycobacterium fluoranthenvivorans*. Therefore, 50% of the OTUs
401 obtained showed a high similarity to two *Pseudomonas* previously detected in oil-related
402 environments and their degradation [64-65]. The rest of the OTUs found were also similar to
403 bacteria related to the same environments, e.g., *Microbacterium* sp. and *Brevundimonas*
404 *mediterranea* strain N7. In addition, these two species were previously identified in the Riutort oil
405 shale mine [30-31]. Table 2 lists the OTUs obtained and the closest relatives identified by BLAST.

406 *Table 2*

407 **3.2. Bioremediation approaches**

408 Surface tension (ST) determinations indicated that both indigenous microbes and the combination
409 of these and exogenous Riutort microorganisms were able to reduce the ST of basal medium

410 supplied with 1% (w/v) oily sludge as the sole source of carbon. In fact, the ST of liquid media was
411 reduced from 62 to 54 and 45 mN·m⁻¹ after 16 days, respectively, without and with the injection of
412 the Riutort consortium. Cell hydrophobicity (BATH assay) of this consortium was 60% under the
413 tested conditions, suggesting that the microbial community has hydrophobic surface properties.
414 Simulated bioremediation of soil polluted with oily sludge from La Libertad Refinery was also
415 evaluated. As shown in Table 3, the greatest TPH degradation after 60 days (50.8%) occurred
416 through a combination of biostimulation and bioaugmentation, notably improving biostimulation
417 (29.8%) and natural attenuation yields (16.5%). The results also revealed the habitual features of
418 differential removal of hydrocarbon fractions [21,25], as in all the trials saturates were degraded
419 more easily and more quickly than aromatics and polar compounds (Table 3). Bioaugmentation
420 showed a general pattern of improved results for all the fractions. For instance, the minimum and
421 maximum removal of saturated compounds (24.5 and 64.2%) was achieved via natural attenuation,
422 and bioaugmentation + biostimulation, respectively, in the 60-day experiment. Similar observations
423 were made for aromatics (16.4 vs. 41.2%) and polar compounds (2.8% vs. 37.4%). All chemical
424 data were coherent with microbial counts, which revealed a strong increase (several orders of
425 magnitude) in the bioaugmentation testing when compared with the other two trials (see Table 4).

426 *Table 3*

427 *Table 4*

428 **3.3. Sand-pack study**

429 In this research, when the exogenous Riutort consortium and nutrients were injected into ANC-0008
430 crude oil, the emulsifying activity (E24) of the bioproducts generated was confirmed. It was noted
431 that emulsification power against *n*-hexadecane increased and reached a maximum value of 69%.
432 Also, the interfacial tension (IFT) of the crude-oil water system decreased from 38 to around 21
433 mN·m⁻¹, thus strongly suggesting microbial production of metabolites such as biosurfactants and

434 bioemulsifiers. Another important outcome was that the viscosity of the crude oil decreased 1.25-
435 fold after biological treatment with the exogenous Riutort microbial population (2.51 mPa·s).

436

437 Core-flooding experiments were also conducted to evaluate the potential application of the natural
438 Riutort consortium and their bioproducts for MEOR. Crude oil recovery after formation water
439 flooding was about 40% v/v of original oil in place, whereas a significant 7% v/v of additional oil
440 over the water flooding residual oil saturation was recovered from the column flooded with the
441 Riutort consortium and nutrients. Both lab-scale sand-pack columns can be considered a two-
442 dimensional model to calculate crude oil recovery [23].

443

444 **4. Discussion**

445 **4.1. Microbial communities**

446 We collected a total of 17 samples for microbial diversity analyses from the water pools present on
447 the gallery floor. Our results showed a typical community from a petroleum reservoir, including
448 microorganisms with aerobic, anaerobic, and facultative metabolisms (see Supplementary Material).
449 The microbial degradation of hydrocarbons from crude oil occurs under both oxic and anoxic
450 conditions. Oil degradation was long considered to be an aerobic process because oxygen was
451 deemed to be critical for hydrocarbon activation. This view was dismissed in the late 1980s after the
452 isolation of bacteria capable of growing on hydrocarbons using alternate electron acceptors, and the
453 description of a consortium capable of water-mediated conversion of hydrocarbons to methane and
454 carbon dioxide. In recent years, it has become apparent that oxygen consumption in most reservoirs
455 is rapid thus causing aerobic metabolisms to be replaced by anaerobic ones [66-69]. In the case of
456 the Riutort oil shale mine, previous data indicated predominantly aerobic degradation. However,
457 many of the genera detected are characterized as having strictly anaerobic metabolisms. Most
458 likely, these two processes occur almost simultaneously and the rapid consumption of oxygen

459 brings the anaerobes into action. External factors such as the occasional opening of the mine and
460 tourist visits increase the oxygen concentration in the pools and aerobic metabolisms recover.

461

462 Our observations demonstrate evident metabolic differences between the pools. Biodegradation
463 occurred in all the pools, but the microbial communities in 3D, 6D, 8D, 9D, 10S, 11S, 13S, 15D
464 and 17D were dominated by aerobic sulfur-oxidizing prokaryotes, mainly *Thermoplasmatales*. The
465 percentage of SRB was also considerable, which suggests that there a combination of SRB and
466 SOB (sulfur-oxidant bacteria) populations complete the sulfur cycle at these sites. SOB or SRB
467 activity will depend on the oxygen concentration at any given time. Similar results were found in
468 other oil reservoirs [70-71]. Furthermore, we can highlight 9D and 10S, where the high percentage
469 of *Methylobacter* points to aerobic oxidation of methane produced by the high numbers of
470 methanogens. This is another characteristic reaction in this type of reservoir [72]. Samples 1S, 2S
471 and 6D showed the most relative abundance of methanogenic archaea and these pools were the sites
472 where consortium and syntrophic associations with hydrocarbon-utilizing organisms such as
473 *Smithella*, *Pirellula*, and *Parcubacteria* probably occurred [62,73-75]. As already mentioned, the
474 lack of electron acceptors promotes this type of association to achieve the anaerobic biodegradation
475 of oil [66-69].

476

477 It is also interesting to note the high presence of archaea of the *Nitrosopumilaceae* group,
478 ammonium oxidizers, in 4S, 7D, 12W and 16I. These aerobic archaea [76] may favor aerobic
479 degradation. This group is isolated from marine oxic waters, but it has previously been related to oil
480 degradation [77]. Ammonium oxidation by this group may contribute to the generation of new
481 electron acceptors for anaerobic degradation during periods of oxygen depletion. The presence of
482 the ammonium-oxidizing archaeal group ANME-1, which has been associated with SRB on
483 numerous occasions, could be explained in a similar way. The difference between ANME-1 and

484 *Nitrosopumilaceae* is that the former are strict anaerobes. At samples where ANME-1
485 predominated, such as 2S, 3D, 5S, 10S, and especially 14S, where ammonium oxidation is
486 anaerobic and will be coupled to sulfate reduction, anaerobic biodegradation will presumably
487 predominate. ANME-1 and *Nitrosopumilaceae* were also detected in 4S, 7D and 16I, so these sites
488 may show alternation between anaerobic and aerobic biodegradation. Sample 12W was the only
489 place where the relatively more abundant archaea belonged to the *Nitrosopumilaceae* group. Further
490 analysis of 12W shows that it stands out from the other sites. The metabolisms detected were
491 related to the aerobic degradation of oil, and it may be the only place in the mine where aerobic
492 degradation continuously prevails. Another singular pool is 5S, where we detected a large number
493 of bacteria from which no major conclusions can be drawn (*Gammaproteobacteria* unclassified,
494 *Planctomycetota* unclassified, and *Burkholderiales* unclassified). The presence of *Planctomyces* and
495 *Burkholdreriales* suggests aerobic hydrocarbon degradation metabolisms. There was also a high
496 percentage of *Thiothrix*, an aerobic sulfide oxidizer. The presence of some strict anaerobes, such as
497 ANME-1, and the SRB *Desulfocapsa*, indicate that there would also be an alternation between
498 aerobic and anaerobic biodegradation at this site. Indeed, this site may reflect a transition to
499 anaerobiosis due to the rapid consumption of oxygen by previous aerobic degradation. Oxygen
500 recycling in the mine gallery is complicated since there are no photosynthetic organisms and the
501 gallery is permanently closed. Of all the samples analyzed, the microbial community of Riutort-16I
502 was particularly remarkable and of potential interest for biotechnological applications. Bacterial
503 genera such as *Pseudomonas* and *Brevundimonas* have proven highly effective at oil biodegradation
504 [64-65]. The rest of the microorganisms identified were related to oil or oil-derivate environments.
505 Therefore, the Riutort-16I sample is a potential biodegradation consortium. The differences
506 between Riutort-16I and the other samples might be due to the lack of hydrocarbon flow rate and
507 water washing [78].

508

509 **4.2. Bioremediation and core flooding experiments**

510 Natural attenuation revealed very little capacity to remediate oily sludge-polluted soil over 60 days
511 as only achieved a 15% decrease in TPH levels in soil cultures and, consequently, it is not a
512 promising strategy for bioremediation [79]. In contrast, the combination of the Riutort consortium
513 and indigenous populations, if including nutrition, led to a decrease in ST and notably accelerated
514 the biodegradation rate of the oily sludge. This notion is in agreement with previous results obtained
515 by Jiang and co-workers [80]. Of note, exogenous and indigenous microbial populations showed
516 compatibility to grow in soil containing oily sludge from La Libertad Refinery. It was also observed
517 that saturated compounds were the easiest to remove in the three bioremediation approaches [81];
518 although, the addition of exogenous microbes also increased the biodegradation rate of aromatic and
519 polar fractions [38]. A set of *Pseudomonas* and *Brevundimonas* strains present in the natural Riutort
520 consortium might produce glycolipids and lipopeptides to collapse the oily sludge [82]. Moreover,
521 given that the criterion employed for selecting biosurfactant producers is capacity to reduce ST to
522 below $40 \text{ mN}\cdot\text{m}^{-1}$ [83], in our case microbial degradation of oily sludge was not solely related to
523 high biosurfactant production as evidenced by ST measurements but also to the emulsifying and
524 metabolic capabilities of the microbes and environmental conditions.

525

526 Nutrients and counts of total microbial degraders are two main factors influencing the rate of oil
527 degradation in soil. Indeed, in a context of a total hydrocarbon-degrading microbial count $< 10^5$
528 CFU/g accompanied by a lack of nutrients, biostimulation and bioaugmentation are necessary for
529 rapid bioremediation [84]. In our case, the initial indigenous population of oily sludge-degrading
530 microorganisms excluding nutrition was less than 10^4 CFU/g of soil. Therefore, bioaugmentation
531 and biostimulation were needed. Our results corroborated that the growth of the microbial
532 population was marginal through natural attenuation and that the addition of nutrients alone
533 (biostimulation), and bioaugmentation plus biostimulation led to higher initial microbial counts

534 (3·10⁴ and 10⁵ CFU/g), which were rapidly and strongly increased during the first month
535 bioremediation trials only in the bioaugmentation test. Our findings also showed that the aromatic
536 and polar fractions were more recalcitrant to biodegradation than saturated hydrocarbons. Of note,
537 the degradation of polar compounds was remarkable in the trial including the Riutort consortium. In
538 this regard, asphaltene degradation by commonly effective hydrocarbon-degrading bacteria such as
539 *Pseudomonas* and *Brevundimonas* strains isolated from petroleum-rich environments has been
540 previously reported [85-86]. This notable degradation of polar fractions may be attributable in part
541 to the abovementioned emulsifying capacity of the Riutort consortium [87]. In general, our results
542 are comparable with those reported by Gholami-Shiri and co-workers in 2017 using synthetic
543 bacterial consortia.

544

545 Here we have also demonstrated the effectiveness of MEOR processes involving the injection of the
546 exogenous Riutort consortium and nutrients into the cores of a shallow well at the Ancón oilfield.
547 These microorganisms can biodegrade petroleum fractions and produce surface active agents that
548 are compatible with the relatively oxic conditions of the Socorro reservoir. Even though exogenous
549 microbes usually alter reservoir biogeochemistry [88], bioaugmentation and biostimulation can be
550 combined to enhance oil recovery [89]. The results show that decrease of interfacial tension and oil
551 viscosity reduction are key mechanisms of oil recovery in this case study. Thus, the increase in the
552 emulsification index (E24) and decrease in IFT are due to the production of bio-products, thereby
553 suggesting the presence of bacteria that are phylogenetically related to species such as
554 *Pseudomonas aeruginosa* and *Brevundimonas* [55,65]. In fact, rhamnolipid-producing
555 *Pseudomonas* and lipopeptide-producing *Brevundimonas* species have been successfully applied for
556 bioremediation and MEOR [38,90]. Accordingly, our observations regarding cell surface
557 hydrophobicity and IFT results suggest that the petroleum-degrading microbes in the Riutort
558 consortium have a significant potential to adhere to hydrocarbons and to produce surface-active

559 metabolites. In this regard, the capacity of microorganisms to adhere to crude oil is associated with
560 hydrocarbon biodegradation potential [82]. It has also been reported that selective microbial
561 degradation of crude oil constituents reduces viscosity and improves mobility in porous systems,
562 overcoming capillary forces by two mechanisms: a decrease in the average molecular weight of the
563 hydrocarbon mixture and a change in the physical characteristics of crude oil [91]. Therefore,
564 optimization of culture and injection conditions could improve the yields of the initial MEOR
565 studies performed.

566

567 **5. Conclusions**

568 Our results showed the presence of diverse anaerobic and aerobic bacteria and archaea in the
569 Riutort oil shale mine, including strains with interesting hydrocarbon degradation potential. A
570 marked prokaryotic diversity was revealed as different groups, mainly bacteria but also archaea,
571 with hydrocarbon-degrading activity were found. Aerobic microorganisms were prevalent in most
572 samples but the presence of facultative microorganisms and a transition to strict anaerobic
573 conditions in some areas, including methanogens and sulfate-reducing bacteria, were also observed.
574 Therefore the Riutort mine environment is a unique site for the study of the microbial ecology of oil
575 degradation. In particular, the Riutort-16I consortium presented an abundance of hydrocarbon-
576 degrader aerobes, such as *Pseudomonas* spp., able to notably reduce TPH (including recalcitrant
577 fractions) in oily sludge-contaminated soils through biostimulation plus bioaugmentation, thus
578 revealing an excellent potential for bioremediation applications. Indeed, a non-subsurface
579 consortium such as Riutort-16I was also tested successfully in the laboratory for the first time and
580 could offer applications in MEOR schemes at relatively low cost in shallow mature oil fields.

581

582 **CRedit author statement**

583 **Elena González-Toril**: Conceptualization, Writing - Original Draft, Methodology, Investigation;
584 **Albert Permanyer**: Writing - Review & Editing, Investigation, Validation, Formal analysis; **José**
585 **Luis Rodríguez Gallego**: Visualization, Resources; **Gonzalo Márquez**: Writing - Original Draft,
586 Supervision, Funding acquisition, Data Curation; **Erika Lorenzo**: Investigation, Visualization;
587 **Ángeles Aguilera**: Data Curation, Formal analysis.

588

589 **Declaration of competing interest**

590 The authors declare that they have no known competing financial interests or personal relationships
591 that could have influenced the work reported in this paper. There are no conflicts of interest to
592 declare.

593

594 **Acknowledgments**

595 The authors are grateful to Cristina Amills, owner of the Riutort oil shale mine, for access to pool
596 samples. They also thank the company Pacificpetrol SA for facilitating access to the crude oil,
597 formation water, and refinery oily-sludge samples. This work was supported by the CGL2015-
598 67508-R and PID2019-104205GB-C22 grants, awarded by the Government of Spain.

599

600 **References**

601 [1] Larter, S., Wilhelms, A., Head, I., Koopmans, M., Aplin, A., Di Primio, R., Zwach, C.,
602 Erdmann, M., Telnaes, N., 2003. The controls on the composition of biodegraded oils in the deep
603 subsurface—part 1: biodegradation rates in petroleum reservoirs. *Org. Geochem.* 34, 601–613.
604 [https://doi.org/10.1016/S0146-6380\(02\)00240-1](https://doi.org/10.1016/S0146-6380(02)00240-1).

605

606 [2] Milner, C.W.D., Rogers, M.A., Evans, C.R., 1977. Petroleum transformations in reservoirs. *J.*
607 *Geochem. Explor.* 7, 101–153. [https://doi.org/10.1016/0375-6742\(77\)90079-6](https://doi.org/10.1016/0375-6742(77)90079-6).

608

609 [3] Connan, J., 1984. Biodegradation of crude oils in reservoirs. In: Brooks, J., Welte, D.H. (Eds.),
610 *Advances in Petroleum Geochemistry 1*. Academic Press, London, pp. 299–335.

611

612 [4] Stetter, K.O. 1996. Hyperthermophilic prokaryotes. *FEMS Microbiol. Rev.* 18, 149–158.
613 <https://doi.org/10.1111/j.1574-6976.1996.tb00233.x>

614

615 [5] Wenger, L.M., Davis, C.L., Isaksen, G.H., 2002. Multiple controls on petroleum biodegradation
616 and impact on oil quality. *SPE Reserv. Eval. Eng.* 5, 375–383. <https://doi.org/10.2118/80168-PA>.

617

618 [6] Palmer, S.E., 1993. Effect of biodegradation and water washing on crude oil composition. In:
619 Engel, M.H. and Macko, S.A. (Eds.), *Organic Geochemistry Principles and Applications*, Plenum
620 Press, New York, 511–533. https://doi.org/10.1007/978-1-4615-2890-6_23.

621

622 [7] Bernard, F.P., Connan, J., 1992. Indigenous microorganisms in connate waters of many
623 oilfields: a new tool in exploration and production techniques. SPE 24811. In: 67th Annual
624 Technical Conference and Exhibition of the Society of Petroleum Engineers, Washington, DC,
625 October 1992, pp. 467–476.

626

627 [8] Head, I.M., Jones, D.M., Larter, S.R., 2003. Biological activity in the deep subsurface and the
628 origin of heavy oil. *Nature* 426, 344–352. <https://doi.org/10.1038/nature02134>.

629

630 [9] Bennett, P.C., Siegel, D.E., Baedeker, M.J., Hult, 1993. Crude oil degradation in a shallow
631 sand and gravel aquifer—I. Hydrogeology and inorganic geochemistry. *Appl. Geochem.* 11, 529–
632 549. [https://doi.org/10.1016/0883-2927\(93\)90012-6](https://doi.org/10.1016/0883-2927(93)90012-6).

633

634 [10] Zengler, K., Richnow, H. H., Rossello-Mora, R., Michaelis, W., Widdel, F., 1999. Methane
635 formation from long-chain alkanes by anaerobic microorganisms. *Nature* 401, 266–269.
636 <https://doi.org/10.1038/45777>.

637

638 [11] Röling, W.F.M., Head, I.M., Larter, S.R., 2003. The microbiology of hydrocarbon degradation
639 in subsurface petroleum reservoirs: perspectives and prospects. *Res. Microbiol.* 154, 321–328.
640 [https://doi.org/10.1016/S0923-2508\(03\)00086-x](https://doi.org/10.1016/S0923-2508(03)00086-x).

641

642 [12] Holba, A.G., Dzou, L. I. P., Hickey, J. J., Franks, S. G., May, S. J., Lenney, T., 1996.
643 Reservoir geochemistry of South Pass 61 field Gulf of Mexico: Compositional heterogeneities
644 reflecting field filling history and biodegradation. *Org. Geochem.* 24, 1179–1198.
645 [https://doi.org/10.1016/S0146-6380\(96\)00101-5](https://doi.org/10.1016/S0146-6380(96)00101-5).

646

647 [13] Esquinas, N., Rodríguez-Valdés, E., Márquez, G., Gallego, J. R., 2017. Diagnostic ratios for
648 the rapid evaluation of natural attenuation of heavy fuel oil pollution along shores. *Chemosphere*
649 184, 1089–1098. <https://doi.org/doi:10.1016/j.chemosphere.2017.06.087>.

650

651 [14] Wilkes, H., 1995. Compositional changes of crude oils upon anaerobic degradation by sulphate
652 reducing bacteria. In: Grimalt, J.O. and Dorronsoro, C. (eds.), *Organic geochemistry, developments*
653 *and applications to energy, climate, environment and human history*, 19th International Meeting on
654 Organic Geochemistry, Donostia-San Sebastian, Spain, 321.

655

656 [15] Peters, K.E., Walters, C., Moldowan, J.M., 2005. *The Biomarker Guide: Biomarkers and*
657 *Isotopes in Petroleum Systems and Earth history*, 2nd edition. Cambridge University Press, 1132 p.

658

659 [16] Masterson, W.D., Dzou, L.I.P., Holba, A.G., Fincannon, A.L., Ellis, L., 2001. Evidence for
660 biodegradation and evaporative fractionation in West Sak, Kuparuk and Prudhoe Bay field areas,
661 North Slope, Alaska. *Org. Geochem.* 32, 411–441. [https://doi.org/10.1016/S0146-6380\(00\)00187-x](https://doi.org/10.1016/S0146-6380(00)00187-x).

662

663 [17] Meredith, W., Kelland, S.-J., Jones, D.M., 2000. Influence of biodegradation on crude oil
664 acidity and carboxylic acid composition. *Org. Geochem.* 31, 1059–1073.
665 [https://doi.org/10.1016/S0146-6380\(00\)00136-4](https://doi.org/10.1016/S0146-6380(00)00136-4).

666

667 [18] Tomczyk, N.A., Winans, R.E., Shinn, J.H., Robinson, R.C., 2001. On the nature and origin of
668 acidic species in petroleum, 1. Detailed acid type distribution in a California Crude Oil. *Energy*
669 *Fuels* 15, 1498–1504. <https://doi.org/10.1021/ef010106v>.

670

671 [19] Vasileva-Tonkova, E., Galabova, D., Stoimenova, E., Lalchev, Z., 2008. Characterization of
672 bacterial isolates from industrial wastewater according to probable modes of hexadecane uptake.
673 *Microbiol. Res.* 163, 481–486. <https://doi.org/10.1016/j.micres.2006.07.015>.

674

675 [20] Nazina, T.N., Sokolova, D.S., Grigoryan, A.A., Shestakova, N.M., Mikhailova, E.M.,
676 Poltarus, A.B., Tourova, T.P., Lysenko, A.M., Osipov, G.A., Belyarev, S.S., 2005. *Geobacillus*
677 *jurassicus* sp. nov., a new thermophilic bacterium isolated from a high temperature petroleum
678 reservoir, and the validation of the *Geobacillus* species. *Syst. Appl. Microbiol.* 28, 43–53.
679 <https://doi.org/10.1016/j.syapm.2004.09.001>.

680

- 681 [21] Gallego, J.R., Fernández, J. R., Díez-Sanz, F., Ordoñez, S., Sastre, H., González-Rojas, E.,
682 Peláez, A.I., Sánchez, J., 2007a. Bioremediation for shoreline cleanup: In situ vs. on-site treatments.
683 *Environ. Eng Sci.* 24, 493–504. <https://doi.org/10.1089/ees.2006.0091>.
684
- 685 [22] Brown, D.M., Okoro, S., van Gils, J., van Spanning, R., Bonte, M., Hutchings, T., Linden, O.,
686 Egbuche, U., Bruun, K.B., Smith, J.W.N. 2017. Comparison of landfarming amendments to
687 improve bioremediation of petroleum hydrocarbons in Niger Delta soils. *Sci. Total Environ.*
688 596/597, 284–292. <https://doi.org/10.1016/j.scitotenv.2017.04.072>.
689
- 690 [23] Bhattacharya, M., Guchhait, S., Biswasa, D., Singh, R., 2019. Evaluation of a microbial
691 consortium for crude oil spill bioremediation and its potential uses in enhanced oil recovery.
692 *Biocatalysis and Agr. Biotechnol.* 18, 101034. <https://doi.org/10.1016/j.bcab.2019.101034>.
693
- 694 [24] Verma, S., Bhargavaa, R., Pruthi, V., 2006. Oily sludge degradation by bacterial from
695 ankleshwar India. *Int. Biodeter. Biodegr.* 57, 207–213.
696 <http://dx.doi.org/10.1016/j.ibiod.2006.02.004>.
697
- 698 [25] Gallego, J.R., Peña-Álvarez, V., Lara, L.M., Baragaño, D., Forján, R. Colina, A- Prosenkov, A.
699 Peláez, A.I., 2022. Effective bioremediation of soil from the Burgan oil field (Kuwait) using
700 compost: a comprehensive hydrocarbon and DNA fingerprinting study. *Ecotox. Environ. Safe* 247,
701 114267. <https://doi.org/10.1016/j.ecoenv.2022.114267>.
702
- 703 [26] Permanyer, A., Vallès, D., Dorronsoro, C., 1988. Source Rocks potential of an Eocene
704 carbonate ramps: the Armàncies Formation of the southern Pyrenean Basin, northeast Spain. In:
705 AAPG Mediterranean Basin Conference, Nice. AAPG Bulletin 72, 1019.

706

707 [27] Riba, O., 1985. El petroli. In: R. Folch i Guillèn (ed.), *Història natural dels Països Catalans*,
708 Vol. 3. Grup Enciclopèdia Catalana, Barcelona, pp. 159–196.

709

710 [28] Permanyer, A., 2000. Natural biodegradation on oil seepage from south-eastern Pyrenees. In:
711 Trindade, L.A., Macedo, A.C., Barbanti, S.M. (Eds.), *New Perspectives on Organic Geochemistry*
712 for the Third Millennium, 7th Latin American Congress on Organic Geochemistry, Foz do Iguaçu,
713 Brasil, pp. 204–207.

714

715 [29] Permanyer, A., Caja, M.A., 2005. Quantification of biodegradation: Applied Example on Oil
716 Seeps in Armàncies Fm, South-eastern Pyrenees. *Geogaceta* 38, 135–138.

717

718 [30] Permanyer, A., Gallego, J.L.R., Dessort, D., 2006. Oil Seep Biodegradation: Quantification
719 and characterization of bacterial mats at the Riutort Mine (SE Pyrenees, Spain). 10th Norwegian
720 Meeting on Organic Geochemistry (NMOG), pp. 76–79.

721

722 [31] Permanyer, A., Gallego, J.L.R., Caja, M.A., Dessort, D., 2010. Crude oil biodegradation and
723 environmental factors at the Riutort Oil Shale Mine, SE Pyrenees. *J. Petrol. Geol.* 33, 123–140.
724 <https://doi.org/10.1111/j.1747-5457.2010.00469.x>.

725

726 [32] Pannekens, M., Kroll, L., Müller, H., Mbow, F.T., Meckenstock, U., 2019. Oil reservoirs, an
727 exceptional habitat for microorganisms. *New Biotechnol.* 49, 1–9. [https://doi.org/10.1016/j.](https://doi.org/10.1016/j.nbt.2018.11.006)
728 [nbt.2018.11.006](https://doi.org/10.1016/j.nbt.2018.11.006).

729

730 [33] Head, I.M., Gray, N.D, Larter, S.R., 2014. Life in the slow lane; biogeochemistry of
731 biodegraded petroleum containing reservoirs and implications for energy recovery and carbon
732 management. *Front. Microbiol.* 5, 566. <https://doi.org/10.3389/fmicb.2014.00566>.
733

734 [34] Shekhar, S.K., Godheja, J., Modi, D.R., 2020. Molecular technologies for assessment of
735 bioremediation and characterization of microbial communities at pollutant-contaminated sites. In:
736 *Bioremediation of Industrial Waste for Environmental Safety*. Springer, Singapore, pp. 437–474.
737

738 [35] Amrouche, F., Gomari, S.R., Islam, M., Xu, D., 2019. New insights into the application of a
739 magnetic field to enhance oil recovery from oil-wet carbonate reservoirs. *Energy Fuels* 33, 10602–
740 10610. <https://doi.org/10.1021/acs.energyfuels.9b02296>.
741

742 [36] Amrouche, F., Xu, D., Short, M., Iglauer, S., Vinogradov, J., Blunt, M.J., 2022. Experimental
743 study of electrical heating to enhance oil production from oil-wet carbonate reservoirs. *Fuel* 324,
744 124559. <https://doi.org/10.1016/.fuel.2022.124559>.
745

746 [37] Gallego, J.R., García-Martínez, M.J., Llamas, J.F., Belloch, C., Peláez, A.I., Sánchez, J.,
747 2007b. Biodegradation of oil tank bottom sludge using microbial consortia. *Biodegradation* 18,
748 269–281. <https://doi.org/10.1007/s10532-006-9061-y>.
749

750 [38] Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001a. Evaluation of inoculum addition to stimulate
751 in situ bioremediation of oily-sludge-contaminated soil. *Appl. Environ. Microbiol.* 67, 1675–1681.
752 <https://doi.org/10.1128/AEM.67.4.1675-1681.2001>.
753

- 754 [39] Gholami-Shiri, J., Mowla, D., Dehghani, S., Setoodeh, P., 2017. Exploitation of novel
755 synthetic bacterial consortia for biodegradation of oily-sludge TPH of Iran gas and oil refineries. *J.*
756 *Environ. Chem. Eng.* 5, 2964–2975. <https://doi.org/10.1016/j.jece.2017.05.056>.
- 757
- 758 [40] Nelson, D.W., Sommers, L.E., 1982. Total carbon, organic carbon and organic matter. In: A.L.
759 Page, R.H. Miller and D.R. Keeney (Eds.), *Methods of Soil Analysis, Part 2: Chemical and*
760 *Microbiological Methods*. Soil Science Society of America, Wisconsin, pp. 539–579.
- 761
- 762 [41] Thomas, G.W., 1996. Soil pH and soil acidity. In: D.L. Sparks (Ed.), *Methods of Soil Analysis,*
763 *Part 2: Chemical Methods*. Soil Science Society of America, Wisconsin, pp. 475–490.
- 764
- 765 [42] Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O.,
766 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
767 generation sequencing-based diversity studies. *Nucleic Acids Res.* 41(1), e1.
768 <https://doi.org/10.1093/nar/gks808>.
- 769
- 770 [43] Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,
771 Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G.,
772 Van Horn, D.J., Weber, C.F., 2015. Introducing mothur: open-source, platform-independent,
773 community-supported software for describing and comparing microbial communities. *Appl.*
774 *Environ. Microbiol.* 75, 7537-7541. <https://doi.org/10.1128/AEM.01541-09>.
- 775
- 776 [44] Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development
777 of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on

778 the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120.
779 <https://doi.org/10.1128/AEM.01043-13>.

780

781 [45] Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
782 search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).

783

784 [46] Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies,
785 J., Ludwig, W., Glöckner, F.O. 2014. The SILVA and “All-species Living Tree Project (LTP)”
786 taxonomic frameworks. *Nucl. Acids Res.* 42, D643-D648. <https://doi.org/10.1093/nar/gkt1209>.

787

788 [47] Parks, D.H., Chuvochina, M., Chaumeil, P.A., Rinke, C., Mussig, A.J., Hugenholtz, P., 2020.
789 A complete domain-to-species taxonomy for Bacteria and Archaea. *Nat. Biotechnol.* 38, 1079–
790 1086. <https://doi.org/10.1038/s41587-020-0501-8>.

791

792 [48] Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. Palaeontological statistics software package
793 for education and data analysis. *Palaeontol. Electron.* 4(1): 9 p. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

795

796 [49] Lane, D.J., 1991. 16S/23S sequencing. In: E Stackebrandt & M Goodfellow (eds) *Nucleic Acid*
797 *Techniques in Bacterial Systematics*. Wiley, New York, pp. 115–175.

798

799 [50] Reysenbach, A.L., Giver, L.J., Wickham, G.S., Pace, N.R., 1992. Differential amplification of
800 rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58, 3417–3418.
801 <https://doi.org/10.1128/aem.58.10.3417-3418.1992>.

802

803 [51] Braak, C.J.F., Smilauer, P., 2002. CANOCO Reference Manual and CanoDraw for Windows
804 User's Guide: Software for Canonical Community Ordination (version 4.5). (Microcomputer
805 Power). www.canoco.com. <https://edepot.wur.nl/405659>.
806

807 [52] Youssef, N., Simpson, D.R., McInerney, M.J., Duncan, K.E., 2013. In-situ lipopeptide
808 biosurfactant production by *Bacillus* strains correlates with improved oil recovery in two oil wells
809 approaching their economic limit of production. *Int. Biodeter. Biodegr.* 81, 127–132.
810 <https://doi.org/10.1016/j.ibiod.2012.05.010>.
811

812 [53] Joshi, S., Bharucha, C., Desai, A.J., 2008. Production of biosurfactant and antifungal
813 compound by fermented food isolate *Bacillus subtilis* 20B. *Bioresource Technol.* 99, 4603–4608.
814 <https://doi.org/10.1016/j.biortech.2007.07.030>.
815

816 [54] Jarvis, B., Wilrich, C., Wilrich, P.-T., 2010. Reconsideration of the derivation of Most
817 Probable Numbers, their standard deviations, confidence bounds and rarity values. *J. Appl.*
818 *Microbiol.* 109, 1660–1667. <https://doi.org/10.1111/j.1365-2672.2010.04792.x>.
819

820 [55] Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S. Nabi, I., 2008. Evaluation of different
821 carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*.
822 *Desalination* 223, 143–151. <https://doi.org/10.1016/j.desal.2007.01.198>.
823

824 [56] Gaytán, I., Mejía, M.A., Hernández-Gama, R., Torres, L.G., Escalante, C.A., Muñoz-Colunga,
825 A., 2015. Effects of indigenous microbial consortia for enhanced oil recovery in a fragmented
826 calcite rocks system. *J. Petrol. Sci. Eng.* 128, 65–72. <https://doi.org/10.1016/j.petrol.2015.02.028>.
827

828 [57] Datta, P., Tiwari, P., Pandey, L.M., 2018. Isolation and characterization of biosurfactant
829 producing and oil degrading *Bacillus subtilis* MG495086 from formation water of Assam oil
830 reservoir and its suitability for enhanced oil recovery. *Bioresource Technol.* 270, 439–448.
831 <https://doi.org/10.1016/j.biortech.2018.09.047>.

832

833 [58] Silva-Castro, G.A., Rodriguez-Calvo, A., Laguna, J. Gonzalez-Lopez, J., Calvo, C., 2016.
834 Autochthonous microbial responses and hydrocarbons degradation in polluted soil during
835 biostimulating treatments under different soil moisture: Assay in pilot plant. *Int. Biodeter. Biodegr.*
836 108, 91–98. <http://dx.doi.org/10.1016/j.ibiod.2015.12.009>.

837

838 [59] Gao, P.K., Li, G.Q., Dai, X.C., Dai, L.B., Wang, H.B., Zhao, L.X., Chen, Y.H., Ma, T., 2013.
839 Nutrients and oxygen alter reservoir biochemical characters and enhance oil recovery during
840 biostimulation. *World J. Microb. Biot.* 29, 2045–2054.

841

842 [60] Al-Sulaimani, H., Al-Wahaibi, Y., Al-Bahry, S., Elshafle, A., Al-Bemani, A., Joshi, S.,
843 Zargari, S., 2011. Optimization and partial characterization of biosurfactants produced by *Bacillus*
844 species and their potential for ex-situ enhanced oil recovery. *SPE J.* 16, 672–682.
845 <https://doi.org/10.2118/129228-PA>.

846

847 [61] Aboelkhair, H., Diaz, P., Attia, A., 2022. Biosurfactant production using Egyptian oil fields
848 indigenous bacteria for microbial enhanced oil recovery. *J. Petrol. Sci. Eng.* 208, 109601.
849 <https://doi.org/10.1016/j.petrol.2021.109601>.

850

851 [62] Qin, Q.S., Feng, D.S., Liu, P.F., He, Q., Li, X., Liu, A.M., Zhang, H., Hu, G.Q., Cheng, L.,
852 2017. Metagenomic characterization of *Candidatus Smithella cisternae* strain M82_1, a syntrophic

853 alkane-degrading bacteria, enriched from the Shengli Oil Field. *Microbes Environ.* 32, 234–243.
854 <https://doi.org/10.1264/jsme2.ME17022>.

855

856 [63] Lazar, C.S., Baker, B.J., Seitz, K.W., Teske, A.P., 2017. Genomic reconstruction of multiple
857 lineages of uncultured benthic archaea suggests distinct biogeochemical roles and ecological
858 niches. *ISME J.* 11, 1118–1129. <https://doi.org/10.1038/ismej.2016.189>.

859

860 [64] Kim, J.S., Crowley, D.E., 2007. Microbial diversity in natural asphalts of the Rancho La Brea
861 Tar Pits. *Appl. Environ. Microbiol.* 73, 4579–4591. <https://doi.org/10.1128/AEM.01372-06>.

862

863 [65] Hidalgo, K.J., Sierra-Garcia, I.N., Dellagnezze, B.M., de Oliveira, V.M., 2020. Metagenomic
864 insights into the mechanisms for biodegradation of polycyclic aromatic hydrocarbons in the Oil
865 Supply Chain. *Front Microbiol.* 18, 11:561506. <https://doi.org/10.3389/fmicb.2020.561506>.

866

867 [66] Shelton, J.L., Akob, D.M., McIntosh, J.C., Fierer, N., Spear, J.R., Warwick, P.D., McCray, J.E.
868 2016. Environmental drivers of differences in microbial community structure in crude oil reservoirs
869 across a methanogenic gradient. *Front. Microbiol.* 28, 1535.
870 <https://doi.org/10.3389/fmicb.2016.01535>.

871

872 [67] Varjani, S.J., Gnansounou, E. 2017. Microbial dynamics in petroleum oilfields and their
873 relationship with physiological properties of petroleum oil reservoirs. *Bioresource Technol.* 245,
874 1258–1265. <https://doi.org/10.1016/j.biortech.2017.08.028>.

875

876 [68] Roy, A., Sar, P., Sarkar, J., Dutta, A., Sarkar, P., Gupta, A., Mohapatra, B., Pal, S., Kazy, S.K.,
877 2018. Petroleum hydrocarbon rich oil refinery sludge of North-East India harbours anaerobic,

878 fermentative, sulfate-reducing, syntrophic and methanogenic microbial populations. *BMC*
879 *Microbiol.* 18(1):151. <https://doi.org/10.1186/s12866-018-1275-8>.

880

881 [69] Vilcáez, J., York, J., Youssef, N., Elshahed, M., 2018. Stimulation of methanogenic crude oil
882 biodegradation in depleted oil reservoirs. *Fuel* 232, 581–590. [https://doi.org/10.1016/j.fuel.2018.06.](https://doi.org/10.1016/j.fuel.2018.06.018)
883 018.

884

885 [70] Okabe, S., Odagiri, M., Ito, T., and Satoh, H. 2007. Succession of sulfur-oxidizing bacteria in
886 the microbial community on corroding concrete in sewer systems. *Appl. Environ. Microbiol.* 73,
887 971–980. <https://doi.org/10.1128/AEM.02054-06>.

888

889 [71] Tian, H., Gao, P., Chen, Z., Li, Y., Li, Y., Wang, Y., Zhou, J., Li, G., Ma, T. 2017.
890 Compositions and abundances of sulfate-reducing and sulfur-oxidizing microorganisms in water-
891 flooded petroleum reservoirs with different temperatures in China. *Front. Microbiol.* 8, 143.
892 <https://doi.org/10.3389/fmicb.2017.00143>.

893

894 [72] Rojas-Gätjens, D., Fuentes-Schweizer, P., Rojas-Jiménez, K. Pérez-Pantoja, D., Avendaño, R.,
895 Alpízar, R., Coronado, C., Chavaría, M., 2022. Methylotrophs and hydrocarbon-degrading bacteria
896 are key players in the microbial community of an abandoned century-old oil exploration well.
897 *Microb. Ecol.* 83, 83–99. <https://doi.org/10.1007/s00248-021-01748-1>.

898

899 [73] Hu, P., Tom, L., Singh, A., Thomas, B.C., Baker, B.J., Piceno, Y.M., Andersen, G.L.,
900 Banfield, J.F. 2016. Genome-Resolved Metagenomic Analysis Reveals Roles for Candidate Phyla
901 and Other Microbial Community Members in Biogeochemical Transformations in Oil Reservoirs.
902 *mBio.* 19; 7(1): e01669-15. <https://doi.org/10.1128/mBio.01669-15>.

903

904 [74] Lenchi, N., Inceoğlu, O., Kebbouche-Gana, S., Gana, M.L., Llíros, M., Servais, P., García-
905 Armisen, T. 2013. Diversity of microbial communities in production and injection waters of
906 Algerian oilfields revealed by 16S rRNA gene amplicon 454 pyrosequencing. *PLoS One*
907 21;8(6):e66588. <https://doi.org/10.1371/journal.pone.0066588>.

908

909 [75] Yun, Y., Gui, G., Xie, J., Chen, Y., Tian, X., Li, G., Gu, J.D., Ma, T. 2021. Stochastic
910 assembly process dominates bacterial succession during a long-term microbial enhanced oil
911 recovery. *Sci. Total Environ.* 790, 148203. <https://doi.org/10.1016/j.scitotenv.2021.148203>.

912

913 [76] Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A.
914 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437(7058), 543–546.
915 <https://doi.org/10.1038/nature03911>.

916

917 [77] Campeão, M.E., Reis, L., Leomil, L., de Oliveira, L., Otsuki, K., Gardinali, P., Pelz, O., Valle,
918 R., Thompson, F.L., Thompson, C.C. 2017. The Deep-Sea Microbial Community from the
919 Amazonian Basin Associated with Oil Degradation. *Front. Microbiol.* 13, 1019.
920 <http://doi.org/10.3389/fmicb.2017.01019>.

921

922 [78] Galarraga, F., Urbani, F., Escobar, M., Márquez, G., Martínez, M., Tocco, R., 2010. Main
923 factors controlling the compositional variability of seepage oils from Trujillo State, Western
924 Venezuela. *J. Petrol. Geol.* 33, 255–268. <https://doi.org/10.1111/j.1747-5457.2010.00477.x>.

925

926 [79] Wang, Sh., Wang, W., Zhang, Ch., Li, F. Guo, G., 2016. Bioremediation of oil sludge
927 contaminated soil by landfarming with added cotton stalks, *Int. Biodeter. Biodegr.* 106, 150–156.
928 <http://dx.doi.org/10.1016/j.ibiod.2015.10.014>.
929

930 [80] Jiang, Y., Brassington, K.J., Prpich, G., Paton, G.I., Semple, K.T., Pollard, S.J., Coulon, F.,
931 2016. Insights into the biodegradation of weathered hydrocarbons in contaminated soils by
932 bioaugmentation and nutrient stimulation. *Chemosphere* 161, 300-307. [https://doi.org/10.1016/j.](https://doi.org/10.1016/j.Chemosphere.2016.07.032)
933 [Chemosphere.2016.07.032](https://doi.org/10.1016/j.Chemosphere.2016.07.032).
934

935 [81] Ubani, O., Atanaga, H.I., Thantsha, M.S., 2013. Biological degradation of oil sludge: A review
936 of the current state of development. *Afr. J. Biotechnol.* 12, 6544–6567. [https://doi.org/10.5897/](https://doi.org/10.5897/AJB11.1139)
937 [AJB11.1139](https://doi.org/10.5897/AJB11.1139).
938

939 [82] Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative
940 bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and
941 bioaugmentation. *Bioresource Technol.* 96, 1049–1055. [http://dx.doi.org/10.1016/j.biortech.2004.](http://dx.doi.org/10.1016/j.biortech.2004.09.008)
942 [09.008](http://dx.doi.org/10.1016/j.biortech.2004.09.008).
943

944 [83] Kebbouche-Gana, S., Gana, M.L., Khemili, S., Fazouane-Naimi, F., Bouanane, N.A.,
945 Penninckx, M., hacene, H., 2009. Isolation and characterization of halophilic Archaea able to
946 produce biosurfactants. *J. Ind. Microbiol. Biotechnol.* 36, 727–738. [https://doi.org/10.1007/s10295-](https://doi.org/10.1007/s10295-009-0545-8)
947 [009-0545-8](https://doi.org/10.1007/s10295-009-0545-8).
948

949 [84] Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001b. In situ bioremediation potential of an oily
950 sludge-degrading bacterial consortium. *Curr. Microbiol.* 43, 328–335.
951 <http://dx.doi.org/10.1007/s002840010311>.
952

953 [85] Martirani-Von Abercron, S.M., Marín, P., Solsona-Ferraz, M., Castañeda-Cataña, M.A.,
954 Marqués, S., 2017. Naphthalene biodegradation under oxygen-limiting conditions: community
955 dynamics and the relevance of biofilm-forming capacity. *Microb. Biotechnol.* 10, 1781–1796.
956 <https://doi.org/10.1111/1751-7915.12842>.
957

958 [86] Gao, H., Zhang, J.H., Lai, H.X., Xue, Q.H., 2017. Degradation of asphaltenes by two
959 *Pseudomonas aeruginosa* strains and their effects on physicochemical properties of crude oil. *Int.*
960 *Biodeter. Biodegr.* 122, 12–22. <https://doi.org/10.1016/j.ibiod.2017.04.010>.
961

962 [87] Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A. H. A., Tamayo-Ramos, J. A.,
963 Rad, C., Gallego, J.R., Barros, R., 2022. Evaluation of biostimulation, bioaugmentation, and
964 organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil.
965 *Chemosphere*, 307, 135638. <https://doi:10.1016/j.chemosphere.2022.135638>.
966

967 [88] Bennett, B., Adams, J.J., Gray, N.D., Sherry, A., Oldenburg, T.B.P., Huang, H., Larter, S.R.,
968 Head, I.M., 2013. The controls on the composition of biodegraded oils in the deep subsurface - Part
969 3. The impact of microorganism distribution on petroleum geochemical gradients in biodegraded
970 petroleum reservoirs. *Org. Geochem.* 56, 94–105. <http://dx.doi: 10.1016/j.orggeochem.2012.12.011>.
971

972 [89] Ortega, M.F., Guerrero, D.E., García-Martínez, M.J., Bolonio, D., Llamas, J.F., Canoira, L.,
973 Gallego, J.L.R., 2018. Optimization of landfarming amendments based on soil texture and crude oil
974 concentration. *Water Air Soil Pollut.* 229, 234. <https://doi.org/10.1007/s11270-018-3891-1>.

975

976 [90] Bher A, Mayekar PC, Auras RA, Schvezov CE., Biodegradation of Biodegradable Polymers in
977 Mesophilic Aerobic Environments. *Int J Mol Sci.* 2022 Oct 12;23(20):12165. doi:
978 10.3390/ijms232012165. PMID: 36293023; PMCID: PMC9603655.

979

980 [91] Lavania, M., Cheema, S., Sarma, P.M., Mandal, A.K., Lal, B., 2012. Biodegradation of asphalt
981 by *Garciaella petrolearia* TERIG02 for viscosity reduction of heavy oil. *Biodegradation* 23, 15–24.
982 <https://doi.org/10.1007/s10532-011-9482-0>.

983

984

985 Figure 1. Location map of the Riutort oil shale mine and structural features of the SE Pyrenees.

986

987 Figure 2. (a) Schematic map of the Riutort oil shale mine showing the study gallery (modified from Riba, 1985). (b)
988 Detailed map of the study gallery showing the position of the samples.

989

990 Figure 3. Flow chart of the designed experiments in the present study.

991

992 Figure 4. Relative abundance of bacterial dominant genus (> 3%), detected by Illumina sequencing of 16S rRNA gene
993 amplicons. The size of the solid circle indicates the relative abundance.

994

995 Figure 5. Principal Component Analyses (PCA) on the relative proportion of archaeal OTUs (at a 0.03 distance). a) 3D
996 plot of the first 3 components showing the clustering of all samples on the basis of archaeal diversity. Labels indicate
997 the origin of the samples. b) Hierarchical clustering dendrogram based on principal component analysis of archaeal
998 OTUs. It shows the similarity in archaeal community composition across sampling sites. Red bubbles indicate
999 representative clusters.

1000

1001 Figure 6. Relative abundance of archaeal dominant genus (>3%), detected by Illumina sequencing of 16S rRNA gene
1002 amplicons. The size of the solid circle indicates the relative abundance.

1003

1 **Metagenomic analysis of the microbial community at the Riutort oil** 2 **shale mine (NE Spain): Potential applications in bioremediation and** 3 **enhanced oil recovery**

4
5 E. GONZÁLEZ-TORIL^a, A. PERMANYER^b, J.R. GALLEGOC, G. MÁRQUEZ^{d,*}, E. LORENZO^e and A. AGUILERA^a
6

7 ^aCenter for Astrobiology (INTA-CSIC), Carretera de Ajalvir km 4, Torrejón de Ardoz, 28850 Madrid, Spain

8 ^bDepartment of Mineralogy, Petrology and Applied Geology, Universitat de Barcelona, 08028 Barcelona, Spain

9 ^c INDUROT and Environmental Biogeochemistry & Raw Materials Group, University of Oviedo, 33600 Mieres, Spain

10 ^dCenter for Research in Sustainable Chemistry (CIQSO), University of Huelva, 21006 Huelva, Spain

11 ^eSchool of Engineering Sciences, State University Santa Elena Peninsula, 240204 La Libertad, Ecuador
12

13 **Abstract**

14 Preservation of the environment of the Riutort oil shale mine for more than a century has favored the presence of a
15 paradigmatic ecosystem of oil-degrading microorganisms. After extensive sampling and analysis by 16S rRNA
16 sequencing, a marked prokaryotic community comprising diverse groups of bacteria (genus such as *Methylobacter*,
17 *Thiothrix*, and *Desulfobacca*) and archaea (e.g., *Methanobrevibacter* genus) with hydrocarbon-degrading activity was
18 found. Aerobic microorganisms were predominant in several samples but facultative microorganisms were also present,
19 and there was an interesting transition to strict anaerobic conditions in some areas. One of the samples contained oil
20 degrading aerobic bacteria such as *Pseudomonas* spp. and *Brevundimonas* spp. Of the microbes studied, we conducted a
21 laboratory assessment of the capacity of this specific consortium for bioremediation of petroleum-polluted soil and
22 microbial enhanced oil recovery processes. To this end, we used oily sludge-contaminated soil from La Libertad
23 Refinery and cores from the Ancón Field, respectively, both sites in southwestern Ecuador. The Riutort consortium
24 degraded 50.8% of total petroleum hydrocarbons, 64.2% of saturates, 41.3% of aromatics, and 37.4% of polar
25 compounds after a 60-day incubation using oily sludge as the sole source of carbon. The performance of this consortium
26 reflects its notable potential for bioremediation purposes. In turn, flooding with the natural Riutort consortium and its
27 metabolites achieved a 7.2% (v/v) incremental recovery of crude oil through a sand-pack assay. These results are
28 comparable to those reported using synthetic bacterial consortia, and thus reveal the great interest of the study seepages,
29 not only for understanding microbial activities in oil degradation but also their use in biotechnological applications.
30

31 **Keywords:** Riutort microbial consortium, hydrocarbon-degraders, 16S rRNA sequencing, bioremediation, MEOR.
32

33 **1. Introduction**

34 The biodegradation of hydrocarbons alters the properties of these compounds and can bring about
35 significant economic losses for the petroleum industry [1]. Microbial oxidation of hydrocarbons

Corresponding author: G. Márquez (gonzalo.marquez@diq.uhu.es)

36 (petroleum biodegradation) generally occurs at temperatures below 75–80°C; i.e. above this range
37 there is scarce microbial life and only hyperthermophilic bacteria and archaea grow [2-5]. Meteoric
38 water influx can cause aerobic biodegradation processes and also remove more water-soluble
39 compounds [6]. However, anaerobic hydrocarbon breakdown is the common process of
40 biodegradation in most petroleum reservoirs [7-8]. Prior studies have verified the predominance of
41 bacteria and archaea in anaerobic processes controlling subsurface hydrocarbon degradation in
42 reservoirs. These processes are linked mostly to methanogenesis and iron reduction [9-11] or, when
43 free sulfate is abundant, to sulfate reduction [12]. Conversely, aerobic microbes are the main agents
44 of surface biodegradation processes after oil spills [13], but also in oil reservoirs whenever they are
45 shallow and contain oxygenated waters. However, anaerobic bacteria may initiate biodegradation
46 under certain conditions [14]. In addition to free or combined oxygen, microbes in shallow
47 environments need water with salinity levels < 100-150‰ and enough nutrients to metabolize
48 hydrocarbons [5] as the degradation of these compounds is frequently limited by phosphorus or
49 nitrogen [9]. Irrespective of whether the process is aerobic or anaerobic, the impact of microbial
50 degradation on crude oil can be notable [3,15]. As biodegradation progresses, it leads to a decrease
51 in oil quality, diminishing its economic value as API gravity decreases, while increasing viscosity
52 and density [5]. Interestingly, the compositional changes caused by biodegradation of petroleum in
53 reservoirs are similar to those observed in surface oil seeps and oil spills [9,16]. They result in a
54 lowering of saturated hydrocarbons and, to a lesser extent, aromatic compounds, thereby
55 concentrating resins and asphaltenes in the residual oil, while also enriching metal and sulfur
56 content [17]. In addition, biodegradation causes an increase in the acidity of the oil [18].

57

58 Hydrocarbons are magnificent growth media for microorganisms [19], and one of the challenges in
59 petroleum microbiology is to identify the microbial diversity in oilfields and hydrocarbon-
60 containing habitats. Although a major research focus has been placed on anaerobes, microbial

61 populations in shallow oil reservoirs also include aerobic microbes introduced through aqueous
62 fluid injection, drilling muds, and natural flows of surface water and groundwater [20]. Aerobic
63 hydrocarbon-degrading and biosurfactant-producing microbial populations are, for the most part,
64 advantageous for bioremediation processes, both in marine and terrestrial spills [21-22], and for
65 microbial enhanced oil recovery (MEOR) operations [23]. In both technologies, microbial consortia
66 can give yields that are greater than those achieved by a single species, as consortia benefit from
67 interrelations between distinct types of microbes [24-25].

68

69 In the SE Pyrenees (Cadí thrust sheet, NE Spain), the organic-rich materials of the Armàncies unit
70 (Eocene) are the most important oil source rock, cropping out for more than 100 km from near the
71 Terrades sector in the east to beyond the village of Bagá in the west (Fig. 1). Part of the Armàncies
72 Formation is characterized by a significant period of anoxia, which induced sedimentation and
73 conservation of organic matter [26]. The abundance of oil shows in outcropping materials from the
74 Cadí thrust sheet generated interest in this area for oil exploration. In this regard, the Riutort oil
75 shale mine was opened in 1912 and operated for around 10-15 years, producing approximately 3400
76 tons of rock during this time [27]. The main underground gallery (74 m long, still accessible) cuts
77 into the Armàncies Formation that has a thickness of 58 m, of which 50 m are oil shales. The slow
78 percolation of groundwater through the mine galleries has resulted in pools of water of various
79 sizes. Oil is present in some of these pools and is accompanied by the growth of microbial mats [28-
80 30]. The biodegradation of seepage oil at level 3-4 on the PM scale [15] can be observed in situ, as
81 attested by the presence of hydrocarbon-degrading bacteria [31]. Increasing levels of CO₂ in the air
82 towards the bottom of the galleries account for predominantly aerobic biodegradation processes,
83 while darkness has excluded the growth of photosynthetic microbes. The environment of the Riutort
84 oil shale mine has allowed oil biodegradation processes to take place for more than a century, and
85 thus the microbial ecology of this site is of great interest [31]. In this regard, the characterization of

86 microbial communities in oil-containing ecosystems can be used to elucidate the role of microbes in
87 relevant biogeochemical processes [32] and may also be useful to enhance bioremediation and
88 MEOR technologies [33-34].

89 *Figure 1*

90 Given the preceding considerations, here we sought to: (i) study in detail the microbial communities
91 involved in the biodegradation processes at the Riutort oil shale mine; ii) examine the impact of
92 using the Riutort microbes for hydrocarbon bioremediation; and (iii) evaluate the potential of the
93 natural Riutort microbial consortium for MEOR in shallow reservoirs. The use of non-subsurface
94 microbial populations such as the Riutort consortia can provide a novel and eco-friendly option for
95 improving hydrocarbon production from mature oilfields during the energy transition [35-36].

96

97 **2. Materials and methods**

98 **2.1. Sample collection and initial characterization**

99 Several underground galleries were identified in the Riutort oil shale mine (Fig. 2a). In particular,
100 the “study” gallery (Fig. 2b), located around 70 m from the mine entrance with 12 °C of constant
101 temperature, was selected as a sampling site due to the preservation of oil seeps (in oily-water
102 pools). To analyze microbial diversity within the pools on the gallery floor, we collected 17 water
103 samples belonging to four types (see Fig. 2b): 8 were surface pool samples (S), 7 deeper pool
104 samples (D), 1 water sample (W), and 1 isolated sample (I) from a pool located away from the
105 gallery walls and characterized by zero seepage flow rate and a lower proportion of water. In all
106 cases, 50 ml of sample was taken in sterile tubes and kept at -20°C until processing.

107

Figure 2

108 For bioremediation trials, oily sludge samples were taken from La Libertad Oil Refinery (longitude
109 80°53’56” W, latitude 2°13’32” S) in southwestern Ecuador, where the average temperature is
110 around 25 °C. Oily sludge is a typical waste of oil refineries, and it consists of a mixture of

111 hydrocarbons, water, and sediments [37]. The amount of total petroleum hydrocarbons (TPH)
112 recovered was determined gravimetrically as described by Mishra and co-workers [38] and
113 accounted for approximately 60% of total TPH, 34% water, and 6% sediments. TPH were
114 fractionated into saturates, aromatics, asphaltenes, and resins (SARA fractioning) by liquid
115 chromatography in a column containing silica gel (activated in an oven at 105 °C for 12 h). In brief,
116 asphaltenes were separated as the insoluble fraction by dissolving 1 g of TPH in *n*-pentane and
117 using 0.45 µm filters. The saturated, aromatic, and resin fractions were then eluted with *n*-hexane,
118 dichloromethane:hexane (4:1, v/v), and dichloromethane:methanol (1:1, v/v), respectively [13].
119 Solvents were evaporated at room temperature under a nitrogen stream in a fume hood, and the
120 SARA fractions were determined gravimetrically (45%, 29%, 5%, and 21% respectively). All
121 measurements were conducted in triplicate. The soil used in the bioremediation trials described in
122 section 2.3.3 was collected from the surface top layer (15 cm depth) in the surroundings of the
123 refinery mentioned above. Samples were homogenized through a 1-mm sieve to remove roots and
124 larger particles [39]. Organic carbon (0.97%), total nitrogen (0.03%), and bulk density (1.6 g/ml)
125 were then determined using standard methods [40-41]. A pH value of 8.0 was also measured in a
126 mixture of soil and water (1:1).

127

128 In turn, for the MEOR tests, rock cores, formation water, and crude oil samples were collected at a
129 depth of 343 m from the ANC-0008 well (80°51'55" W, 2°19'08" S) of the Ancón oilfield in
130 southwestern Ecuador. The physico-chemical properties of the formation fluids were examined
131 using 200-ml samples, which were first filtered. Organic compounds were then extracted using Sep-
132 Pak C18 solid phase extraction cartridges (Waters Chromatography). Following the procedure
133 described above, the initial SARA composition of the crude oil (32°API and viscosity of 3.15 mPa·s
134 at 37°C) from the ANC-0008 well was 69% saturates (SAT), 22% aromatic hydrocarbons (ARO),
135 and polar (POL) compounds (3% resins and 6% asphaltenes). Crude oil viscosity was measured

136 using an Oswald viscometer maintained at a constant temperature (37°C) in a water bath. Three
137 repeated measurements were performed at a 100 s⁻¹ shear rate. Finally, salinity, pH, and electrical
138 conductivity of the formation water were measured with a TPH 02154 device, a BASIC 20 pH
139 meter, and an EC Meter BASIC 30+, and the main ions were determined in an ion chromatograph
140 883 Basic IC Plus (Metrohm). As shown in Table 1, our data indicated low salinity and slightly
141 alkaline pH for these formation waters (Socorro reservoir in the Ancón field).

142 *Table 1*

143 **2.2 Assessment of microbial communities**

144 *2.2.1 DNA extraction, amplification and sequencing, and OTU detection*

145 Genomic DNA was extracted from 1 ml of representative homogenized subsamples using the
146 FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC) following the manufacturer's instructions
147 and it was quantified by PICOGREEN®. The extracted DNA was used in a first PCR of 27 cycles
148 with Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs) in the presence of 100
149 nM primers for 16S rRNA amplification. The primers used amplify the V3-V4 region of 16S rRNA
150 Archaea or Bacteria domain [42]. Each sample was amplified and, after the first PCR, a second
151 PCR of 14 cycles was performed with Q5® Hot Start High-Fidelity DNA Polymerase (New
152 England Biolabs) in the presence of 400 nM of the primers (5' AATGATACGGCGACCACCGAG
153 ATCTACACTGACGACA TGGTTCTACA-3' and 5'-CAAGCAGAAGACGGCATACGAG AT-
154 [barcode]-TACGGTAGCA GAGACTTGGT CT-3') of the Access Array Barcode Library for
155 Illumina Sequencers (Fluidigm). Finally, the pool of amplicons was denatured before being seeded
156 on a flow cell at a density of 10 pM, where clusters were formed and sequenced using a “MiSeq
157 Reagent Kit v3”, in a 2x300 pair-end sequencing run on a MiSeq sequencer.

158

159 For the detection and classification of OTUs (operational taxonomic units), sequence processing,
160 alignment and analysis were carried out with Mothur v.1.38.1 [43-44] and The Basic Local

161 Alignment Search Tool (BLAST) [45]. The databases used were SILVA-SEED (v138) [46] and the
162 Genome Taxonomy Database [47]. Chao 1 estimator and Shannon index were calculated to predict
163 community richness using PAST software 50 [48].

164

165 2.2.2. 16S rRNA gene amplification and library construction

166 The microbial species of the Riutort-16I sample were further studied by 16SrRNA gene
167 amplification and cloning. Amplification of nearly full-length bacterial and archaeal 16S rRNA
168 genes was carried out with the primer sets 8F/1492R [49] and 25F/1492R [50]. Each 50- μ L reaction
169 tube contained 20–30 ng of template DNA, 1 \times PCR reaction buffer (Promega Biotech Iberica,
170 Spain), 2.5 μ M of each dNTP (Amersham Biosciences, UK), 2.5 mM of MgCl₂, 1 mg mL⁻¹ of
171 bovine serum albumin, 500 mM of the forward and reverse primers, and 0.025 U μ L⁻¹ of DNA Taq
172 polymerase (Promega Biotech). PCR reactions were performed in a Perkin Elmer Thermocycler
173 with the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of
174 denaturation at 95°C for 1 min, annealing at 49°C for the bacterial primer set and 52°C for the
175 archaeal primer set, and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was
176 performed. Amplified 16S rRNA gene products (> 1400 bp) were cloned using the TOPO TA
177 Cloning Kit (Invitrogen, CA) and sequenced using a Big-Dye kit (Applied Biosystems), following
178 the manufacturer's instructions. Taxonomic assignation was performed by BLAST
179 (<https://blast.ncbi.nlm.nih.gov/>).

180

181 2.2.3. Statistical analyses

182 Principal Component Analyses (PCA) on the relative proportion of OTUs (at a distance of 0.03)
183 among the samples were performed. Using the three components as new variables, hierarchical
184 dendrograms by Euclidean squared distance method were also calculated (IBM SPSS Statistics 24
185 package). Using relative percentages, new PCA analyses were performed by CANOCO 4.5

186 (Microcomputer Power, Ithaca, NY, USA) [51]. The program CANODRAW 4.0 (in the Canoco
187 package) was used for graphical presentation of ordination results. Distribution analyses of OTU
188 abundance were done in Tableau Software (www.tableau.com).

189

190 **2.3 Biotechnological applications**

191 *2.3.1. Preparation of inoculums*

192 The microbial populations present in the Riutort-16I water pool sample (see results) were picked up
193 from oil agar plates and added to 50 ml basal medium [(grams per liter of brine): glucose (10),
194 sodium nitrate (1)]; and [(mg per liter of brine): manganese sulfate (0.25), magnesium sulfate (2.5),
195 zinc sulfate (0.25), copper sulfate (0.025), ferrous sulfate (0.25), aluminum potassium sulfate
196 (0.025), sodium molybdate (0.025), sodium selenate (0.013), nickel chloride (0.075) and boric acid
197 (0.025)]. They were incubated under aerobic conditions in a shaker at 37°C and 150 rpm [52]. Cell
198 growth was monitored by measuring the optical density at 600 nm through a UV-2550 Shimadzu
199 spectrophotometer [53]. When the culture reached the late exponential phase of growth, the cells
200 were resuspended in sterile basal medium, which was incubated at 37°C (rotatory shaker, 150 rpm)
201 for 24 h. Agitation and aeration were maintained using glass gas dispersion tubes with fritted
202 cylinders (Fisher Scientific). Microbial populations were counted using the most probable number
203 (MPN) method [54]. Each dilution was placed on a nutrient agar plate at 25°C. The number of
204 CFUs (Colony-Forming Units) at each dilution rate was counted after incubation and the average
205 CFU/ml or g of soil was determined. Figure 3 shows the overall experimental workflow.

206

Figure 3

207 *2.3.2. Consortium properties*

208 To determine surface tension (ST) reduction caused by the selected consortium, 1% (v/v) of
209 Riutort-16I consortium was added to a 250 ml Erlenmeyer flask containing 100 ml of sterile basal
210 medium supplemented with 1% (w/v) of La Libertad Refinery's oily sludge and placed in a shaker

211 incubator at 25°C and 150 rpm. Aliquots of this culture broth were sampled at different intervals
212 over 16 days. Cells were harvested by centrifugation at 9000 rpm and 4 °C for 15 min and then
213 discarded. Finally, the surface tensions of the supernatants were measured by a Gibertini
214 tensiometer (TSD 132389), following the method described by Abouseoud and co-workers [55].
215 Interfacial tension (IFT) determinations were performed against 1% (v/v) of ANC-0008 crude oil in
216 the same way at 37 °C (the well's bottom-hole temperature). Measurements were carried out in
217 triplicate.

218

219 The hydrophobicity of hydrocarbonoclastic microbial cells from the Riutort-16I sample was
220 measured through the Bacterial adhesion to hydrocarbons (BATH) assay, following the method
221 previously reported [56]. Phosphate urea magnesium sulfate (PUM) buffer [pH equals 7.1 and
222 composition (g per liter): $K_2HPO_4 \cdot 3H_2O$ (22.2), KH_2PO_4 (7.26), urea (1.8) and $MgSO_4 \cdot 7H_2O$ (0.2)]
223 was used to wash the cells twice and suspend them to obtain an optical density (OD) value of 0.6 at
224 600 nm. Thus, 2 ml of the cell suspension was mixed with 3 ml of *n*-hexadecane, and then the
225 mixture was vortexed for 2 min. Finally, *n*-hexadecane and the aqueous phase were held at room
226 temperature for 30 min to separate the phases. OD at 600 nm was quantified in the latter phase.
227 Hydrophobicity is expressed as the percentage of adherence to hexadecane, and it was calculated as
228 follows: $100 \cdot [(1 - OD_{600} \text{ of the aqueous phase}) / (OD_{600} \text{ of the initial cell suspension})]$. This analysis
229 was done in triplicate.

230

231 Emulsification activity was also used as an indicator of biosurfactant production in the culture broth
232 to determine a relationship between ANC-0008 crude oil biodegradation and biosurfactant
233 production [23]. The emulsion stability of the culture samples was determined by adding 3 ml of *n*-
234 hexadecane to 2 ml of culture supernatant in glass test tubes, shaking vigorously using a vortex for

235 2 min, and later incubating at 37 °C for 24 h. The emulsification index (E24) was calculated as the
236 percentage of the emulsified layer height divided by the total height of the liquid column [57].

237

238 2.3.3. *Bioremediation trials*

239 The biodegradation of the oily sludge from La Libertad Refinery was examined using three
240 approaches: natural attenuation, biostimulation, and combined biostimulation-bioaugmentation.

241 These trials were carried out in 250-ml sterile Erlenmeyer flasks containing 100 ml of soil spiked
242 with 1% (w/v) oily sludge and mixed thoroughly for 72 h. The natural attenuation trial was done
243 without any amendment, whereas biostimulation was carried out by adding 5% (v/v) sterile basal
244 medium, and biostimulation+bioaugmentation with 5% (v/v) basal medium including the microbial
245 inoculum from Riutort-16I. The experiments were done at 25°C (Santa Elena Peninsula's annual
246 mean temperature). As soil moisture content influences the biodegradation of petroleum compounds
247 [11,58], it was maintained around 60% throughout the experiment. TPH extraction and SARA
248 fractionation were done at 30 and 60 days. All tests were performed three times.

249

250 2.3.4. *Oil recovery tests*

251 Core-flooding experiments were performed to study the efficacy of the Riutort-16I microbial
252 consortium to enhance oil recovery when compared to conventional water flooding. Lab-scale
253 simulations for oil recovery were conducted following the procedure described by Gao and co-
254 workers [59]. Two core tubes, 50 cm long and 2.5 cm inner diameter, were filled with sandstone of
255 the Socorro Formation of the Ancón Field by mechanical loading. After packing with sand,
256 cleaning, and drying [60], each core tube was saturated with formation water from the ANC-0008
257 well, at a flow rate of $0.25 \text{ cm}^3 \cdot \text{s}^{-1}$, then a 0.7 pore volume (PV) was determined using the dry-
258 weight and wet-weight of the cores. Formation water was previously passed through a 0.45- μm
259 Millipore Filtration Unit [61]. Each core was then flooded with dehydrated ANC-0008 crude oil to

260 irreducible water saturation, as observed in petroleum reservoirs; the total volume of injected crude
261 oil was recorded. Both core tubes were allowed to soak at 37 °C for a week. One of the cores was
262 then injected with several pore volumes of the ANC-0008 formation water to simulate the
263 secondary oil recovery stage until reaching a water-cut level of 98%. The volume of oil recovered
264 by the formation water flooding was then determined. Finally, to simulate the tertiary recovery
265 process, microbial flooding was carried out by injecting 0.3 PV of the natural Riutort-16I
266 consortium (10^7 CFU/ml) mixed with nutrient basal medium (1.0%) in water into the second core
267 tube, which was then closed for 4 days. After that, the core was injected with water until no more
268 oil was released. The additional oil recovered by the microbial flooding was recorded. The
269 secondary and tertiary recoveries were calculated as a percentage of the amount of oil recovered in
270 each case in relation to the total mass of crude oil injected. The tests were carried out at 37 °C to
271 simulate the bottom-hole temperature of the Ancón-0008 well, with a displacement pressure of
272 about 4 MPa. All tests were done in triplicate.

273

274 **3. Results**

275 **3.1. Microbial diversity**

276 *3.1.1. Sequencing analysis and estimated richness*

277 The collections of amplicons were sequenced, and the number of reads is summarized in Appendix.
278 Using bacterial-specific primers, a total of $\sim 2 \times 10^6$ reads were obtained (about 120,000 per sample
279 on average), whereas using archaeal-specific primers a total of $\sim 4 \times 10^6$ reads were achieved
280 ($\sim 250,000$ per sample). Analysis of the read length distributions indicated a modal distribution
281 between 442 and 444 nucleotides. After quality control checks, chimera sequence removal, and the
282 subtraction of control sequences, a total of $\sim 1.5 \times 10^6$ sequences remained, about 86,000 per sample
283 on average in the case of the Bacteria domain, and 700,000 with about 40,000 per sample on
284 average for archaeal-specific primers. The number of bacterial OTUs detected at a distance of 0.03

285 ranged from 255 to 477 per sample and from 4 to 16 per sample in the case of archaeal OTUs.
286 Nevertheless, Chao1 estimations suggest that we were able to identify between 52 and 79% of the
287 total bacterial OTUs and between 53 and 97% of the total archaeal OTUs.

288

289 The bacterial community along the gallery showed a high diversity (Shannon index > 3; Appendix)
290 and multiple phenotypes. Shannon indices higher than 4 were detected in every pool sampled
291 through the gallery. Sample 16I showed the lowest value (4.42) and 11S the highest (5.04), with a
292 low deviation (0.16). With respect to *Archaea*, the Shannon indices showed a lower diversity. The
293 lowest value was detected in 5S (1.21) and the highest in 6D (2.47), with a deviation of 0.3.
294 Correlation networks of microbial populations and metabolic role of *Bacteria* and *Archaea* related
295 with oily-water pools in the Riutort oil shale mine were also studied (see Supplementary Material).

296

297 3.1.2. Bacterial community profiles

298 Taxonomic analyses of bacterial sequences were analyzed using the SILVA classifier algorithm
299 against SILVA.SEED V138 16S rRNA database. Reads were allotted to OTUs with an identity
300 cutoff of $\geq 97\%$, which were further assigned to higher taxa: 1 domain (*Bacteria*), 41 phyla, 119
301 classes, 287 orders, 460 families, and 746 genera. Most of the *Bacteria* were distributed among the
302 phyla *Proteobacteria*, *Planctomycetota*, *Bacteroidota*, *Verrucomicrobiota*, *Desulfobacterota*,
303 *Nitrospirota*, *Patescibacteria*, *Chloroflexi*, *Acidobacteriota*, *Firmicutes*, *Actinobacteriota*,
304 *Myxococcota* and *Dependentiae*, which accounted for $\geq 80\%$ of the total *Bacteria* within each
305 sampling site (Fig. 2). *Proteobacteria* were the most relative abundant group in all pools, except for
306 6D, where *Desulfobacterota* was predominant. The *Gammaproteobacteria* class dominated among
307 the *Proteobacteria* phylum and, to a lesser extent, *Alphaproteobacteria*. More precisely,
308 *Gammaproteobacteria* were predominant in 1S, 2S, 4S, 5S, 8D, 9D, 10S, 11S, 13S, 15D, and 16I
309 (between 14 and 37%). *Verrucomicrobiota* was the most relatively abundant phylum in 7D (15%),

310 14S (13%), and 17D (15%). *Planctomycetota* was the most abundant in 3D (15%) and 12W (23%).
311 Finally, only in 6D (25%) was *Desulfobacterota* the phylum with the most relative abundance.
312 Further analysis of the *Proteobacteria* community revealed a high proportion of unidentified
313 *Gammaproteobacteria* (between 3 and 17% in the different pools) and *Alphaproteobacteria* (1-
314 9%). With regard to *Alphaproteobacteria*, it is interesting to note the presence of *Brevundimonas* in
315 16I. Within the *Gammaproteobacteria* that had been identified, there were members of the
316 *Burkholderiales* order, the *Comamonadaceae* and *Methylomonadaceae* families, and the genera
317 *Methylobacter*, *Pseudomonas* and *Thiothrix*. The *Methylobacter* genus includes aerobic
318 methanotrophic bacteria and was detected (1-3%) mostly at the beginning of the gallery (8D, 9D,
319 10S, 11S, 12W, 13S, 15D, 16I, and 17D). Up to 7% of the *Pseudomonas* genus was detected in pool
320 16I, but was negligible in the other pools. The OTUs of *Thiothrix*, an aerobic sulfur-oxidizing
321 bacterium, were detected in only two pools, namely 5S (6%) and 11S (1%). A large number of
322 *Planctomycetota* resulted unclassified (1-5%), and between 1 and 3% of OTUs of the *Pirellulaceae*
323 family were also detected. With respect to *Desulfobacterota*, the third most abundant phylum, again
324 a good part of the sequences remained unclassified (between 1 and 6%). A high percentage (1-3%)
325 of unidentified microorganisms was also found at the *Desulfosarcinaceae* family level. Finally,
326 *Desulfobacca*, *Desulfocapsa*, and *Smithella* were the dominant genera of *Desulfobacterota*. The
327 sulfate-reducing bacteria (SRB) *Desulfobacca* was detected at between 1 to 7% in 1S, 2S, 3D, 5S,
328 6D, 7D, 8D, 9D, 10S, 11S, 14S, 15D, and 17D. *Desulfocapsa* was detected in high numbers (1-3%)
329 of sequences in 1S, 2S, 5S, 6F, 7F, 11S, and 13S, and the anaerobic propionate-degrading
330 syntrophic *Smithella* was found in important numbers (between 1-3%) in only 5 pools: 1S, 3D, 5S,
331 6D and 7D. This genus has been frequently detected in oil environments [62]. All sequences from
332 the *Nitrospirota* group were related to unclassified *Thermodesulfovibrionia*, which are mainly
333 anaerobic, moderately thermophilic, and SRB; they were detected mostly (1-4%) in 3D, 6D, 8D,
334 9D, 14S and 17D. In summary, 4S was the only sample in which no SRB were detected, and in

335 12W and 16I these bacteria were scarce, with only 1% *Desulfobacterota* and 1%
336 *Desulfosarcinaceae*, respectively. The *Verrucomicrobiota* group included sequences belonging to
337 the *Candidatus* “Omnitrophus” (1-6%) and *Candidatus* “Protochlamydia” (1-6%), and also part of
338 the sequences remained as unclassified *Chlamydiales* (1-4%). For a better understanding, Figure 4
339 summarizes the relative proportions of the most representative bacterial genera (those with a
340 relative abundance >3% in at least one sample).

341 *Figure 4*

342 The PCA for bacterial phyla and *Proteobacteria*-class level was conducted and based on number of
343 genus-level OTUs; three components accounted for 89% of the variance. The point cloud in Figure
344 5a shows how the D points (deep samples) tend to cluster together. In contrast, the S (surface)
345 points are more dispersed, except 14S, which joins 3D and 8D robustly. Various clusters are marked
346 in the dendrogram in Figure 5b.

347 *Figure 5*

348 3.1.3. Archaeal community profiles

349 Archaeal sequences were also taxonomically classified by SILVA.SEED V138 16S rRNA database.
350 Again, reads were allotted to OTUs with an identity cutoff of $\geq 97\%$, and these were further
351 assigned to higher taxa: 1 domain (*Archaea*), 5 phyla, 12 classes, 15 orders, 18 families, and 23
352 genera. Samples 1S (44 OTUs), 2S (51), 4S (13), 5S (17), and 7D (42) showed the lowest data and
353 15D and 6D the highest. In general, deep pools (D) showed the largest number of OTUs. The
354 majority of the archaeal sequences resulted unclassified (53-89%). Overall, a very low number of
355 sequences were identified. Sample 14S showed the most OTUs (314), and 2S, 5S and 4S the fewest
356 (8, 8, and 6 respectively). With the exception of 14S, the deep samples were the ones in which most
357 archaeal OTUs were identified. The OTUs detected belonged to only 5 phyla according to SILVA's
358 taxonomic classification v138 [46], namely *Halobacterota* (377 OTUs in total), *Thermoplasmata*
359 (377), *Crenarchaeota* (186), *Asgardarchaeota* (53) and *Euryarchaeota* (10). Note that although

360 *Halobacterota* and *Thermoplasmatota* showed the same relative abundance, only *Halobacterota*
361 was identified at all the sampling sites. A large proportion of the *Halobacterota* sequences were
362 phylogenetically affiliated to methanogenic archaea. Especially abundant were those affiliated to
363 the genus *Methanoregula*, which occurred in all sampling sites except 5S and 12W. This genus was
364 especially abundant in 2S (25%). The rest of the methanogens of this phylum were related to an
365 unclassified *Methanosarcinales* order (up to 53% in 1S). The rest of the *Halobacterota* sequences
366 were affiliated to the ANME-1 group (genus level). This anaerobic and methanotrophic genus was
367 detected at all the sampling sites except 1S, and it was especially abundant in 14S, where it showed
368 the highest relative abundance (64%). A large number of *Thermoplasmata* resulted unclassified (1-
369 39%). This clade was detected in all samples except 5S. In addition, a significant number of
370 sequences were taxonomically affiliated to *Candidatus Proteinoplasmatales* archaeon SG8-5 (1-
371 38%), an uncultured benthic archaeon, probably methanogenic [63]. Finally, 20% of the archaeal
372 sequences detected in 6D and 6% in 3D were affiliated to *Methanomethylophilaceae*, a
373 methanogenic anaerobic archaeal *Thermoplasmatota*. *Crenarchaeota* sequences were grouped in
374 three clades: *Bathyarchaeia*, *Nitrosopumilales*, and unclassified *Crenarchaeota*. In 1S, 2S and 10S,
375 *Crenarchaeota* were not identified. Representatives of *Bathyarchaeia* were found up to 36% in 7P,
376 while representatives of *Nitrosopumilales* accounted for 80% of the sequences obtained in 12W.
377 Most of the *Asgardarchaeota* sequences were related to *Lokiarchaeia*; this group was detected in 11
378 pools. In the case of 5S, 50% of the sequences were affiliated to *Lokiarchaeia*, although it should be
379 noted that very few archaeal OTUs (17) were obtained in this sample. In addition to *Lokiarchaeia*, a
380 significant number of *Asgardarchaeota* remained unclassified (up to 25%, again in 5S).
381 Furthermore, 2% of the sequences obtained in 6D were classified as *Odinarchaeia*. Members of
382 phyla *Euryarchaeota* were found only in 1S, 2S, 6D, 8D, 9D, 13S, and 15D. All sequences were
383 phylogenetically affiliated to the methanogenic genus, most of them to *Methanobrevibacter* and a
384 minority to *Methanobacterium* and *Methanofollis* (only 1% in 8D). Figure 6 summarizes the

385 relative proportions of the most representative archaeal genera (those with a relative abundance
386 >3% in at least one sample).

387 *Figure 6*

388 3.1.4. 16S rRNA gene amplification and library construction

389 Upon completing the study of the microbial communities, we focused on the selection of an
390 appropriate consortium for biotechnological applications. In this regard, the high percentage of
391 *Pseudomonas* (7%) detected in sample 16I from the NGS results was outstanding, as the potential
392 of these bacteria in oil biodegradation is well known [64-65]. We therefore chose sample 16I.

393
394 To precisely identify species of Riutort-16I, bacterial 16S rRNA gene amplification and cloning
395 was carried out. A total of 96 clones were sequenced and classified using BLAST. 31% of the
396 OTUs showed similarity to *Pseudomonas marincola* strain 002-Na3, 19% to an uncultured
397 bacterium clone (*Pseudomonas* sp.), 13% to *Brevundimonas mediterranea* strain N7, 12% to
398 uncultured *Desulfobacula* sp. clone LU2-210 (sulfate-reducing bacteria), 12% to the uncultured
399 *Gammaproteobacteria* clone 91-13 (probably sulfur-oxidizing bacteria), 6% to *Microbacterium* sp.
400 BA45(2011), and finally 6% to *Mycobacterium fluoranthenvivorans*. Therefore, 50% of the OTUs
401 obtained showed a high similarity to two *Pseudomonas* previously detected in oil-related
402 environments and their degradation [64-65]. The rest of the OTUs found were also similar to
403 bacteria related to the same environments, e.g., *Microbacterium* sp. and *Brevundimonas*
404 *mediterranea* strain N7. In addition, these two species were previously identified in the Riutort oil
405 shale mine [30-31]. Table 2 lists the OTUs obtained and the closest relatives identified by BLAST.

406 *Table 2*

407 **3.2. Bioremediation approaches**

408 Surface tension (ST) determinations indicated that both indigenous microbes and the combination
409 of these and exogenous Riutort microorganisms were able to reduce the ST of basal medium

410 supplied with 1% (w/v) oily sludge as the sole source of carbon. In fact, the ST of liquid media was
411 reduced from 62 to 54 and 45 mN·m⁻¹ after 16 days, respectively, without and with the injection of
412 the Riutort consortium. Cell hydrophobicity (BATH assay) of this consortium was 60% under the
413 tested conditions, suggesting that the microbial community has hydrophobic surface properties.
414 Simulated bioremediation of soil polluted with oily sludge from La Libertad Refinery was also
415 evaluated. As shown in Table 3, the greatest TPH degradation after 60 days (50.8%) occurred
416 through a combination of biostimulation and bioaugmentation, notably improving biostimulation
417 (29.8%) and natural attenuation yields (16.5%). The results also revealed the habitual features of
418 differential removal of hydrocarbon fractions [21,25], as in all the trials saturates were degraded
419 more easily and more quickly than aromatics and polar compounds (Table 3). Bioaugmentation
420 showed a general pattern of improved results for all the fractions. For instance, the minimum and
421 maximum removal of saturated compounds (24.5 and 64.2%) was achieved via natural attenuation,
422 and bioaugmentation + biostimulation, respectively, in the 60-day experiment. Similar observations
423 were made for aromatics (16.4 vs. 41.2%) and polar compounds (2.8% vs. 37.4%). All chemical
424 data were coherent with microbial counts, which revealed a strong increase (several orders of
425 magnitude) in the bioaugmentation testing when compared with the other two trials (see Table 4).

426 *Table 3*

427 *Table 4*

428 **3.3. Sand-pack study**

429 In this research, when the exogenous Riutort consortium and nutrients were injected into ANC-0008
430 crude oil, the emulsifying activity (E24) of the bioproducts generated was confirmed. It was noted
431 that emulsification power against *n*-hexadecane increased and reached a maximum value of 69%.
432 Also, the interfacial tension (IFT) of the crude-oil water system decreased from 38 to around 21
433 mN·m⁻¹, thus strongly suggesting microbial production of metabolites such as biosurfactants and

434 bioemulsifiers. Another important outcome was that the viscosity of the crude oil decreased 1.25-
435 fold after biological treatment with the exogenous Riutort microbial population (2.51 mPa·s).

436

437 Core-flooding experiments were also conducted to evaluate the potential application of the natural
438 Riutort consortium and their bioproducts for MEOR. Crude oil recovery after formation water
439 flooding was about 40% v/v of original oil in place, whereas a significant 7% v/v of additional oil
440 over the water flooding residual oil saturation was recovered from the column flooded with the
441 Riutort consortium and nutrients. Both lab-scale sand-pack columns can be considered a two-
442 dimensional model to calculate crude oil recovery [23].

443

444 **4. Discussion**

445 **4.1. Microbial communities**

446 We collected a total of 17 samples for microbial diversity analyses from the water pools present on
447 the gallery floor. Our results showed a typical community from a petroleum reservoir, including
448 microorganisms with aerobic, anaerobic, and facultative metabolisms (see Supplementary Material).
449 The microbial degradation of hydrocarbons from crude oil occurs under both oxic and anoxic
450 conditions. Oil degradation was long considered to be an aerobic process because oxygen was
451 deemed to be critical for hydrocarbon activation. This view was dismissed in the late 1980s after the
452 isolation of bacteria capable of growing on hydrocarbons using alternate electron acceptors, and the
453 description of a consortium capable of water-mediated conversion of hydrocarbons to methane and
454 carbon dioxide. In recent years, it has become apparent that oxygen consumption in most reservoirs
455 is rapid thus causing aerobic metabolisms to be replaced by anaerobic ones [66-69]. In the case of
456 the Riutort oil shale mine, previous data indicated predominantly aerobic degradation. However,
457 many of the genera detected are characterized as having strictly anaerobic metabolisms. Most
458 likely, these two processes occur almost simultaneously and the rapid consumption of oxygen

459 brings the anaerobes into action. External factors such as the occasional opening of the mine and
460 tourist visits increase the oxygen concentration in the pools and aerobic metabolisms recover.

461

462 Our observations demonstrate evident metabolic differences between the pools. Biodegradation
463 occurred in all the pools, but the microbial communities in 3D, 6D, 8D, 9D, 10S, 11S, 13S, 15D
464 and 17D were dominated by aerobic sulfur-oxidizing prokaryotes, mainly *Thermoplasmatales*. The
465 percentage of SRB was also considerable, which suggests that there a combination of SRB and
466 SOB (sulfur-oxidant bacteria) populations complete the sulfur cycle at these sites. SOB or SRB
467 activity will depend on the oxygen concentration at any given time. Similar results were found in
468 other oil reservoirs [70-71]. Furthermore, we can highlight 9D and 10S, where the high percentage
469 of *Methylobacter* points to aerobic oxidation of methane produced by the high numbers of
470 methanogens. This is another characteristic reaction in this type of reservoir [72]. Samples 1S, 2S
471 and 6D showed the most relative abundance of methanogenic archaea and these pools were the sites
472 where consortium and syntrophic associations with hydrocarbon-utilizing organisms such as
473 *Smithella*, *Pirellula*, and *Parcubacteria* probably occurred [62,73-75]. As already mentioned, the
474 lack of electron acceptors promotes this type of association to achieve the anaerobic biodegradation
475 of oil [66-69].

476

477 It is also interesting to note the high presence of archaea of the *Nitrosopumilaceae* group,
478 ammonium oxidizers, in 4S, 7D, 12W and 16I. These aerobic archaea [76] may favor aerobic
479 degradation. This group is isolated from marine oxic waters, but it has previously been related to oil
480 degradation [77]. Ammonium oxidation by this group may contribute to the generation of new
481 electron acceptors for anaerobic degradation during periods of oxygen depletion. The presence of
482 the ammonium-oxidizing archaeal group ANME-1, which has been associated with SRB on
483 numerous occasions, could be explained in a similar way. The difference between ANME-1 and

484 *Nitrosopumilaceae* is that the former are strict anaerobes. At samples where ANME-1
485 predominated, such as 2S, 3D, 5S, 10S, and especially 14S, where ammonium oxidation is
486 anaerobic and will be coupled to sulfate reduction, anaerobic biodegradation will presumably
487 predominate. ANME-1 and *Nitrosopumilaceae* were also detected in 4S, 7D and 16I, so these sites
488 may show alternation between anaerobic and aerobic biodegradation. Sample 12W was the only
489 place where the relatively more abundant archaea belonged to the *Nitrosopumilaceae* group. Further
490 analysis of 12W shows that it stands out from the other sites. The metabolisms detected were
491 related to the aerobic degradation of oil, and it may be the only place in the mine where aerobic
492 degradation continuously prevails. Another singular pool is 5S, where we detected a large number
493 of bacteria from which no major conclusions can be drawn (*Gammaproteobacteria* unclassified,
494 *Planctomycetota* unclassified, and *Burkholderiales* unclassified). The presence of *Planctomyces* and
495 *Burkholdreriales* suggests aerobic hydrocarbon degradation metabolisms. There was also a high
496 percentage of *Thiothrix*, an aerobic sulfide oxidizer. The presence of some strict anaerobes, such as
497 ANME-1, and the SRB *Desulfocapsa*, indicate that there would also be an alternation between
498 aerobic and anaerobic biodegradation at this site. Indeed, this site may reflect a transition to
499 anaerobiosis due to the rapid consumption of oxygen by previous aerobic degradation. Oxygen
500 recycling in the mine gallery is complicated since there are no photosynthetic organisms and the
501 gallery is permanently closed. Of all the samples analyzed, the microbial community of Riutort-16I
502 was particularly remarkable and of potential interest for biotechnological applications. Bacterial
503 genera such as *Pseudomonas* and *Brevundimonas* have proven highly effective at oil biodegradation
504 [64-65]. The rest of the microorganisms identified were related to oil or oil-derivate environments.
505 Therefore, the Riutort-16I sample is a potential biodegradation consortium. The differences
506 between Riutort-16I and the other samples might be due to the lack of hydrocarbon flow rate and
507 water washing [78].

508

509 **4.2. Bioremediation and core flooding experiments**

510 Natural attenuation revealed very little capacity to remediate oily sludge-polluted soil over 60 days
511 as only achieved a 15% decrease in TPH levels in soil cultures and, consequently, it is not a
512 promising strategy for bioremediation [79]. In contrast, the combination of the Riutort consortium
513 and indigenous populations, if including nutrition, led to a decrease in ST and notably accelerated
514 the biodegradation rate of the oily sludge. This notion is in agreement with previous results obtained
515 by Jiang and co-workers [80]. Of note, exogenous and indigenous microbial populations showed
516 compatibility to grow in soil containing oily sludge from La Libertad Refinery. It was also observed
517 that saturated compounds were the easiest to remove in the three bioremediation approaches [81];
518 although, the addition of exogenous microbes also increased the biodegradation rate of aromatic and
519 polar fractions [38]. A set of *Pseudomonas* and *Brevundimonas* strains present in the natural Riutort
520 consortium might produce glycolipids and lipopeptides to collapse the oily sludge [82]. Moreover,
521 given that the criterion employed for selecting biosurfactant producers is capacity to reduce ST to
522 below $40 \text{ mN}\cdot\text{m}^{-1}$ [83], in our case microbial degradation of oily sludge was not solely related to
523 high biosurfactant production as evidenced by ST measurements but also to the emulsifying and
524 metabolic capabilities of the microbes and environmental conditions.

525

526 Nutrients and counts of total microbial degraders are two main factors influencing the rate of oil
527 degradation in soil. Indeed, in a context of a total hydrocarbon-degrading microbial count $< 10^5$
528 CFU/g accompanied by a lack of nutrients, biostimulation and bioaugmentation are necessary for
529 rapid bioremediation [84]. In our case, the initial indigenous population of oily sludge-degrading
530 microorganisms excluding nutrition was less than 10^4 CFU/g of soil. Therefore, bioaugmentation
531 and biostimulation were needed. Our results corroborated that the growth of the microbial
532 population was marginal through natural attenuation and that the addition of nutrients alone
533 (biostimulation), and bioaugmentation plus biostimulation led to higher initial microbial counts

534 (3·10⁴ and 10⁵ CFU/g), which were rapidly and strongly increased during the first month
535 bioremediation trials only in the bioaugmentation test. Our findings also showed that the aromatic
536 and polar fractions were more recalcitrant to biodegradation than saturated hydrocarbons. Of note,
537 the degradation of polar compounds was remarkable in the trial including the Riutort consortium. In
538 this regard, asphaltene degradation by commonly effective hydrocarbon-degrading bacteria such as
539 *Pseudomonas* and *Brevundimonas* strains isolated from petroleum-rich environments has been
540 previously reported [85-86]. This notable degradation of polar fractions may be attributable in part
541 to the abovementioned emulsifying capacity of the Riutort consortium [87]. In general, our results
542 are comparable with those reported by Gholami-Shiri and co-workers in 2017 using synthetic
543 bacterial consortia.

544

545 Here we have also demonstrated the effectiveness of MEOR processes involving the injection of the
546 exogenous Riutort consortium and nutrients into the cores of a shallow well at the Ancón oilfield.
547 These microorganisms can biodegrade petroleum fractions and produce surface active agents that
548 are compatible with the relatively oxic conditions of the Socorro reservoir. Even though exogenous
549 microbes usually alter reservoir biogeochemistry [88], bioaugmentation and biostimulation can be
550 combined to enhance oil recovery [89]. **The results show that decrease of interfacial tension and oil**
551 **viscosity reduction are key mechanisms of oil recovery in this case study.** Thus, the increase in the
552 emulsification index (E24) and decrease in IFT are due to the production of bio-products, thereby
553 suggesting the presence of bacteria that are phylogenetically related to species such as
554 *Pseudomonas aeruginosa* and *Brevundimonas* [55,65]. In fact, rhamnolipid-producing
555 *Pseudomonas* and lipopeptide-producing *Brevundimonas* species have been successfully applied for
556 bioremediation and MEOR [38,90]. Accordingly, our observations regarding cell surface
557 hydrophobicity and IFT results suggest that the petroleum-degrading microbes in the Riutort
558 consortium have a significant potential to adhere to hydrocarbons and to produce surface-active

559 metabolites. In this regard, the capacity of microorganisms to adhere to crude oil is associated with
560 hydrocarbon biodegradation potential [82]. It has also been reported that selective microbial
561 degradation of crude oil constituents reduces viscosity and improves mobility in porous systems,
562 overcoming capillary forces by two mechanisms: a decrease in the average molecular weight of the
563 hydrocarbon mixture and a change in the physical characteristics of crude oil [91]. Therefore,
564 optimization of culture and injection conditions could improve the yields of the initial MEOR
565 studies performed.

566

567 **5. Conclusions**

568 Our results showed the presence of diverse anaerobic and aerobic bacteria and archaea in the
569 Riutort oil shale mine, including strains with interesting hydrocarbon degradation potential. A
570 marked prokaryotic diversity was revealed as different groups, mainly bacteria but also archaea,
571 with hydrocarbon-degrading activity were found. Aerobic microorganisms were prevalent in most
572 samples but the presence of facultative microorganisms and a transition to strict anaerobic
573 conditions in some areas, including methanogens and sulfate-reducing bacteria, were also observed.
574 Therefore the Riutort mine environment is a unique site for the study of the microbial ecology of oil
575 degradation. In particular, the Riutort-16I consortium presented an abundance of hydrocarbon-
576 degrader aerobes, such as *Pseudomonas* spp., able to notably reduce TPH (including recalcitrant
577 fractions) in oily sludge-contaminated soils through biostimulation plus bioaugmentation, thus
578 revealing an excellent potential for bioremediation applications. Indeed, a non-subsurface
579 consortium such as Riutort-16I was also tested successfully in the laboratory for the first time and
580 could offer applications in MEOR schemes at relatively low cost in shallow mature oil fields.

581

582 **CRedit author statement**

583 **Elena González-Toril**: Conceptualization, Writing - Original Draft, Methodology, Investigation;
584 **Albert Permanyer**: Writing - Review & Editing, Investigation, Validation, Formal analysis; **José**
585 **Luis Rodríguez Gallego**: Visualization, Resources; **Gonzalo Márquez**: Writing - Original Draft,
586 Supervision, Funding acquisition, Data Curation; **Erika Lorenzo**: Investigation, Visualization;
587 **Ángeles Aguilera**: Data Curation, Formal analysis.

588

589 **Declaration of competing interest**

590 The authors declare that they have no known competing financial interests or personal relationships
591 that could have influenced the work reported in this paper. There are no conflicts of interest to
592 declare.

593

594 **Acknowledgments**

595 The authors are grateful to Cristina Amills, owner of the Riutort oil shale mine, for access to pool
596 samples. They also thank the company Pacificpetrol SA for facilitating access to the crude oil,
597 formation water, and refinery oily-sludge samples. This work was supported by the CGL2015-
598 67508-R and PID2019-104205GB-C22 grants, awarded by the Government of Spain.

599

600 **References**

601 [1] Larter, S., Wilhelms, A., Head, I., Koopmans, M., Aplin, A., Di Primio, R., Zwach, C.,
602 Erdmann, M., Telnaes, N., 2003. The controls on the composition of biodegraded oils in the deep
603 subsurface—part 1: biodegradation rates in petroleum reservoirs. *Org. Geochem.* 34, 601–613.
604 [https://doi.org/10.1016/S0146-6380\(02\)00240-1](https://doi.org/10.1016/S0146-6380(02)00240-1).

605

606 [2] Milner, C.W.D., Rogers, M.A., Evans, C.R., 1977. Petroleum transformations in reservoirs. *J.*
607 *Geochem. Explor.* 7, 101–153. [https://doi.org/10.1016/0375-6742\(77\)90079-6](https://doi.org/10.1016/0375-6742(77)90079-6).

608

609 [3] Connan, J., 1984. Biodegradation of crude oils in reservoirs. In: Brooks, J., Welte, D.H. (Eds.),
610 *Advances in Petroleum Geochemistry 1*. Academic Press, London, pp. 299–335.

611

612 [4] Stetter, K.O. 1996. Hyperthermophilic prokaryotes. *FEMS Microbiol. Rev.* 18, 149–158.
613 <https://doi.org/10.1111/j.1574-6976.1996.tb00233.x>

614

615 [5] Wenger, L.M., Davis, C.L., Isaksen, G.H., 2002. Multiple controls on petroleum biodegradation
616 and impact on oil quality. *SPE Reserv. Eval. Eng.* 5, 375–383. <https://doi.org/10.2118/80168-PA>.

617

618 [6] Palmer, S.E., 1993. Effect of biodegradation and water washing on crude oil composition. In:
619 Engel, M.H. and Macko, S.A. (Eds.), *Organic Geochemistry Principles and Applications*, Plenum
620 Press, New York, 511–533. https://doi.org/10.1007/978-1-4615-2890-6_23.

621

622 [7] Bernard, F.P., Connan, J., 1992. Indigenous microorganisms in connate waters of many
623 oilfields: a new tool in exploration and production techniques. SPE 24811. In: 67th Annual
624 Technical Conference and Exhibition of the Society of Petroleum Engineers, Washington, DC,
625 October 1992, pp. 467–476.

626

627 [8] Head, I.M., Jones, D.M., Larter, S.R., 2003. Biological activity in the deep subsurface and the
628 origin of heavy oil. *Nature* 426, 344–352. <https://doi.org/10.1038/nature02134>.

629

630 [9] Bennett, P.C., Siegel, D.E., Baedeker, M.J., Hult, 1993. Crude oil degradation in a shallow
631 sand and gravel aquifer—I. Hydrogeology and inorganic geochemistry. *Appl. Geochem.* 11, 529–
632 549. [https://doi.org/10.1016/0883-2927\(93\)90012-6](https://doi.org/10.1016/0883-2927(93)90012-6).

633

634 [10] Zengler, K., Richnow, H. H., Rossello-Mora, R., Michaelis, W., Widdel, F., 1999. Methane
635 formation from long-chain alkanes by anaerobic microorganisms. *Nature* 401, 266–269.
636 <https://doi.org/10.1038/45777>.

637

638 [11] Röling, W.F.M., Head, I.M., Larter, S.R., 2003. The microbiology of hydrocarbon degradation
639 in subsurface petroleum reservoirs: perspectives and prospects. *Res. Microbiol.* 154, 321–328.
640 [https://doi.org/10.1016/S0923-2508\(03\)00086-x](https://doi.org/10.1016/S0923-2508(03)00086-x).

641

642 [12] Holba, A.G., Dzou, L. I. P., Hickey, J. J., Franks, S. G., May, S. J., Lenney, T., 1996.
643 Reservoir geochemistry of South Pass 61 field Gulf of Mexico: Compositional heterogeneities
644 reflecting field filling history and biodegradation. *Org. Geochem.* 24, 1179–1198.
645 [https://doi.org/10.1016/S0146-6380\(96\)00101-5](https://doi.org/10.1016/S0146-6380(96)00101-5).

646

647 [13] Esquinas, N., Rodríguez-Valdés, E., Márquez, G., Gallego, J. R., 2017. Diagnostic ratios for
648 the rapid evaluation of natural attenuation of heavy fuel oil pollution along shores. *Chemosphere*
649 184, 1089–1098. <https://doi.org/doi:10.1016/j.chemosphere.2017.06.087>.

650

651 [14] Wilkes, H., 1995. Compositional changes of crude oils upon anaerobic degradation by sulphate
652 reducing bacteria. In: Grimalt, J.O. and Dorronsoro, C. (eds.), *Organic geochemistry, developments*
653 *and applications to energy, climate, environment and human history*, 19th International Meeting on
654 Organic Geochemistry, Donostia-San Sebastian, Spain, 321.

655

656 [15] Peters, K.E., Walters, C., Moldowan, J.M., 2005. *The Biomarker Guide: Biomarkers and*
657 *Isotopes in Petroleum Systems and Earth history*, 2nd edition. Cambridge University Press, 1132 p.

658

659 [16] Masterson, W.D., Dzou, L.I.P., Holba, A.G., Fincannon, A.L., Ellis, L., 2001. Evidence for
660 biodegradation and evaporative fractionation in West Sak, Kuparuk and Prudhoe Bay field areas,
661 North Slope, Alaska. *Org. Geochem.* 32, 411–441. [https://doi.org/10.1016/S0146-6380\(00\)00187-x](https://doi.org/10.1016/S0146-6380(00)00187-x).

662

663 [17] Meredith, W., Kelland, S.-J., Jones, D.M., 2000. Influence of biodegradation on crude oil
664 acidity and carboxylic acid composition. *Org. Geochem.* 31, 1059–1073.
665 [https://doi.org/10.1016/S0146-6380\(00\)00136-4](https://doi.org/10.1016/S0146-6380(00)00136-4).

666

667 [18] Tomczyk, N.A., Winans, R.E., Shinn, J.H., Robinson, R.C., 2001. On the nature and origin of
668 acidic species in petroleum, 1. Detailed acid type distribution in a California Crude Oil. *Energy*
669 *Fuels* 15, 1498–1504. <https://doi.org/10.1021/ef010106v>.

670

671 [19] Vasileva-Tonkova, E., Galabova, D., Stoimenova, E., Lalchev, Z., 2008. Characterization of
672 bacterial isolates from industrial wastewater according to probable modes of hexadecane uptake.
673 *Microbiol. Res.* 163, 481–486. <https://doi.org/10.1016/j.micres.2006.07.015>.

674

675 [20] Nazina, T.N., Sokolova, D.S., Grigoryan, A.A., Shestakova, N.M., Mikhailova, E.M.,
676 Poltarus, A.B., Tourova, T.P., Lysenko, A.M., Osipov, G.A., Belyarev, S.S., 2005. *Geobacillus*
677 *jurassicus* sp. nov., a new thermophilic bacterium isolated from a high temperature petroleum
678 reservoir, and the validation of the *Geobacillus* species. *Syst. Appl. Microbiol.* 28, 43–53.
679 <https://doi.org/10.1016/j.syapm.2004.09.001>.

680

- 681 [21] Gallego, J.R., Fernández, J. R., Díez-Sanz, F., Ordoñez, S., Sastre, H., González-Rojas, E.,
682 Peláez, A.I., Sánchez, J., 2007a. Bioremediation for shoreline cleanup: In situ vs. on-site treatments.
683 *Environ. Eng Sci.* 24, 493–504. <https://doi.org/10.1089/ees.2006.0091>.
684
- 685 [22] Brown, D.M., Okoro, S., van Gils, J., van Spanning, R., Bonte, M., Hutchings, T., Linden, O.,
686 Egbuche, U., Bruun, K.B., Smith, J.W.N. 2017. Comparison of landfarming amendments to
687 improve bioremediation of petroleum hydrocarbons in Niger Delta soils. *Sci. Total Environ.*
688 596/597, 284–292. <https://doi.org/10.1016/j.scitotenv.2017.04.072>.
689
- 690 [23] Bhattacharya, M., Guchhait, S., Biswasa, D., Singh, R., 2019. Evaluation of a microbial
691 consortium for crude oil spill bioremediation and its potential uses in enhanced oil recovery.
692 *Biocatalysis and Agr. Biotechnol.* 18, 101034. <https://doi.org/10.1016/j.bcab.2019.101034>.
693
- 694 [24] Verma, S., Bhargavaa, R., Pruthi, V., 2006. Oily sludge degradation by bacterial from
695 ankleshwar India. *Int. Biodeter. Biodegr.* 57, 207–213.
696 <http://dx.doi.org/10.1016/j.ibiod.2006.02.004>.
697
- 698 [25] Gallego, J.R., Peña-Álvarez, V., Lara, L.M., Baragaño, D., Forján, R. Colina, A- Prosenkov, A.
699 Peláez, A.I., 2022. Effective bioremediation of soil from the Burgan oil field (Kuwait) using
700 compost: a comprehensive hydrocarbon and DNA fingerprinting study. *Ecotox. Environ. Safe* 247,
701 114267. <https://doi.org/10.1016/j.ecoenv.2022.114267>.
702
- 703 [26] Permanyer, A., Vallès, D., Dorronsoro, C., 1988. Source Rocks potential of an Eocene
704 carbonate ramps: the Armàncies Formation of the southern Pyrenean Basin, northeast Spain. In:
705 AAPG Mediterranean Basin Conference, Nice. AAPG Bulletin 72, 1019.

706

707 [27] Riba, O., 1985. El petroli. In: R. Folch i Guillèn (ed.), *Història natural dels Països Catalans*,
708 Vol. 3. Grup Enciclopèdia Catalana, Barcelona, pp. 159–196.

709

710 [28] Permanyer, A., 2000. Natural biodegradation on oil seepage from south-eastern Pyrenees. In:
711 Trindade, L.A., Macedo, A.C., Barbanti, S.M. (Eds.), *New Perspectives on Organic Geochemistry*
712 for the Third Millennium, 7th Latin American Congress on Organic Geochemistry, Foz do Iguaçu,
713 Brasil, pp. 204–207.

714

715 [29] Permanyer, A., Caja, M.A., 2005. Quantification of biodegradation: Applied Example on Oil
716 Seeps in Armàncies Fm, South-eastern Pyrenees. *Geogaceta* 38, 135–138.

717

718 [30] Permanyer, A., Gallego, J.L.R., Dessort, D., 2006. Oil Seep Biodegradation: Quantification
719 and characterization of bacterial mats at the Riutort Mine (SE Pyrenees, Spain). 10th Norwegian
720 Meeting on Organic Geochemistry (NMOG), pp. 76–79.

721

722 [31] Permanyer, A., Gallego, J.L.R., Caja, M.A., Dessort, D., 2010. Crude oil biodegradation and
723 environmental factors at the Riutort Oil Shale Mine, SE Pyrenees. *J. Petrol. Geol.* 33, 123–140.
724 <https://doi.org/10.1111/j.1747-5457.2010.00469.x>.

725

726 [32] Pannekens, M., Kroll, L., Müller, H., Mbow, F.T., Meckenstock, U., 2019. Oil reservoirs, an
727 exceptional habitat for microorganisms. *New Biotechnol.* 49, 1–9. <https://doi.org/10.1016/j.nbt.2018.11.006>.

729

730 [33] Head, I.M., Gray, N.D, Larter, S.R., 2014. Life in the slow lane; biogeochemistry of
731 biodegraded petroleum containing reservoirs and implications for energy recovery and carbon
732 management. *Front. Microbiol.* 5, 566. <https://doi.org/10.3389/fmicb.2014.00566>.
733

734 [34] Shekhar, S.K., Godheja, J., Modi, D.R., 2020. Molecular technologies for assessment of
735 bioremediation and characterization of microbial communities at pollutant-contaminated sites. In:
736 *Bioremediation of Industrial Waste for Environmental Safety*. Springer, Singapore, pp. 437–474.
737

738 [35] Amrouche, F., Gomari, S.R., Islam, M., Xu, D., 2019. New insights into the application of a
739 magnetic field to enhance oil recovery from oil-wet carbonate reservoirs. *Energy Fuels* 33, 10602–
740 10610. <https://doi.org/10.1021/acs.energyfuels.9b02296>.
741

742 [36] Amrouche, F., Xu, D., Short, M., Iglauer, S., Vinogradov, J., Blunt, M.J., 2022. Experimental
743 study of electrical heating to enhance oil production from oil-wet carbonate reservoirs. *Fuel* 324,
744 124559. <https://doi.org/10.1016/j.fuel.2022.124559>.
745

746 [37] Gallego, J.R., García-Martínez, M.J., Llamas, J.F., Belloch, C., Peláez, A.I., Sánchez, J.,
747 2007b. Biodegradation of oil tank bottom sludge using microbial consortia. *Biodegradation* 18,
748 269–281. <https://doi.org/10.1007/s10532-006-9061-y>.
749

750 [38] Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001a. Evaluation of inoculum addition to stimulate
751 in situ bioremediation of oily-sludge-contaminated soil. *Appl. Environ. Microbiol.* 67, 1675–1681.
752 <https://doi.org/10.1128/AEM.67.4.1675-1681.2001>.
753

- 754 [39] Gholami-Shiri, J., Mowla, D., Dehghani, S., Setoodeh, P., 2017. Exploitation of novel
755 synthetic bacterial consortia for biodegradation of oily-sludge TPH of Iran gas and oil refineries. *J.*
756 *Environ. Chem. Eng.* 5, 2964–2975. <https://doi.org/10.1016/j.jece.2017.05.056>.
- 757
- 758 [40] Nelson, D.W., Sommers, L.E., 1982. Total carbon, organic carbon and organic matter. In: A.L.
759 Page, R.H. Miller and D.R. Keeney (Eds.), *Methods of Soil Analysis, Part 2: Chemical and*
760 *Microbiological Methods*. Soil Science Society of America, Wisconsin, pp. 539–579.
- 761
- 762 [41] Thomas, G.W., 1996. Soil pH and soil acidity. In: D.L. Sparks (Ed.), *Methods of Soil Analysis,*
763 *Part 2: Chemical Methods*. Soil Science Society of America, Wisconsin, pp. 475–490.
- 764
- 765 [42] Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O.,
766 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
767 generation sequencing-based diversity studies. *Nucleic Acids Res.* 41(1), e1.
768 <https://doi.org/10.1093/nar/gks808>.
- 769
- 770 [43] Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,
771 Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G.,
772 Van Horn, D.J., Weber, C.F., 2015. Introducing mothur: open-source, platform-independent,
773 community-supported software for describing and comparing microbial communities. *Appl.*
774 *Environ. Microbiol.* 75, 7537-7541. <https://doi.org/10.1128/AEM.01541-09>.
- 775
- 776 [44] Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development
777 of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on

778 the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120.
779 <https://doi.org/10.1128/AEM.01043-13>.

780

781 [45] Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
782 search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).

783

784 [46] Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies,
785 J., Ludwig, W., Glöckner, F.O. 2014. The SILVA and “All-species Living Tree Project (LTP)”
786 taxonomic frameworks. *Nucl. Acids Res.* 42, D643-D648. <https://doi.org/10.1093/nar/gkt1209>.

787

788 [47] Parks, D.H., Chuvochina, M., Chaumeil, P.A., Rinke, C., Mussig, A.J., Hugenholtz, P., 2020.
789 A complete domain-to-species taxonomy for Bacteria and Archaea. *Nat. Biotechnol.* 38, 1079–
790 1086. <https://doi.org/10.1038/s41587-020-0501-8>.

791

792 [48] Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. Palaeontological statistics software package
793 for education and data analysis. *Palaeontol. Electron.* 4(1): 9 p. [http://palaeo-electronica.org/2001_](http://palaeo-electronica.org/2001_1/past/issue1_01.htm)
794 [1/past/issue1_01.htm](http://palaeo-electronica.org/2001_1/past/issue1_01.htm).

795

796 [49] Lane, D.J., 1991. 16S/23S sequencing. In: E Stackebrandt & M Goodfellow (eds) *Nucleic Acid*
797 *Techniques in Bacterial Systematics*. Wiley, New York, pp. 115–175.

798

799 [50] Reysenbach, A.L., Giver, L.J., Wickham, G.S., Pace, N.R., 1992. Differential amplification of
800 rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58, 3417–3418.
801 <https://doi.org/10.1128/aem.58.10.3417-3418.1992>.

802

803 [51] Braak, C.J.F., Smilauer, P., 2002. CANOCO Reference Manual and CanoDraw for Windows
804 User's Guide: Software for Canonical Community Ordination (version 4.5). (Microcomputer
805 Power). www.canoco.com. <https://edepot.wur.nl/405659>.

806

807 [52] Youssef, N., Simpson, D.R., McInerney, M.J., Duncan, K.E., 2013. In-situ lipopeptide
808 biosurfactant production by *Bacillus* strains correlates with improved oil recovery in two oil wells
809 approaching their economic limit of production. *Int. Biodeter. Biodegr.* 81, 127–132.
810 <https://doi.org/10.1016/j.ibiod.2012.05.010>.

811

812 [53] Joshi, S., Bharucha, C., Desai, A.J., 2008. Production of biosurfactant and antifungal
813 compound by fermented food isolate *Bacillus subtilis* 20B. *Bioresource Technol.* 99, 4603–4608.
814 <https://doi.org/10.1016/j.biortech.2007.07.030>.

815

816 [54] Jarvis, B., Wilrich, C., Wilrich, P.-T., 2010. Reconsideration of the derivation of Most
817 Probable Numbers, their standard deviations, confidence bounds and rarity values. *J. Appl.*
818 *Microbiol.* 109, 1660–1667. <https://doi.org/10.1111/j.1365-2672.2010.04792.x>.

819

820 [55] Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S. Nabi, I., 2008. Evaluation of different
821 carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*.
822 *Desalination* 223, 143–151. <https://doi.org/10.1016/j.desal.2007.01.198>.

823

824 [56] Gaytán, I., Mejía, M.A., Hernández-Gama, R., Torres, L.G., Escalante, C.A., Muñoz-Colunga,
825 A., 2015. Effects of indigenous microbial consortia for enhanced oil recovery in a fragmented
826 calcite rocks system. *J. Petrol. Sci. Eng.* 128, 65–72. <https://doi.org/10.1016/j.petrol.2015.02.028>.

827

828 [57] Datta, P., Tiwari, P., Pandey, L.M., 2018. Isolation and characterization of biosurfactant
829 producing and oil degrading *Bacillus subtilis* MG495086 from formation water of Assam oil
830 reservoir and its suitability for enhanced oil recovery. *Bioresource Technol.* 270, 439–448.
831 <https://doi.org/10.1016/j.biortech.2018.09.047>.

832

833 [58] Silva-Castro, G.A., Rodriguez-Calvo, A., Laguna, J. Gonzalez-Lopez, J., Calvo, C., 2016.
834 Autochthonous microbial responses and hydrocarbons degradation in polluted soil during
835 biostimulating treatments under different soil moisture: Assay in pilot plant. *Int. Biodeter. Biodegr.*
836 108, 91–98. <http://dx.doi.org/10.1016/j.ibiod.2015.12.009>.

837

838 [59] Gao, P.K., Li, G.Q., Dai, X.C., Dai, L.B., Wang, H.B., Zhao, L.X., Chen, Y.H., Ma, T., 2013.
839 Nutrients and oxygen alter reservoir biochemical characters and enhance oil recovery during
840 biostimulation. *World J. Microb. Biot.* 29, 2045–2054.

841

842 [60] Al-Sulaimani, H., Al-Wahaibi, Y., Al-Bahry, S., Elshafle, A., Al-Bemani, A., Joshi, S.,
843 Zargari, S., 2011. Optimization and partial characterization of biosurfactants produced by *Bacillus*
844 species and their potential for ex-situ enhanced oil recovery. *SPE J.* 16, 672–682.
845 <https://doi.org/10.2118/129228-PA>.

846

847 [61] Aboelkhair, H., Diaz, P., Attia, A., 2022. Biosurfactant production using Egyptian oil fields
848 indigenous bacteria for microbial enhanced oil recovery. *J. Petrol. Sci. Eng.* 208, 109601.
849 <https://doi.org/10.1016/j.petrol.2021.109601>.

850

851 [62] Qin, Q.S., Feng, D.S., Liu, P.F., He, Q., Li, X., Liu, A.M., Zhang, H., Hu, G.Q., Cheng, L.,
852 2017. Metagenomic characterization of *Candidatus Smithella cisternae* strain M82_1, a syntrophic

853 alkane-degrading bacteria, enriched from the Shengli Oil Field. *Microbes Environ.* 32, 234–243.
854 <https://doi.org/10.1264/jsme2.ME17022>.

855

856 [63] Lazar, C.S., Baker, B.J., Seitz, K.W., Teske, A.P., 2017. Genomic reconstruction of multiple
857 lineages of uncultured benthic archaea suggests distinct biogeochemical roles and ecological
858 niches. *ISME J.* 11, 1118–1129. <https://doi.org/10.1038/ismej.2016.189>.

859

860 [64] Kim, J.S., Crowley, D.E., 2007. Microbial diversity in natural asphalts of the Rancho La Brea
861 Tar Pits. *Appl. Environ. Microbiol.* 73, 4579–4591. <https://doi.org/10.1128/AEM.01372-06>.

862

863 [65] Hidalgo, K.J., Sierra-Garcia, I.N., Dellagnezze, B.M., de Oliveira, V.M., 2020. Metagenomic
864 insights into the mechanisms for biodegradation of polycyclic aromatic hydrocarbons in the Oil
865 Supply Chain. *Front Microbiol.* 18, 11:561506. <https://doi.org/10.3389/fmicb.2020.561506>.

866

867 [66] Shelton, J.L., Akob, D.M., McIntosh, J.C., Fierer, N., Spear, J.R., Warwick, P.D., McCray, J.E.
868 2016. Environmental drivers of differences in microbial community structure in crude oil reservoirs
869 across a methanogenic gradient. *Front. Microbiol.* 28, 1535.
870 <https://doi.org/10.3389/fmicb.2016.01535>.

871

872 [67] Varjani, S.J., Gnansounou, E. 2017. Microbial dynamics in petroleum oilfields and their
873 relationship with physiological properties of petroleum oil reservoirs. *Bioresource Technol.* 245,
874 1258–1265. <https://doi.org/10.1016/j.biortech.2017.08.028>.

875

876 [68] Roy, A., Sar, P., Sarkar, J., Dutta, A., Sarkar, P., Gupta, A., Mohapatra, B., Pal, S., Kazy, S.K.,
877 2018. Petroleum hydrocarbon rich oil refinery sludge of North-East India harbours anaerobic,

878 fermentative, sulfate-reducing, syntrophic and methanogenic microbial populations. *BMC*
879 *Microbiol.* 18(1):151. <https://doi.org/10.1186/s12866-018-1275-8>.

880

881 [69] Vilcáez, J., York, J., Youssef, N., Elshahed, M., 2018. Stimulation of methanogenic crude oil
882 biodegradation in depleted oil reservoirs. *Fuel* 232, 581–590. [https://doi.org/10.1016/j.fuel.2018.06.](https://doi.org/10.1016/j.fuel.2018.06.018)
883 018.

884

885 [70] Okabe, S., Odagiri, M., Ito, T., and Satoh, H. 2007. Succession of sulfur-oxidizing bacteria in
886 the microbial community on corroding concrete in sewer systems. *Appl. Environ. Microbiol.* 73,
887 971–980. <https://doi.org/10.1128/AEM.02054-06>.

888

889 [71] Tian, H., Gao, P., Chen, Z., Li, Y., Li, Y., Wang, Y., Zhou, J., Li, G., Ma, T. 2017.
890 Compositions and abundances of sulfate-reducing and sulfur-oxidizing microorganisms in water-
891 flooded petroleum reservoirs with different temperatures in China. *Front. Microbiol.* 8, 143.
892 <https://doi.org/10.3389/fmicb.2017.00143>.

893

894 [72] Rojas-Gätjens, D., Fuentes-Schweizer, P., Rojas-Jiménez, K. Pérez-Pantoja, D., Avendaño, R.,
895 Alpízar, R., Coronado, C., Chavaría, M., 2022. Methylotrophs and hydrocarbon-degrading bacteria
896 are key players in the microbial community of an abandoned century-old oil exploration well.
897 *Microb. Ecol.* 83, 83–99. <https://doi.org/10.1007/s00248-021-01748-1>.

898

899 [73] Hu, P., Tom, L., Singh, A., Thomas, B.C., Baker, B.J., Piceno, Y.M., Andersen, G.L.,
900 Banfield, J.F. 2016. Genome-Resolved Metagenomic Analysis Reveals Roles for Candidate Phyla
901 and Other Microbial Community Members in Biogeochemical Transformations in Oil Reservoirs.
902 *mBio.* 19; 7(1): e01669-15. <https://doi.org/10.1128/mBio.01669-15>.

903

904 [74] Lenchi, N., Inceoğlu, O., Kebbouche-Gana, S., Gana, M.L., Llirós, M., Servais, P., García-
905 Armisen, T. 2013. Diversity of microbial communities in production and injection waters of
906 Algerian oilfields revealed by 16S rRNA gene amplicon 454 pyrosequencing. *PLoS One*
907 21;8(6):e66588. <https://doi.org/10.1371/journal.pone.0066588>.

908

909 [75] Yun, Y., Gui, G., Xie, J., Chen, Y., Tian, X., Li, G., Gu, J.D., Ma, T. 2021. Stochastic
910 assembly process dominates bacterial succession during a long-term microbial enhanced oil
911 recovery. *Sci. Total Environ.* 790, 148203. <https://doi.org/10.1016/j.scitotenv.2021.148203>.

912

913 [76] Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A.
914 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437(7058), 543–546.
915 <https://doi.org/10.1038/nature03911>.

916

917 [77] Campeão, M.E., Reis, L., Leomil, L., de Oliveira, L., Otsuki, K., Gardinali, P., Pelz, O., Valle,
918 R., Thompson, F.L., Thompson, C.C. 2017. The Deep-Sea Microbial Community from the
919 Amazonian Basin Associated with Oil Degradation. *Front. Microbiol.* 13, 1019.
920 <http://doi.org/10.3389/fmicb.2017.01019>.

921

922 [78] Galarraga, F., Urbani, F., Escobar, M., Márquez, G., Martínez, M., Tocco, R., 2010. Main
923 factors controlling the compositional variability of seepage oils from Trujillo State, Western
924 Venezuela. *J. Petrol. Geol.* 33, 255–268. <https://doi.org/10.1111/j.1747-5457.2010.00477.x>.

925

926 [79] Wang, Sh., Wang, W., Zhang, Ch., Li, F. Guo, G., 2016. Bioremediation of oil sludge
927 contaminated soil by landfarming with added cotton stalks, *Int. Biodeter. Biodegr.* 106, 150–156.
928 <http://dx.doi.org/10.1016/j.ibiod.2015.10.014>.
929

930 [80] Jiang, Y., Brassington, K.J., Prpich, G., Paton, G.I., Semple, K.T., Pollard, S.J., Coulon, F.,
931 2016. Insights into the biodegradation of weathered hydrocarbons in contaminated soils by
932 bioaugmentation and nutrient stimulation. *Chemosphere* 161, 300-307. [https://doi.org/10.1016/j.](https://doi.org/10.1016/j.Chemosphere.2016.07.032)
933 [Chemosphere.2016.07.032](https://doi.org/10.1016/j.Chemosphere.2016.07.032).
934

935 [81] Ubani, O., Atanaga, H.I., Thantsha, M.S., 2013. Biological degradation of oil sludge: A review
936 of the current state of development. *Afr. J. Biotechnol.* 12, 6544–6567. [https://doi.org/10.5897/](https://doi.org/10.5897/AJB11.1139)
937 [AJB11.1139](https://doi.org/10.5897/AJB11.1139).
938

939 [82] Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative
940 bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and
941 bioaugmentation. *Bioresource Technol.* 96, 1049–1055. [http://dx.doi.org/10.1016/j.biortech.2004.](http://dx.doi.org/10.1016/j.biortech.2004.09.008)
942 [09.008](http://dx.doi.org/10.1016/j.biortech.2004.09.008).
943

944 [83] Kebbouche-Gana, S., Gana, M.L., Khemili, S., Fazouane-Naimi, F., Bouanane, N.A.,
945 Penninckx, M., hacene, H., 2009. Isolation and characterization of halophilic Archaea able to
946 produce biosurfactants. *J. Ind. Microbiol. Biotechnol.* 36, 727–738. [https://doi.org/10.1007/s10295-](https://doi.org/10.1007/s10295-009-0545-8)
947 [009-0545-8](https://doi.org/10.1007/s10295-009-0545-8).
948

949 [84] Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., 2001b. In situ bioremediation potential of an oily
950 sludge-degrading bacterial consortium. *Curr. Microbiol.* 43, 328–335.
951 <http://dx.doi.org/10.1007/s002840010311>.
952

953 [85] Martirani-Von Abercron, S.M., Marín, P., Solsona-Ferraz, M., Castañeda-Cataña, M.A.,
954 Marqués, S., 2017. Naphthalene biodegradation under oxygen-limiting conditions: community
955 dynamics and the relevance of biofilm-forming capacity. *Microb. Biotechnol.* 10, 1781–1796.
956 <https://doi.org/10.1111/1751-7915.12842>.
957

958 [86] Gao, H., Zhang, J.H., Lai, H.X., Xue, Q.H., 2017. Degradation of asphaltenes by two
959 *Pseudomonas aeruginosa* strains and their effects on physicochemical properties of crude oil. *Int.*
960 *Biodeter. Biodegr.* 122, 12–22. <https://doi.org/10.1016/j.ibiod.2017.04.010>.
961

962 [87] Curiel-Alegre, S., Velasco-Arroyo, B., Rumbo, C., Khan, A. H. A., Tamayo-Ramos, J. A.,
963 Rad, C., Gallego, J.R., Barros, R., 2022. Evaluation of biostimulation, bioaugmentation, and
964 organic amendments application on the bioremediation of recalcitrant hydrocarbons of soil.
965 *Chemosphere*, 307, 135638. <https://doi:10.1016/j.chemosphere.2022.135638>.
966

967 [88] Bennett, B., Adams, J.J., Gray, N.D., Sherry, A., Oldenburg, T.B.P., Huang, H., Larter, S.R.,
968 Head, I.M., 2013. The controls on the composition of biodegraded oils in the deep subsurface - Part
969 3. The impact of microorganism distribution on petroleum geochemical gradients in biodegraded
970 petroleum reservoirs. *Org. Geochem.* 56, 94–105. <http://dx.doi: 10.1016/j.orggeochem.2012.12.011>.
971

972 [89] Ortega, M.F., Guerrero, D.E., García-Martínez, M.J., Bolonio, D., Llamas, J.F., Canoira, L.,
973 Gallego, J.L.R., 2018. Optimization of landfarming amendments based on soil texture and crude oil
974 concentration. *Water Air Soil Pollut.* 229, 234. <https://doi.org/10.1007/s11270-018-3891-1>.

975

976 [90] Bher A, Mayekar PC, Auras RA, Schvezov CE., Biodegradation of Biodegradable Polymers in
977 Mesophilic Aerobic Environments. *Int J Mol Sci.* 2022 Oct 12;23(20):12165. doi:
978 10.3390/ijms232012165. PMID: 36293023; PMCID: PMC9603655.

979

980 [91] Lavania, M., Cheema, S., Sarma, P.M., Mandal, A.K., Lal, B., 2012. Biodegradation of asphalt
981 by *Garciaella petrolearia* TERIG02 for viscosity reduction of heavy oil. *Biodegradation* 23, 15–24.
982 <https://doi.org/10.1007/s10532-011-9482-0>.

983

984

985 Figure 1. Location map of the Riutort oil shale mine and structural features of the SE Pyrenees.

986

987 Figure 2. (a) Schematic map of the Riutort oil shale mine showing the study gallery (modified from Riba, 1985). (b)
988 Detailed map of the study gallery showing the position of the samples.

989

990 Figure 3. Flow chart of the designed experiments in the present study.

991

992 Figure 4. Relative abundance of bacterial dominant genus (> 3%), detected by Illumina sequencing of 16S rRNA gene
993 amplicons. The size of the solid circle indicates the relative abundance.

994

995 Figure 5. Principal Component Analyses (PCA) on the relative proportion of archaeal OTUs (at a 0.03 distance). a) 3D
996 plot of the first 3 components showing the clustering of all samples on the basis of archaeal diversity. Labels indicate
997 the origin of the samples. b) Hierarchical clustering dendrogram based on principal component analysis of archaeal
998 OTUs. It shows the similarity in archaeal community composition across sampling sites. Red bubbles indicate
999 representative clusters.

1000

1001 Figure 6. Relative abundance of archaeal dominant genus (>3%), detected by Illumina sequencing of 16S rRNA gene
1002 amplicons. The size of the solid circle indicates the relative abundance.

1003

Table 1: Parameters of Ancón formation waters.

Conductivity (mS/cm)	17.8
Salinity (ppm)	11,695
pH	7.2
Chloride (Cl ⁻)	2469.41
Sodium (Na ⁺)	1612.32
Magnesium (Mg ²⁺)	183.50
Calcium (Ca ²⁺)	236.27
Sulfate (SO ₄ ²⁻)	105.72
Bicarbonate (HCO ₃ ⁻)	124.63

Note: ionic concentrations in mg/l.

Table 2. Closest BLAST relative of the most representative 16S rRNA gene clones retrieved from sample Riutort-16I.

Closest relative by BLAST	Accession n°	Similarity (%)	Environment
<i>Pseudomonas marincola</i> strain 002-Na3	MG456871	98.87	Oil contaminated sediment [97]
Uncultured bacterium clone N-179 (<i>Pseudomonas</i> sp.)	HQ218621	99.33	Response to naphthalene
<i>Brevundimonas mediterranea</i> strain N7	MF156545	99.86	Naphthalene biodegradation [98]
Uncultured <i>Desulfobacula</i> sp. clone LU2-210	KF059938	98.62	Oil reservoir (unpublished)
Uncultured bacterium clone 91-13 (<i>Gammaproteobacteria</i>)	EF157132	97.13	Natural asphalts [70]
<i>Mycobacterium fluoranthenorans</i>	CP059894	99.38	Isobutane-metabolizing strains, degrading ether contaminants (unpublished)
<i>Microbacterium</i> sp. BA45(2011)	HQ398381	97.46	Caribbean Sponges [99]

Table 3: Percentages of TPH reduction and SARA fraction removal related to bioremediation methods in soil media supplemented with 1% (w/v) La Libertad Refinery's oily sludge at T=25°C and initial pH=7.

Biodegradation approaches	Time (day)							
	30 days after incubation				60 days after incubation			
	TPH	SAT	ARO	POL	TPH	SAT	ARO	POL
Natural attenuation	11.9	17.2	14.1	0.2	16.5	24.5	16.4	2.8
Biostimulation	17.6	23.9	18.1	6.0	29.8	29.1	26.1	9.3
Bioaugmentation + biostimulation	32.3	38.4	30.1	23.9	50.8	64.2	41.3	37.4

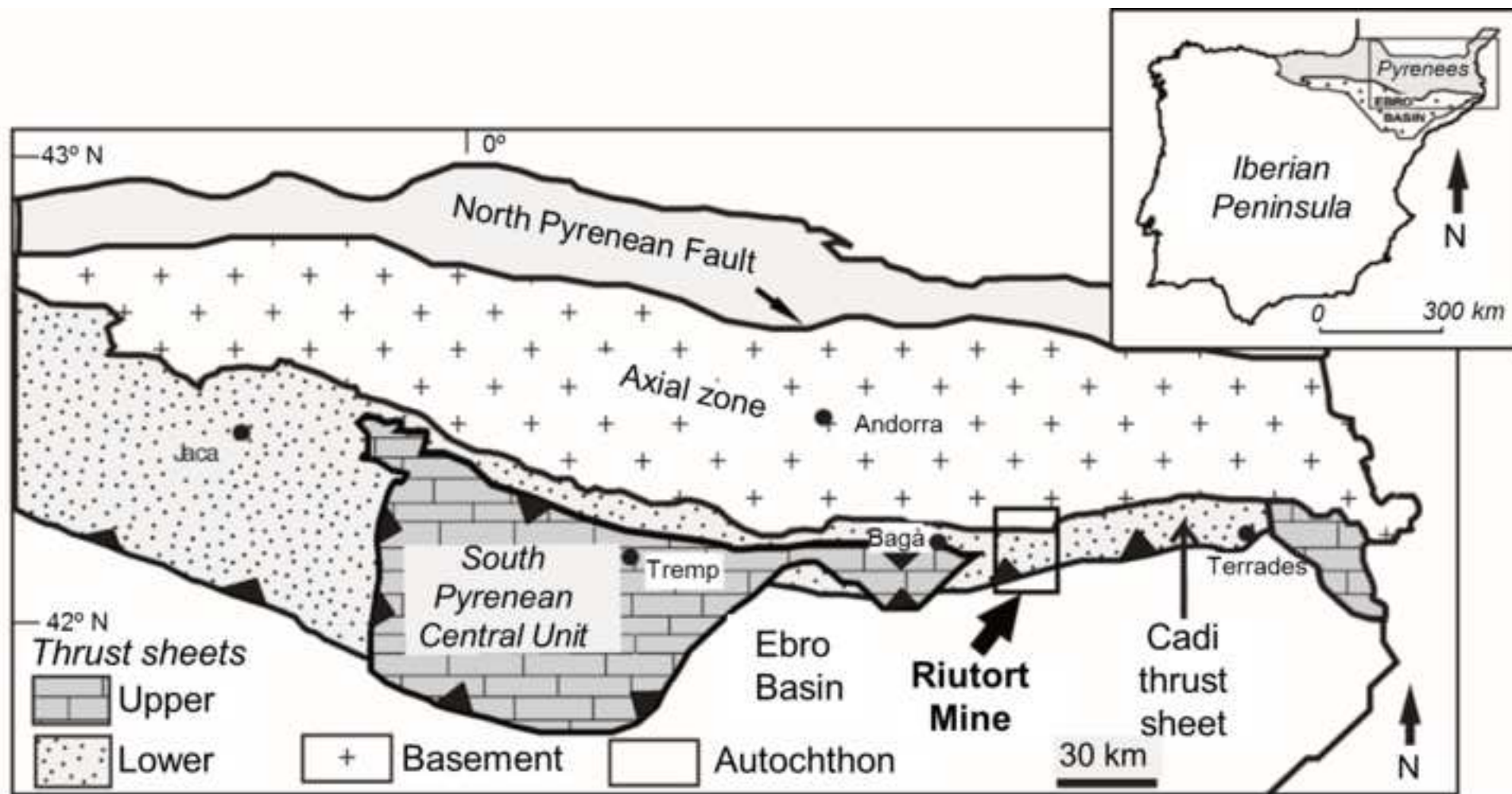
Table 4: Total microbial population (CFU/g of soil) related to bioremediation techniques in soil cultures at T=25°C and initial pH=7.

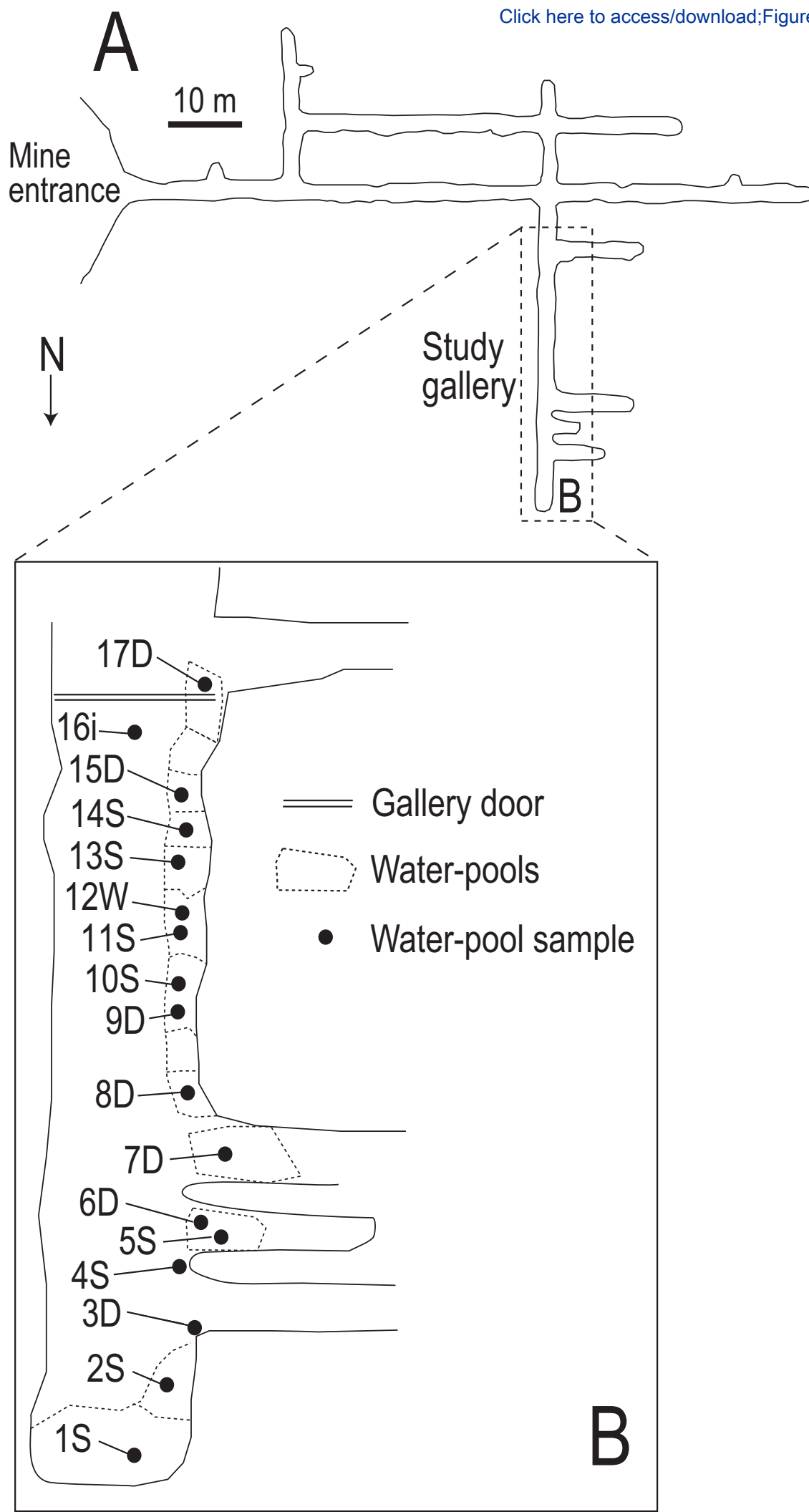
Biodegradation approaches	Time (day)		
	Onset	30 days after incubation	60 days after incubation
Natural attenuation	$6.54 \cdot 10^3$	$8.81 \cdot 10^3$	$9.62 \cdot 10^3$
Biostimulation	$30.15 \cdot 10^4$	$55.73 \cdot 10^4$	$42.95 \cdot 10^4$
Bioaugmentation + biostimulation	$3.26 \cdot 10^5$	$2.14 \cdot 10^8$	$1.45 \cdot 10^8$

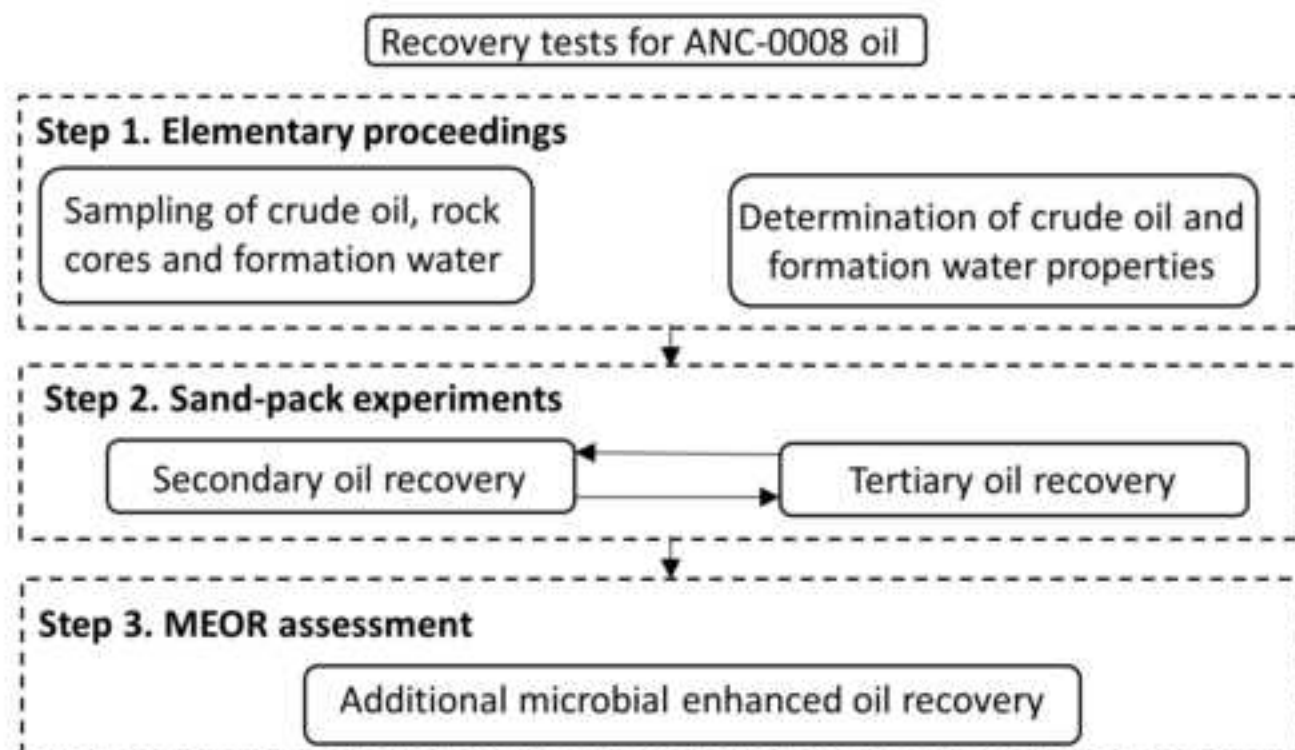
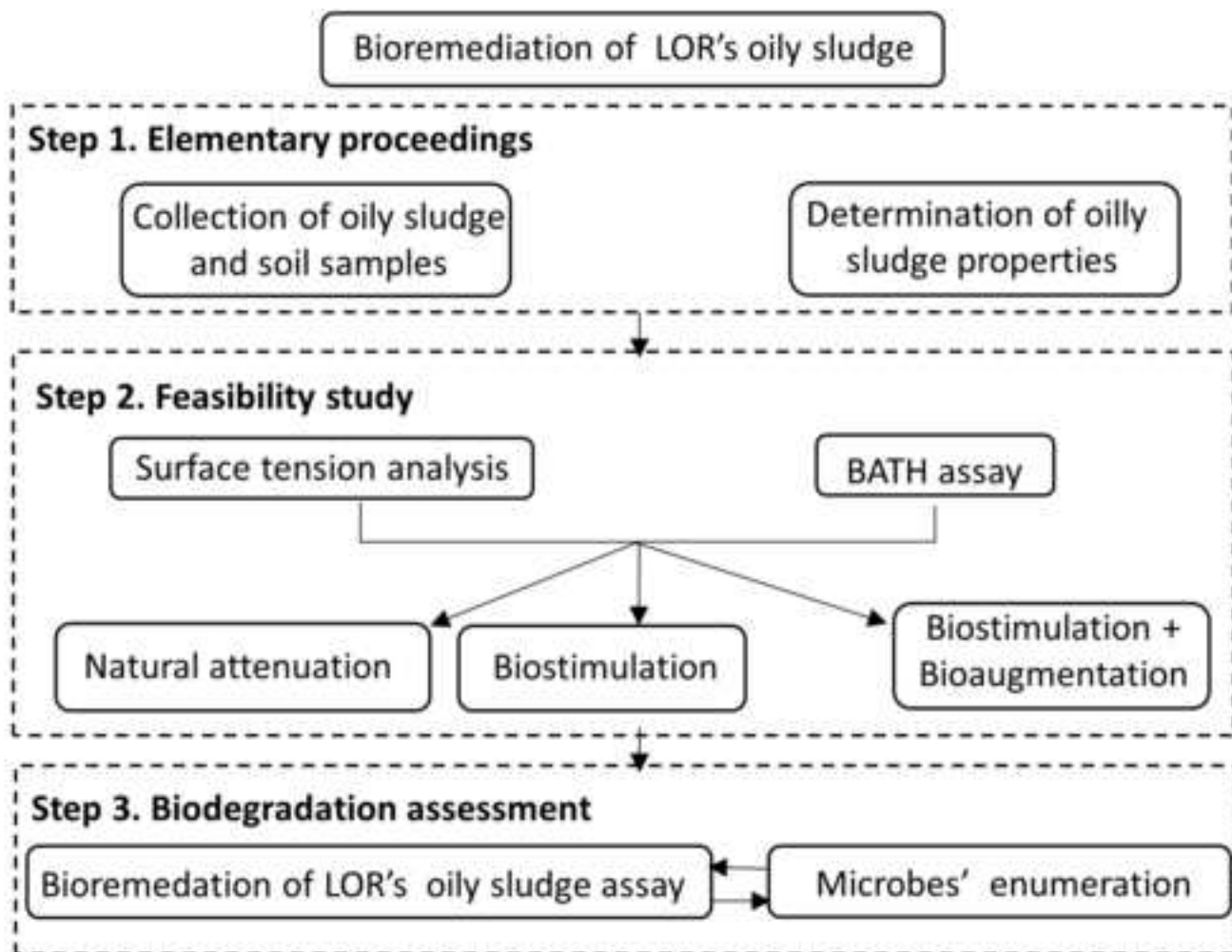
Appendix: NGS data; total numbers of reads accumulated for each sample in the original datasets and after filtering and classification; number of total OTUs and Shannon diversity (H) index.

	Sample	Initial sequences	Removed chimeras (%)	Final sequences	OTUs	Chao-1	H index
Bacteria	1S	90655	0.9	65418	296	507.7	4.8
	2S	122391	1.1	84417	355	501.7	4.8
	3D	110550	1.3	75586	332	484.0	4.7
	4S	96118	1.5	67774	376	520.1	4.7
	5S	136157	2.1	84417	272	518.6	4.6
	6D	128081	1.8	100228	255	439.5	4.5
	7D	120398	1.5	87929	297	421.1	4.9
	8D	122316	1.2	85379	396	541.0	4.9
	9D	88024	1.2	61676	355	486.6	4.9
	10S	112177	1.1	79678	384	491.0	4.8
	11S	132484	1.2	94537	424	580.8	5.0
	12W	101454	1.1	70299	337	450.2	4.5
	13S	169355	1.3	120515	477	632.8	5.0
	14S	131431	1.3	89391	369	523.7	4.7
	15D	180651	1.7	123676	416	523.9	4.8
	16I	155373	3.6	110762	362	489.1	4.4
	17D	94893	1.2	65827	441	569.3	5.0
Archaea	1S	211796	1.2	4492	8	11.3	1.8
	2S	295652	2.7	4840	5	6.5	1.5
	3D	268519	0.8	79176	10	16.0	1.8
	4S	331463	2.9	178	5	8.0	1.6
	5S	204855	2.1	2098	4	4.5	1.3
	6D	278640	2.0	53164	16	19.7	2.5
	7D	280132	2.0	5355	7	10.0	1.7
	8D	202044	1.6	50673	14	21.5	2.8
	9D	229779	0.8	37231	13	15.0	1.9
	10S	237660	2.2	19913	7	9.0	1.7
	11S	254540	0.9	36772	9	10.0	1.7
	12W	236409	2.1	36671	9	12.0	1.5
	13S	257159	0.8	21056	12	22.5	2.0
	14S	340369	0.6	217939	14	20.0	1.4
	15D	288802	0.9	53437	16	16.6	2.1
	16I	256689	2.2	6420	10	10.3	2.2
	17D	253754	0.9	60197	11	12.5	1.6

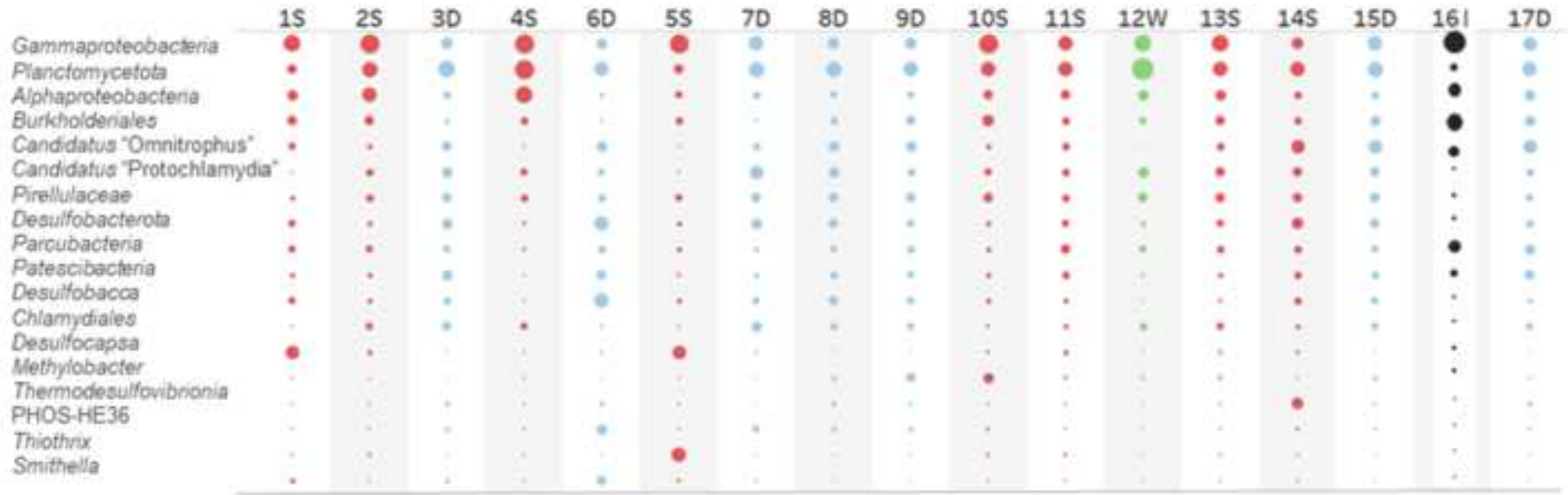
Figure 1







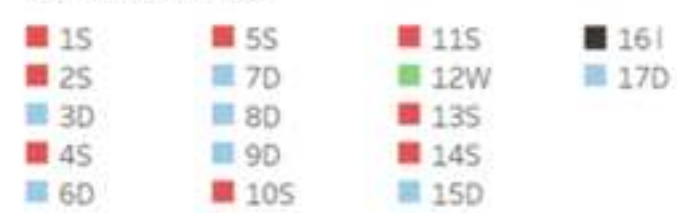
Bacteria > 3%



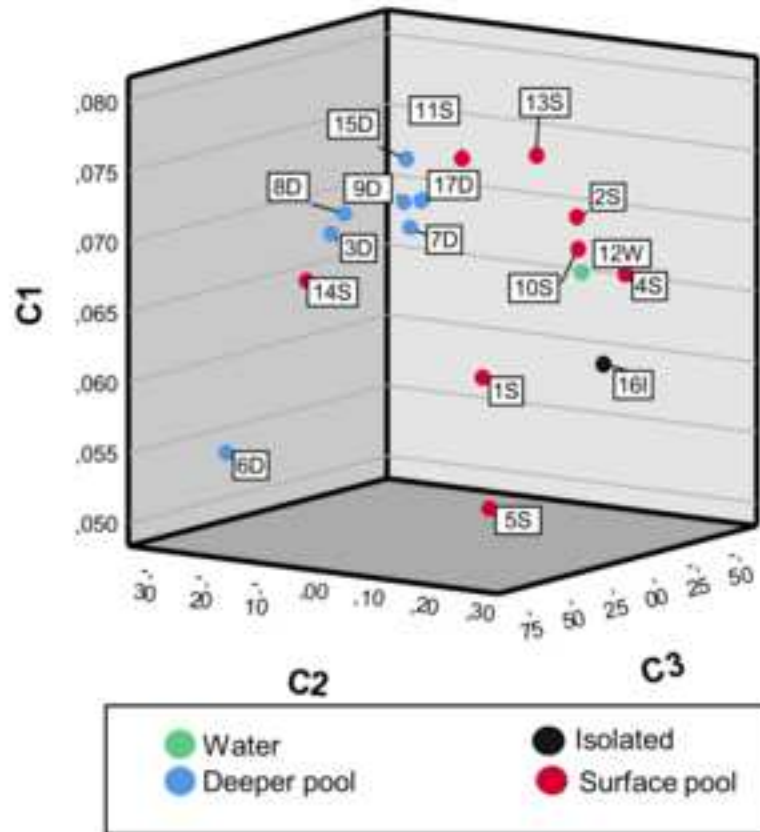
Measurement values



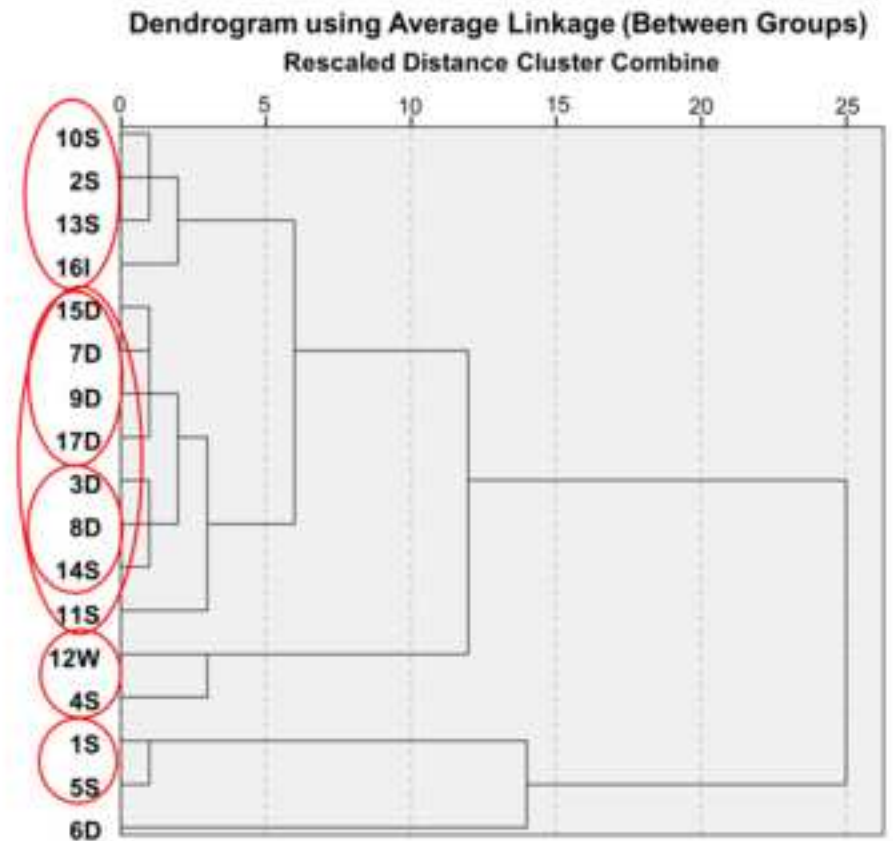
Sampling points



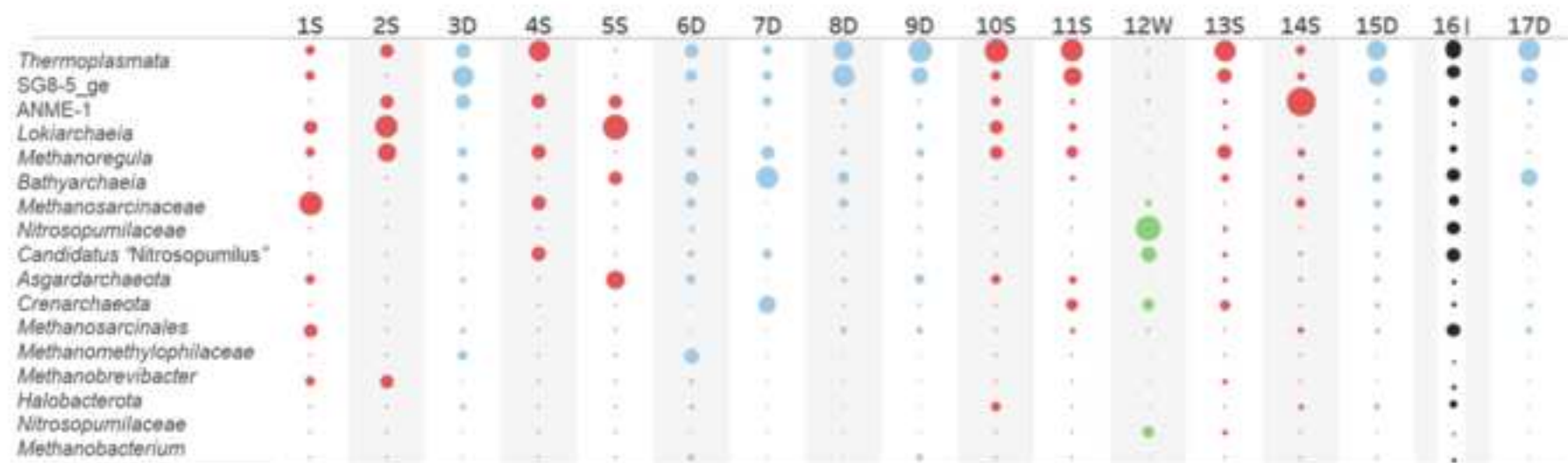
a)



b)



Archaea > 3%

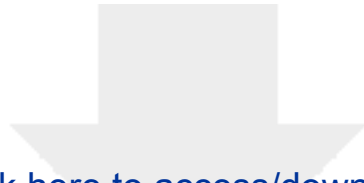


Measurement values

· 0,0000 ● 0,2000 ● 0,4000 ● 0,7000

Sampling points

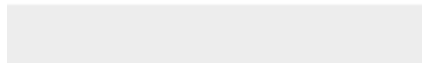
■ 15 ■ 55 ■ 115 ■ 16I
 ■ 25 ■ 70 ■ 12W ■ 170
 ■ 30 ■ 80 ■ 13S
 ■ 45 ■ 90 ■ 145
 ■ 60 ■ 105 ■ 150



[Click here to access/download](#)

Supplementary Material

Microbial correlation network and metabolisms.docx



Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: