Cultivable hydrocarbon degrading bacteria have low phylogenetic diversity but highly versatile functional potential

Marta Barbato¹, Francesca Mapelli¹, Elena Crotti¹, Daniele Daffonchio^{1,2}, Sara Borin^{1*}

¹ Department of Food, Environmental and Nutritional Sciences, University of Milan, 20133 Milan, Italy

² King Abdullah University of Science and Technology (KAUST), Biological and Environmental Sciences and Engineering Division, 23955-6900 Thuwal, Saudi Arabia

* corresponding author: Sara Borin, Department of Food, Environmental and Nutritional Sciences, University of Milan, via Celoria 2, 20133 Milan, Italy. Email: sara.borin@unimi.it

Abstract

Hydrocarbon (HC) pollution is a threat to the marine environment and bioremediation strategies based on microbial degradation have been developed for pollution clean-up. Effectiveness of bioaugmentation, i.e. the addition of suitable HC-degrading microorganisms to the polluted matrix, strongly depends on the metabolic and physiological versatility of **cultivable** HC-degrading microorganisms and on their adaptation capacity. **The aim of this work was to investigate the potential of laboratory enrichment approaches to obtain cultivable HC-degrading bacteria having versatility breadth. Despite we used as inoculum marine samples of different origin and contamination history, and applied different enrichment strategies, we brought into culture 183 hydrocarbonoclastic bacterial strains strongly dominated by only the two genera *Alcanivorax* and *Marinobacter*. These isolates, screened for traits related to HC degradation, biostimulation and abiotic stress tolerance, demonstrated nevertheless to have a diverse functional potential, correlated to the adopted enrichment strategy.** Although the obtained strains resulted phylogenetically similar, we showed that multiple cultivation approaches enhanced their metabolic diversification with potential benefits for bioaugmentation effectiveness.

Keywords

Marine bioremediation, Bioaugmentation, Bacterial diversity, Cultivation strategies, *Alcanivorax*, *Marinobacter*

1. Introduction

As a consequence of the intensive exploitation of underground marine petroleum deposits, the high traffic of oil tankers and the presence of submarine pipes for crude oil and refined product transport, hydrocarbon (HC) pollution is a threat to natural marine ecosystems worldwide (Daffonchio et al., 2012). The risk associated to HC pollution is even higher in semi-enclosed basins such as the Mediterranean Sea or the Red Sea, due to the seawater circulation turnover and the high exposure to oil HC. For instance, 20% of the global oil tanker traffic passes through the Mediterranean Sea, even in proximity of several delicate ecosystems (Daffonchio et al., 2013).

Current remediation strategies of oil spills are mostly based on mechanical techniques, the addition of chemical dispersants and the physical cleanup of the coastlines. However, in the last decades, a range of novel biotechnological remediation approaches have been proposed for water and coast clean-up (Mapelli et al., 2017). Such approaches are defined as *bioremediation* and take advantage of the metabolic potential of microorganisms to degrade pollutants (Sanabria, 2014). Microorganisms play key roles in all biogeochemical cycles and they contribute to the natural attenuation of pollutants not only by the way of the HC catabolic pathways but also as a consequence of their ability to produce molecules that increase the pollutant availability, such as biosurfactants. Bioaugmentation is one of the main approaches sustaining bioremediation of oil spills. It is based on the addition to the contaminated matrix of microorganisms capable to degrade pollutants (Mapelli et al., 2017). In particular, autochthonous bioaugmentation (ABA) technology (Nikolopoulou et al., 2013a) exploits the autochthonous microbial population of a polluted system. It consists in the enrichment and isolation in the laboratory of pollutant-degrading microorganisms, their biomass production and their subsequent inoculation in the polluted matrix. ABA can attenuate the limitations of allochthonous microorganisms dictated by the unpredictability of their adaptation to the environmental conditions of the polluted site, but mandatorily requires the obtainment in culture of degrading microorganisms equipped by additional physiological traits that could enhance pollutant degradation activity and bacterial adaptation to the contaminated environment.

The technological advances in molecular biology and biochemistry and the increased knowledge on the ecological, genetic and metabolic diversity of microorganisms, recently boosted marine HC bioremediation strategies (Daghio et al., 2016; Fodelianakis et al., 2015; Genovese et al., 2014; Viggi et al., 2015) that, however, can be only partially successful under field conditions. One important aspect to maximize bioaugmentation effectivity is the enhancement of the functional diversity of the cultured microbial degraders. The biotechnological potential of strains inhabiting marine environments is still largely unexplored because of their recalcitrance to isolation and/or maintenance under laboratory conditions (Joint et al., 2010; Overmann and Lepleux, 2016). While the study of the microbial community of a polluted site through high-throughput sequencing is very useful for understanding the existing microbial diversity, improving the

isolation and cultivation efficiency is an essential requisite for improving the success potential of bioaugmentation interventions. Different cultivation methods have been proposed to increase strain cultivability, including the optimization of novel media, the use of enrichment media for specific microbial groups, the development of *in situ* cultivation strategies and the mimicking of the conditions of the original environment (for instance by replicating the oligotrophic conditions of the polluted system or by adding specific signaling molecules) (reviewed in L'Haridon et al., 2016; Prakash et al., 2013). Particularly, the use of rich nutrient media, such as Marine Broth (MB), should be prevented since it allows the overgrowth of the heterotrophic, fast-growing and r-strategist microorganisms to the detriment of the slow-growing, K-strategist ones that, dominating the pelagic habitat, have a more relevant role from the ecological point of view. Marine fast-growing bacteria are, indeed, typically referred as weed-type microorganisms and include, among the others, genera such as *Vibrio*, *Pseudomonas*, *Oceanospirillum* and *Alteromonas* (L'Haridon et al., 2016; Prakash et al., 2013). Successful isolation attempts of slow-growing marine microorganisms have been performed when pristine seawater or oligotrophic media have been prepared (Cho and Giovannoni, 2004; Rappe et al., 2002).

In the perspective to develop enrichment strategies for the cultivation of diversified highly-performing microbial consortia for HC bioremediation, the objective of this work was to investigate the potential of laboratory oligotrophic enrichment approaches to cultivate HC-degrading bacteria having multiple adaptive traits with the ultimate goal of expanding the potential effectiveness of bioaugmentation applications. HC natural attenuation depends upon networks of microorganisms, not only capable of degrading the pollutants, but that harbor largely diverse functional adaptive traits such as biosurfactant and bioemulsifier production, cellular surface hydrophobicity and the capability to exploit different nutritional sources (Mapelli et al., 2017; McGenity et al., 2012). In addition, HC degrading bacteria should adapt and thrive in different environmental conditions that may change over time (Coulon et al., 2007; Dash et al., 2013). Here, we focused on a broad range of physiological properties important for HC biodegradation such as i) the ability to cope with different abiotic conditions, ii) motility and the capacity to adhere on solid surface, iii) the potential to interact with hydrophobic molecules and increase HC availability, and iv) the capacity of using different nitrogen sources.

2. Materials and Methods

2.1 Microcosm enrichments and bacteria isolation

Enrichment cultures were established using as inocula different marine environmental matrices (seawater, sediments) collected from different geographical areas. Different HCs were used as carbon source by supplementing to liquid mineral medium (Table 1). All the enrichment cultures were established in 500 mL serum bottle with 250 mL of growth media and 1% v/v (seawater samples) or 1% w/v (sediment samples) of

inoculum. Cycloheximide (0.01% w/v) was added to all the microcosms to avoid eukaryote growth. The enrichments were incubated at 30°C with constant shaking at 150 rpm until turbidity appeared (5-7 days). HC degrading (HD) enrichments were established using ONR7a mineral growth medium and 1% v/v of different HC molecules or mixture as the sole carbon source, as described in Table 1. The HD microcosm n°2 was setup in a two-step enrichment, firstly by using ONR7a growth medium and 1% v/v of crude oil (CO) as the sole carbon source and, after turbidity appearance, by inoculating 1 mL of this enrichment culture in 100 mL of ONR7a medium and 1% w/v pentadecane (C15) as the sole carbon source.

Long-term biostimulation enrichments (LTB; n°9 and 11) were established using ONR7a growth medium supplemented with a vitamin solution (stock solution 100x: Biotin 2.0 mg/L; Folic acid 2.0 mg/L; Thiamine-HCl 5.0mg/L; Calcium pantothenate 5.0 mg/L; Vitamin B12 0.1 mg/L; Riboflavin 5.0 mg/L) and CO as the sole carbon source. LTB enrichments were incubated for six months at 30°C before bacterial strain isolation.

Oil sorbent material enrichments (OSM, n°5-8 and 10) were established using either sterile marine water supplemented with a fertilizing solution (6.98 mM Na₂HPO₄·H₂O, 2.87 Mm K₂HPO₄, 9.35 mM NH₄Cl, Gertler et al., 2009) (n°5, 6 and 10) or Marine Broth (Conda, Spain) diluted 1:5 with sterile marine water (n°7 and 8). CO was supplied through the Oliguard™ fabric (HEIQ, Schlieren, Switzerland), which selectively absorbs lipophilic substances; Oliguard™ fabric was previously cut (1 cm x 2 cm), autoclaved and soaked in CO for 15 minutes. Aiming to enrich the bacterial populations able to grow attached to the Oliguard™ fabric surface, after 5 days of incubation of the primary enrichment (until appearance of medium turbidity), the fabric was washed in sterile physiologic water (0.9% NaCl) and re-inoculated in the same sterile growth medium. The procedure was repeated three times prior to proceed with the bacteria isolation.

For HD (n°1-4) and LTB (n° 9 and 11) enrichment cultures, at the end of the incubation time, bacterial strains were isolated by serial dilution on ONR7a agar plate containing cycloheximide (0.01%w/v) and 35 µL of the same carbon source present in the original enrichment culture. In the case of OSM enrichments (n°5-8 and 10), the isolation of the bacterial strains included an additional step where the fabric was washed in 10 mL sterile physiologic water and vigorously shaken in order to detach the bacteria from its surface. Either 35 µL of CO or a mix of aromatic compounds (naphthalene, p-xylene and m-xylene 1:1:1) were added as sole carbon source to ONR7a, spread on the surface of the solidified medium prior to the inoculum. For all isolation procedures, the plates were incubated at 30°C until visible bacteria colonies formed, then colonies were streaked three times into the same medium to obtain pure bacterial cultures.

2.2 Genotyping and identification of bacterial strains

The bacteria collection was dereplicated by intergenic internal transcribed spacer (ITS) PCR-fingerprinting, performed as previously described using the primers ITSF (3'-GTCGTAACAAGGTAGCCGTA-5') and ITS-R (3'-CTACGGCTACCTTGTTACGA-5') (Vacchini et al., 2017). The collection has been clustered in groups of strains

138 exhibiting the same ITS-fingerprinting profile. At least one representative for each ITS group was identified
139 by partial 16S rRNA sequencing and subsequent alignment of the sequence in NCBI database
140 (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>). The amplification of the bacterial 16S rRNA gene was
141 performed according to Mapelli et al. (2013) using the universal primers 27F (3'-AGAGTTTGATCMTGGCTCAG-
142 5') and 1492R (3'-CTACGGCTACCTTGTACGA-5'). The partial 16S rRNA gene sequences obtained from the
143 bacterial isolates are available at the European Nucleotide Archive (ENA) under accession numbers
144 LT935671-LT935783.

145

146 **2.3 Characterization of isolated bacteria physiological traits**

147

148 2.3.1 Tolerance to abiotic stresses

149 The strains were inoculated on either ONR7a with 1% Na-pyruvate or MB agar plates to assess their tolerance
150 to environmental stresses. The plates were incubated at 4°C, 15°C, 20°C, 30°C, 37°C and 50°C, for a maximum
151 of 15 days, to assess the strain capability to grow at different temperatures. The ability of the isolates to grow
152 at high salt concentration was tested in solid medium added by different NaCl concentration (5%, 10% and
153 15%) and incubating the plates at 30° for 7 days.

154

155 2.3.2 Motility assessment: swimming, swarming and chemotaxis assays

156 The swimming and swarming motility assays were modified by Mi et al. (2015). Plates for the swimming assay
157 contained either ONR7a with 1% Na-pyruvate or MB solidified by 0.3% (w/v) agar, whereas the swarming
158 assay plates were obtained by solidifying the same growth medium with 0.5% agar. Five µl of bacterial culture
159 were spotted on each plate and incubated at 30°C for 24 hours.

160

161 2.3.3 Adhesion test

162 The capacity of the isolates to adhere to solid surfaces was determined by the crystal violet staining assay
163 (Antoniani et al., 2010; Stepanovic et al., 2007). Bacteria were grown for 72 h in liquid marine broth or ONR7a
164 medium added 1% Na-pyruvate, depending on the isolation condition, at 30 °C in 96 wells polystyrene plates.
165 After the growth, the optical density at 600 nm (OD₆₀₀) of the cell culture was measured. The liquid culture
166 was then removed from the wells, and cells attached to the well surface were washed gently with PBS and
167 stained for 10 min with 0.03% crystal violet, thoroughly washed with PBS and water, and dried. The OD₆₀₀ of
168 crystal violet stained biofilm cells was determined and normalized to OD₆₀₀ of cells from the corresponding
169 liquid cultures; this value is defined as "adhesion units". The isolates were considered capable to adhere to
170 solid surfaces if they showed an adhesion unit value of at least 0.12. If the value exceeded 0.24, they were
171 classified as strongly adherent. Strains displaying values between 0.12 and 0.24 were classified as weakly
172 adherent (Amer et al., 2015).

173

174 2.3.4 Emulsification tests

175 The test was performed by modifying the procedure previously described by Batista et al. (2006) and Das et
176 al. (1998). Cells were grown in 20 mL of ONR7a medium added with 1% CO as the sole carbon source, at 30
177 °C under constant shaking. After 1 week of incubation the test was performed on 2 mL of both the whole
178 culture and its cell-free supernatant (obtained by pelleting the cells at 4500 rpm for 10 min at 4 °C). Two mL
179 of mineral oil and the same volume of cell culture/supernatant were vortexed together for 2 min and then
180 equilibrated at room temperature for 30 min before evaluating the height of the emulsion layer (t_0). The
181 emulsification activity was calculated as follows: $EV_{t_0} (\%) = (\text{height of the emulsion layer} / \text{total height}) \times 100$.
182 To evaluate emulsification, the height of the emulsification layer obtained from each sample was compared
183 with positive (SDS 10%) and negative (sterile growth media) controls, while to evaluate the stability of the
184 emulsion the emulsification activity value at t_0 was compared with the emulsification value calculated after
185 48 h ($EV_{t_{48h}}$).

186

187 2.3.5 Microbial adhesion to hydrocarbons (MATH)

188 Cell hydrophobicity was evaluated through the MATH test as previously reported (Barbato et al, 2016).
189 Bacterial strains were grown with either ONR7a or MB liquid media added with CO 1% v/v. Cells were pelleted
190 at 4000 rpm for 10 min and rinsed twice in PUM buffer (22.2 g/L $K_2HPO_4 \cdot 3H_2O$; 7.26 g/L KH_2PO_4 ; 1.8 g/L UREA;
191 0.2 g/L $MgSO_4 \cdot 7H_2O$; pH was adjusted to 7.1). Cells were resuspended in PUM buffer to a final volume of 1.2
192 mL. After measuring the rinsed cells OD at 600 nm (OD_i), cells were supplied with 0.2 mL of mineral oil and
193 incubated at 30°C for 10 min. Afterwards, cells were mixed for 2 min and left to rest for 15 min. Finally, the
194 OD at 600 nm of the aqueous phase was measured again (OD_f). The relative amount of cells bound to mineral
195 oil was calculated as follows: $((OD_i - OD_f) * 100) / OD_i$.

196

197 2.3.6 Uric acid degradation

198 The uricolytic activity of the isolates was tested according to Morales-Jiménez et al., 2013 with minor
199 modifications. Cells were grown in liquid medium (either MB or ONR7a added with 1% Na-pyruvate). Ten µl
200 of each cell culture were spotted in triplicates on the solid medium added with 0.5% uric acid, and incubated
201 at 30 °C for 4 days. The strains able to degrade uric acid showed the formation of a clear halo around the
202 colony.

203

204 **2.4 Statistical analyses**

205

206 A matrix of the presence/absence of the tested activities for each strains was built and similarities between
207 the different bacterial strains was explored by non-metric multi-dimensional scaling (nMDS). Significant

208 differences between diverse species, carbon sources and growth media used during the bacterial strain
209 enrichment were investigated through Permutational Multivariate Analysis of Variance (PERMANOVA,
210 Anderson, 2001). The analysis were conducted using the software PRIMER v. 6.1 (Clarke and Gorley, 2006)
211 and PERMANOVA + for PRIMER routines (Anderson et al., 2008).

212

213 3. Results and Discussion

214

215 3.1 Isolation and identification of hydrocarbon degrading bacteria

216

217 In order to expand the probability to bring onto culture a bacterial collection with wide phylogenetic diversity,
218 different marine samples (different geographical locations, sediments/seawater) were inoculated in different
219 oligotrophic mineral media supplemented by oil hydrocarbons (crude oil or selected hydrocarbon
220 compounds), isolating planktonic bacteria or bacterial cells with the ability to colonize oil sorbent fabric
221 (Table 1). A total of 183 HC-degrading bacterial isolates were obtained (Table S1). They clustered in 61 groups
222 with polymorphic ITS-fingerprints and were affiliated to four phyla: the Gram positive Actinobacteria (n=19)
223 and Firmicutes (n=4) and the Gram negative Bacteroidetes (n=3) and Proteobacteria (n=157). The
224 predominance of Proteobacteria is in accordance with previous studies on microbial communities inhabiting
225 HC polluted environments (Cappello et al., 2016; Mahjoubi et al., 2013). This phylum comprises several
226 hydrocarbon degrading genera involved in the first phases of HC degradation in marine ecosystems (Head et
227 al., 2006).

228 The isolates belonged to 14 genera (*Alcanivorax*, n=39; *Bacillus*, n=4; *Dietzia*, n=2; *Halomonas*, n=30;
229 *Idiomarina*, n=2; *Isoperticola*, n=7; *Marinobacter*, n=61; *Micrococcus*, n=5; *Muricauda*, n=3; *Pseudomonas*,
230 n=10; *Roseovarius*, n=8; *Songiibacter*, n=3; *Streptomyces*, n=5; *Thioclava*, n=4), showing nevertheless the
231 net dominance of *Marinobacter* (33% of the strains) and *Alcanivorax* (21% of the strains). Both these genera,
232 which were isolated from all the enrichment conditions, are ubiquitous in oil contaminated marine
233 environments and generally are the first growing after an oil spill (Mapelli et al., 2017). Given i) the
234 predominance of *Alcanivorax* and *Marinobacter* in our bacterial collection, despite obtained with different
235 enrichment methods applied to different samples, and ii) their importance and ubiquity in marine
236 ecosystems, especially in relation to HC pollution, natural attenuation and bioremediation processes (Head
237 et al., 2006; McGenity et al., 2012), we focused on their physiological characterization, aiming to evaluate if
238 these strains could nevertheless harbor functional diversity, although belonging to the same taxonomic
239 groups.

240 The *Alcanivorax* strains were affiliated to the species *A. borkumensis* (n=20 isolates), *A. dieselolei* (n=5) and
241 *A. venustensis* (n=14) (Table S1). *A. borkumensis* is a deeply characterized marine hydrocarbonoclastic species
242 (Barbato et al., 2016; Bookstaver et al., 2015; Hassanshahian et al., 2014; Naether et al., 2013; Sabirova et

al., 2008; Schneiker et al., 2006; Scoma et al., 2016) able to degrade linear alkanes, cycloalkanes and isoprenoids, and to produce different glycolipid biosurfactants (Gertler et al., 2009; Golyshin et al., 2003; Yakimov et al., 1998). Coherently with its capability to grow attached to oil sorbent materials (Gertler et al., 2009), all *A. borkumensis* strains of this collection were specifically enriched from the OSM microcosms (Table S1, Figure 1a). *A. venustensis* and *A. dieselolei* were isolated from different sites, both from seawater and sediments, in the presence of the carbon sources DS, CO (liquid or supplied through the OSM) or C12 (Table S1, Figure 1 a-c). *A. dieselolei* is known to degrade a wide range of linear and branched alkanes (Barbato et al., 2015; Liu and Shao, 2005; Wang and Shao, 2014).

The *Marinobacter* genus includes marine bacteria with a wider metabolic versatility than *Alcanivorax*. Several *Marinobacter* spp. strains grow on and degrade both alkane and polycyclic aromatic hydrocarbons (PAHs) (Bonin et al., 2015; Cui et al., 2013; Wang and Shao, 2012). The *Marinobacter* strains were classified in four species (Table S1), *M. adhaerens* (n=32), *M. alkaliphilus* (n=11), *M. flavimaris* (n=13) and *M. hydrocarbonoclasticus* (n=5). *M. adhaerens* strains were isolated on CO supplied through the OSM solid tissues, and in liquid DS and C8 media (Table S1, Figure 1a,b). This species is known to grow both in planktonic conditions and on solid surfaces, such as diatoms (Gärdes et al., 2010). *M. alkaliphilus* (Takai et al., 2005) was isolated exclusively from the Augusta Bay seawater and exclusively on C8 and C12 as carbon source (Figure 1b,c). *M. flavimaris* can degrade long chain HCs and PAHs (Bonin et al., 2015) and it was isolated exclusively from OSM enrichments using the Augusta seawater inoculum (Figure 1c). Strains of *M. hydrocarbonoclasticus* were isolated from both seawater and sediments, in presence of CO and C15 (Figure 1 a-c). This species can grow in both planktonic and biofilm forms (Gauthier et al., 1992; Klein et al., 2008; McGenity et al., 2012).

3.2 Physiological characterization of *Alcanivorax* and *Marinobacter* strains

3.2.1 Strain ability to cope with different abiotic conditions

All the *Alcanivorax* and *Marinobacter* strains were able to grow at temperatures ranging between 15 and 37°C, while none grew at higher temperature (50°C). Eighteen percent of the strains were able to grow at 4°C: they were mainly isolated from Augusta seawater (n=17) and they belong to *M. adhaerens* (n=1), *M. alkaliphilus* (n=5) and *M. flavimaris* (n=11) species. Only one *A. venustensis* strain, isolated from Augusta sediments in the long-term biostimulation enrichment, grew at 4°C (Table S2). The tolerance toward low temperature was, nevertheless, not specifically related to the temperature of the samples' collection sites. The water average temperatures of the epi-, meso- and bathypelagic waters are indeed higher than 13°C both in the Eastern and Western Mediterranean Sea (Mapelli et al. 2013) and the average temperature of sediments in the Augusta area was reported to be about 19 °C (Bargiela et al. 2015). Therefore, although the sites' temperature is not a direct selecting factor for the presence of psychrophilic microorganisms, we observed a thermal versatility of the isolates.

278 All the strains tolerated higher salinity than that of seawater. All grew at 5% NaCl and 80% of them tolerated
279 10% NaCl, but no relationships emerged among salinity tolerance and the species, origin of sampling or
280 enrichment technique. The strains able to grow in media containing 15% NaCl belonged only to 4 species, i.e.
281 *A. dieselolei* (n=1), *A. venustensis* (n=4), *M. adhaerens* (n=5) and *M. flavimaris* (n=13) (Table S2).

282

283 3.2.2 Bacteria motility in the environment and adhesion on solid surfaces

284 We assessed the swimming and swarming capability of the strains on the surface of the agarised medium, as
285 an index of the capacity to move towards carbon sources or escape hostile environments (Taktikos et al.,
286 2013). Swimming is the motility in liquid environments related to flagella, while swarming is a more complex
287 movement on semi-solid surfaces that consists in the ability of bacteria to slide one over each other and
288 requires changes in cells morphology (Partridge and Harshey, 2013). The moving capacity appeared to be a
289 taxonomy-related trait, mainly exhibited by *Alcanivorax*. Only 1 *M. adhaerens* and 9 *M. flavimaris* strains
290 were able to move through swimming, with the latter species including also 6 strains able to move by
291 swarming (Table S2). Differently, most of the *A. borkumensis* strains were able to move through swimming
292 (n=17) and swarming (n=18), as well as the *A. dieselolei* strains (n=4). As far as *A. venustensis*, only few of the
293 14 isolates were able to move through swimming (n=2) and swarming (n=1).

294 The adhesion test indicates the strains ability to adhere to solid surfaces, and represents an indirect evidence
295 of their biofilm formation capability (Moreira et al., 2017). Bacteria adhesion on solid surfaces can be useful
296 in bioremediation strategies implementing the use of OSM, on which hydrocarbonoclastic bacteria could
297 grow. Biofilm formation also helps bacteria to protect from hostile conditions, allowing mutualistic
298 interactions between cells that can lead to an easier and more efficient pollutant degradation (Singh et al.,
299 2006). Thirty one percent of the 100 strains tested presented a strong adhesion phenotype, while only 19%
300 showed a weak adhesion capacity. With the only exception of *A. dieselolei* strains, all *Alcanivorax* and
301 *Marinobacter* species showed such capability (Table S2). Noteworthy, 44% of the positive strains were
302 isolated from the OSM enrichments.

303 Biofilm formation can be also related to swarming because both processes require cell-to-cell interaction and
304 efficient communication system to induce the differentiation mechanisms (Deziel et al., 2001). In our
305 collection, 13 strains (*A. borkumensis*, n=10; *M. flavimaris*, n=3) were able to both move through swarming
306 and adhere to solid surfaces (Table S2). All these strains were isolated from the enrichments established with
307 the oil sorbent material using the Augusta Bay seawater as inoculum, suggesting the use of OSM as optimal
308 cultivation strategy for the selection of biofilm-forming HC degrading bacteria.

309

310 3.2.3 Interaction with hydrophobic molecules to promote HC availability

311 To enhance the bioavailability of the hydrophobic HCs, microorganisms evolved different strategies, such as
312 the production of bioemulsifiers, high-molecular weight molecules that, even at low concentration, can

emulsify two immiscible liquids (Uzoigwe et al., 2015). In HC polluted environments, bioemulsifiers stabilize the emulsions and prevent the aggregation of the dispersed HCs, thus increasing HC specific surface area and bioavailability (Uzoigwe et al., 2015). Eighty-three percent of the *Alcanivorax* and *Marinobacter* strains were able to produce bioemulsifiers, as detected testing the whole culture, and 72% of them produced a stable emulsion. All species included strains able to produce emulsifying molecules and, in particular, all the strains of *A. dieselolei*, *M. hydrocarbonoclasticus* and *M. alkaliphilus* were positive (Table S2). The supernatant of the positive strains was tested for the presence of extracellular bioemulsifying molecules, revealing that 79% of the strains were capable to release these molecules in the growth medium (Table S2). Although emulsification makes HCs more bioavailable, to be degraded they have to enter inside the bacterial cell through the cell surface barriers (Xue et al., 2015). HC uptake can be therefore facilitated by a hydrophobic cellular surface. The MATH test is indicative of the bacterial cell surface hydrophobicity, allowing to characterize their capability to adhere to hydrophobic molecules. Regardless the strain taxonomy, sample of origin and enrichment method, 63% of the strains showed different levels of hydrophobicity. Nonetheless, only 24% of the strains (*A. borkumensis* n=12, *A. venustensis* n=2, *M. adhaerens* n=8, *M. alkaliphilus* n=2) showed a rate of cell repartition in the hydrophobic phase higher than 50% (Table S2). In total, 57 strains of the genera *Alcanivorax* and *Marinobacter* presented both bioemulsifier production capacity and a hydrophobic cellular surface (Table S2), an advantageous combination that favors increased HC solubilization and micelle formation and enhances cell interaction with HC (Bouchez Naïtali et al., 1999).

331

332 3.2.4 Use of uric acid as nitrogen source

The unbalanced C/N/P ratio usually encountered in marine sites where oil spills occurred, is a main limiting factor hampering HC degradation (Gertler et al., 2015). The addition of nitrogen and phosphorous fertilizers is usually adopted for biostimulating HC degradation (Gertler et al., 2015; Knezevich et al., 2007; Koren et al., 2003; Nikolopoulou and Kalogerakis, 2008; Nikolopoulou et al., 2013b). Compared to other nitrogen sources, uric acid is highly available, low-cost and more efficient than conventional fertilizers, due to its low water solubility and high adhesion to lipophilic substances, which determine its low dispersion in the environment (Gertler et al., 2015; Ron and Rosenberg, 2014). The possibility to achieve cheap uric acid-based biostimulation techniques in a range of marine sediments has been recently proposed (Gertler et al., 2015) and a metagenomics-based study revealed that it influenced the substrate utilization capacities and preferences by the bacterial community (Bargiela et al., 2015). A specific role for uric acid degradation has been specifically described only for the genus *Halomonas* (Gertler et al., 2015), however, in our collection, 68% of the isolates were able to solubilize uric acid. As indicated in Table S2 these strains were identified as *A. borkumensis* (n=10), *A. dieselolei* (n=4), *A. venustensis* (n=8), *M. adhaerens* (n=29), *M. alkaliphilus* (n=9), *M. flavimaris* (n=3) and *M. hydrocarbonoclasticus* (n=5).

347

3.3 Factors influencing physiological characteristics of *Alcanivorax* and *Marinobacter* strains

The described phenotypes are indicative of the strain adaptation to different environmental conditions and of their biotechnological potential, two aspects that make them promising resources for bioaugmentation strategies. In this work, we aimed to identify among members of the genera *Alcanivorax* and *Marinobacter*, extensively brought into culture from different HC contaminated environments regardless of the cultivation method adopted (Jurelevicius et al., 2013; Raeid et al., 2014), which factors related to the cultivation strategy can lead to obtain strains with diversified and versatile physiological traits of interest for bioaugmentation.

The strains were therefore categorized in classes on the basis of the environmental matrix of origin, their phylogenetic group and the HC delivery method adopted in their original enrichment. PERMANOVA analyses was then applied to evaluate the statistically significant differences in the strain physiological traits within the above-mentioned categories. The strain physiological traits were significantly different according to the method of HC delivery adopted during the bacteria enrichment (PERMANOVA, $F_{1,99}=17.17$; $p=0.0001$, Figure 2a), the environmental matrix used as inoculum (PERMANOVA, $F_{1,99}=6.00$; $p=0.0024$, Figure 2b) and the species (PERMANOVA, $F_{6,99}=18.546$; $p=0.0001$, Figure 2c). PERMANOVA results highlighted that, besides phylogenetic affiliation of the strains, also the enrichment strategy was related to their physiological traits, suggesting the importance of setting up diversified cultivation conditions to obtain in culture isolates with versatile functional properties of interest. Besides cultivation strategy, the strain physiological traits were significantly differentiated in relation to the sample of origin, indicating that also different habitats can be source of strains having diversified functional potential.

Looking at the entire *Alcanivorax* and *Marinobacter* collection, an effect ascribable exclusively to the taxonomic affiliation of the strains did not clearly emerge. Although *A. borkumensis*, *A. dieselolei* and *M. flavimaris* strains were significantly different from those affiliated to all the other species in terms of functional diversity (PERMANOVA, see Table 2), these species have been isolated only under one of the two HC delivery system approaches, either OSM (*A. borkumensis* and *M. flavimaris*) or liquid HC (*A. dieselolei*), thus hampering any speculation about the selecting factor (i.e. the phylogenetic species or the HC delivery method adopted for strain enrichment).

The most relevant traits which contributed to differentiate the functional potential of the strains were the cell movement capacity and temperature and salt tolerance. *A. borkumensis* and *A. dieselolei* representatives were both characterized by a high number of strains capable to perform swimming and swarming, while most of the strains growing under the most extreme abiotic conditions tested in this study (4°C and 15% NaCl) belonged to the *M. flavimaris* species.

To reveal if the functional diversity of the strains depended on the taxonomic classification or on the HC delivery strategy, we focused on the *M. adhaerens* collection, comprising strains isolated from microcosms established both supplying HC in the liquid form and through OSM (Figure 1a). The enrichment method for

the isolates belonging to this genus significantly influenced their functional diversity, indicating that the supplement of HC in the liquid form or vehiculated through the OSM could have induced the selection of strains with diversified physiological characteristics (Pair-wise PERMANOVA; $t=3.0075$, $P=0.0013$). Specifically, *M. adhaerens* strains isolated with the OSM have higher adhesion values with respect to those isolated with liquids HCs, therefore the OSM use could have selected *M. adhaerens* strains potentially more effective in forming biofilms.

A comparison of all strains isolated using the OSM revealed that they are significantly different, in terms of functionality, on the bases of the different species (PERMANOVA, see Table 3a), indicating that OSM use promotes the enrichment of strains with high diversity in terms of physiological traits relevant for bioaugmentation. On the contrary, in the enrichment established using liquid HCs, the *A. venustensis* strains could not be significantly separated from those affiliated to the other species (i.e. *M. adhaerens*, *M. alkaliphilus* and *M. hydrocarbonoclasticus*) (PERMANOVA, see Table 3b), suggesting that the liquid HCs have favored the selection of strains sharing common physiological traits. Interestingly, all these strains have in common a low mobility capacity, which was higher in strains isolated in the presence of OSM. The results lead to speculate that the supply of HC through a solid carrier like OSM selected bacteria able to move reaching the HC source and enrich those able to attach to the substrate growing in the form of biofilm.

399

400 4. Conclusion

Cultivation of new HC-degrading marine bacteria is a fundamental step for the development of bioaugmentation processes but, despite the application of different cultivation approaches and different environmental samples of origin, we were able to bring into culture mainly the well-known *Alcanivorax* and *Marinobacter* genera, routinely isolated from contaminated environments. The characterization of their physiological traits, nevertheless, demonstrated the existence of a significant influence, among other factors, of laboratory cultivation strategies on the selection of functionally diverse bacterial strains having versatile activities relevant in HC bioremediation, especially in autochthonous bioaugmentation. The use of the oil sorbent material as HC vector allowed, in particular, the isolation of strains with the highest level of functional diversity.

410

411 Acknowledgments

This work was granted by the EU FP7 project Kill Spill (G.A. No. 312139) and the EU Horizon 2020 Project INMARE (G.A. No. 634486). DD thanks the support of King Abdullah University of Science and Technology (baseline research funds to DD). The authors acknowledge Dr. Simone Cappello and Dr. Mirko Magagnini and their research groups for providing part of the environmental samples used in this work, and Ms. Valentina Sforza for her contribute in performing the physiological characterization of the strains.

417

418 **Author contributions**

419 The study was conceived by SB and supported by SB and DD. All authors contributed to data collection. MB
420 and FM performed the laboratory work and data analysis. MB, FM, EC and SB interpreted the data and
421 prepared the manuscript. All authors critically reviewed and edited the manuscript and have approved its
422 publication.

423

424 **Competing interests**

425 The authors declare that they have no conflict of interest.

426

427 REFERENCES

- 428 Amer, R.A., Mapelli, F., Gendi, H.M. El, Barbato, M., Goda, D.A., Corsini, A., Cavalca, L., Fusi, M., Borin,
429 S., Daffonchio, D., Abdel-fattah, Y.R., 2015. Bacterial Diversity and Bioremediation Potential of the
430 Highly Contaminated Marine Sediments at El-Max District (Egypt , Mediterranean Sea). Biomed Res.
431 Int.
- 432 Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral
433 Ecol. 26, 32–46. <https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x>
- 434 Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. +for PRIMER: Guide to Software and Statistical
435 Methods, PRIMER-E. Plymouth, UK.
- 436 Antoniani, D., Bocci, P., MacIa²g, A., Raffaelli, N., Landini, P., 2010. Monitoring of diguanylate cyclase
437 activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening
438 of biofilm inhibitors. Appl. Microbiol. Biotechnol. 85, 1095–1104. [https://doi.org/10.1007/s00253-](https://doi.org/10.1007/s00253-009-2199-x)
439 009-2199-x
- 440 Barbato, M., Mapelli, F., Chouaia, B., Crotti, E., Daffonchio, D., Borin, S., 2015. Draft Genome
441 Sequence of the Hydrocarbon-Degrading Bacterium *Alcanivorax dieselolei* KS-293 Isolated from
442 Surface Seawater in the. genomeA 3, 6–7. <https://doi.org/10.1128/genomeA.01424-15>.Copyright
- 443 Barbato, M., Scoma, A., Mapelli, F., De Smet, R., Banat, I.M., Daffonchio, D., Boon, N., Borin, S., 2016.
444 Hydrocarbonoclastic alcanivorax isolates exhibit different physiological and expression responses to
445 N-dodecane. Front. Microbiol. 7, 1–14. <https://doi.org/10.3389/fmicb.2016.02056>
- 446 Bargiela, R., Gertler, C., Magagnini, M., Mapelli, F., Chen, J., Daffonchio, D., Golyshin, P.N., Ferrer, M.,
447 2015. Degradation network reconstruction in uric acid and ammonium amendments in oil-degrading
448 marine microcosms guided by metagenomic data. Front. Microbiol. 6, 1–12.
449 <https://doi.org/10.3389/fmicb.2015.01270>
- 450 Batista, S.B., Mouteer, a. H., Amorim, F.R., Tótola, M.R., 2006. Isolation and characterization of
451 biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Bioresour.
452 Technol. 97, 868–875. <https://doi.org/10.1016/j.biortech.2005.04.020>
- 453 Bonin, P., Vieira, C., Grimaud, R., Militon, C., Cuny, P., Lima, O., Guasco, S., Brussaard, C.P.D.,
454 Michotey, V., 2015. Substrates specialization in lipid compounds and hydrocarbons of *Marinobacter*
455 genus. Environ. Sci. Pollut. Res. 22, 15347–15359. <https://doi.org/10.1007/s11356-014-4009-y>

456 Bookstaver, M., Godfrin, M.P., Bose, A., Tripathi, A., 2015. An insight into the growth of *Alcanivorax*
 457 *borkumensis* under different inoculation conditions. J. Pet. Sci. Eng. 129, 153–158.
 458 <https://doi.org/10.1016/j.petrol.2015.02.038>

459 Bouchez Naïtali, M., Rakatozafy, H., Marchal, R., Leveau, J.Y., Vandecasteele, J.P., 1999. Diversity of
 460 bacterial strains degrading hexadecane in relation to the mode of substrate uptake. J. Appl. Microbiol.
 461 86, 421–428.

462 Cappello, S., Volta, A., Santisi, S., Morici, C., Mancini, G., Quatrini, P., Genovese, M., Yakimov, M.M.,
 463 Torregrossa, M., 2016. Oil-degrading bacteria from a membrane bioreactor (BF-MBR) system for
 464 treatment of saline oily waste: Isolation, identification and characterization of the biotechnological
 465 potential. Int. Biodeterior. Biodegrad. 110, 235–244. <https://doi.org/10.1016/j.ibiod.2015.12.028>

466 Cho, J.C., Giovannoni, S.J. 2004. Cultivation and growth characteristics of a diverse group of
 467 oligotrophic marine Gammaproteobacteria. Appl. Environ. Microbiol. 70, 432–440.
 468 <https://doi.org/10.1128/AEM.70.1.432-440.2004>

469 Clarke, K., Gorley, R., 2006. PRIMER v6. User manual/tutorial. Plymouth routine in multivariate
 470 ecological research.

471 Cui, Z., Gao, W., Li, Q., Xu, G., Zheng, L., 2013. Genome Sequence of the Polycyclic Aromatic
 472 Hydrocarbon-Degrading 1, 5–6. <https://doi.org/10.1128/genomeA.00301-13>. Copyright

473 Daffonchio, D., Ferrer, M., Mapelli, F., Cherif, A., Lafraya, A., Malkawi, H.I., Yakimov, M.M., Abdel-
 474 Fattah, Y.R., Blaghen, M., Golyshin, P.N., Kalogerakis, N., Boon, N., Magagnini, M., Fava, F., 2013.
 475 Bioremediation of Southern Mediterranean oil polluted sites comes of age. N. Biotechnol. 30, 743–
 476 8. <https://doi.org/10.1016/j.nbt.2013.05.006>

477 Daffonchio, D., Mapelli, F., Cherif, A., Malkawi, H.I., Yakimov, M.M., Abdel-Fattah, Y.R., Blaghen, M.,
 478 Golyshin, P.N., Ferrer, M., Kalogerakis, N., Boon, N., Magagnini, M., Fava, F., 2012. ULIXES, unravelling
 479 and exploiting Mediterranean Sea microbial diversity and ecology for xenobiotics' and pollutants'
 480 clean up. Rev. Environ. Sci. Biotechnol. 11, 207–211. <https://doi.org/10.1007/s11157-012-9283-x>

481 Daghighi, M., Vaiopoulou, E., Patil, S.A., Suárez-Suárez, A., Head, I.M., Franzetti, A., Rabaey, K., 2016.
 482 Anodes stimulate anaerobic toluene degradation via sulfur cycling in marine sediments. Appl.
 483 Environ. Microbiol. 82, 297–307. <https://doi.org/10.1128/AEM.02250-15>

484 Das, M., Das, S.K., Mukherjee, R.K., 1998. Surface active properties of the culture filtrates of a
 485 *Micrococcus* species grown on n-alkanes and sugars. *Bioresour. Technol.* 63, 231–235.

486 Deziel, E., Comeau, Y., Villemur, R., 2001. Initiation of Bio Im Formation by. *Am. Soc. Microbiol.* 183,
 487 1195–1204. <https://doi.org/10.1128/JB.183.4.1195>

488 Fodelianakis, S., Antoniou, E., Mapelli, F., Magagnini, M., Nikolopoulou, M., Marasco, R., Barbato, M.,
 489 Tsiola, A., Tsikopoulou, I., Giaccaglia, L., Mahjoubi, M., Jaouani, A., Amer, R., Hussein, E., Al-Horani,
 490 F.A., Benzha, F., Blaghen, M., Malkawi, H.I., Abdel-Fattah, Y., Cherif, A., Daffonchio, D., Kalogerakis,
 491 N., 2015. Allochthonous bioaugmentation in ex situ treatment of crude oil-polluted sediments in the
 492 presence of an effective degrading indigenous microbiome. *J. Hazard. Mater.* 287, 78–86.

493 Fuentes, S., Méndez, V., Aguila, P., Seeger, M., 2014. Bioremediation of petroleum hydrocarbons:
 494 Catabolic genes, microbial communities, and applications. *Appl. Microbiol. Biotechnol.* 98, 4781–
 495 4794. <https://doi.org/10.1007/s00253-014-5684-9>

496 Gärdes, A., Kaepfel, E., Shehzad, A., Seebah, S., Teeling, H., Yarza, P., Glöckner, F.O., Grossart, H.-P.,
 497 Ullrich, M.S., 2010. Complete genome sequence of *Marinobacter adhaerens* type strain (HP15), a
 498 diatom-interacting marine microorganism. *Stand. Genomic Sci.* 3, 97–107.
 499 <https://doi.org/10.4056/sigs.922139>

500 Gauthier, M.J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P., Bertrand, J.C., 1992.
 501 *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant,
 502 hydrocarbon-degrading marine bacterium. *Int. J. Syst. Bacteriol.* 42, 568–576.
 503 <https://doi.org/10.1099/00207713-42-4-568>

504 Genovese, M., Crisafi, F., Denaro, R., Cappello, S., Russo, D., Calogero, R., Santisi, S., Catalfamo, M.,
 505 Modica, A., Smedile, F., Genovese, L., Golyshin, P.N., Giuliano, L., Yakimov, M.M., 2014. Effective
 506 bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill
 507 simulation. *Front. Microbiol.* 5, 1–14. <https://doi.org/10.3389/fmicb.2014.00162>

508 Gertler, C., Bargiela, R., Mapelli, F., Han, X., Chen, J., Hai, T., Amer, R. a., Mahjoubi, M., Malkawi, H.,
 509 Magagnini, M., Cherif, A., Abdel-Fattah, Y.R., Kalogerakis, N., Daffonchio, D., Ferrer, M., Golyshin,
 510 P.N., 2015. Conversion of Uric Acid into Ammonium in Oil-Degrading Marine Microbial Communities:
 511 a Possible Role of Halomonads. *Microb. Ecol.* <https://doi.org/10.1007/s00248-015-0606-7>

512 Gertler, C., Gerdt, G., Timmis, K.N., Golyshin, P.N., 2009. Microbial consortia in mesocosm
513 bioremediation trial using oil sorbents, slow-release fertilizer and bioaugmentation. FEMS Microbiol.
514 Ecol. 69, 288–300. <https://doi.org/10.1111/j.1574-6941.2009.00693.x>

515 Golyshin, P.N., Martins Dos Santos, V. a. P., Kaiser, O., Ferrer, M., Sabirova, Y.S., Lünsdorf, H.,
516 Chernikova, T.N., Golyshina, O. V., Yakimov, M.M., Pühler, A., Timmis, K.N., 2003. Genome sequence
517 completed of *Alcanivorax borkumensis*, a hydrocarbon-degrading bacterium that plays a global role
518 in oil removal from marine systems. J. Biotechnol. 106, 215–220.
519 <https://doi.org/10.1016/j.jbiotec.2003.07.013>

520 Hassanshahian, M., Emtiazi, G., Caruso, G., Cappello, S., 2014. Bioremediation
521 (bioaugmentation/biostimulation) trials of oil polluted seawater: A mesocosm simulation study. Mar.
522 Environ. Res. 95, 28–38. <https://doi.org/10.1016/j.marenvres.2013.12.010>

523 Head, I.M., Jones, D.M., Röling, W.F.M., 2006. Marine microorganisms make a meal of oil. Nat. Rev.
524 Microbiol. 4, 173–82. <https://doi.org/10.1038/nrmicro1348>

525 Joint, I., Mühling, M., Querellou, J., 2010. Culturing marine bacteria — An essential prerequisite for
526 biodiscovery. J Microb. Biotechnol 3, 564–575.

527 Jurelevicius D., Alvarez V.M., Marques J.M., Ribeiro Fonseca de Sousa Lima L., de Almeida Dias F.,
528 Seldin L., 2013, Bacterial Community Response to Petroleum Hydrocarbon Amendments in
529 Freshwater, Marine, and Hypersaline Water-Containing Microcosms. Applied and Environmental
530 Microbiology, 79:19:5927-5935

531 Klein, B., Grossi, V., Bouriat, P., Goulas, P., Grimaud, R., 2008. Cytoplasmic wax ester accumulation
532 during biofilm-driven substrate assimilation at the alkane–water interface by *Marinobacter*
533 *hydrocarbonoclasticus* SP17. Res. Microbiol. 159, 137–144.

534 Knezevich, V., Koren, O., Ron, E., Rosenberg, E., 2007. Petroleum bioremediation in seawater using
535 guano as the fertilizer. Bioremed J 10, 83–91.

536 Koren, O., Knezevic, V., Ron, E.Z., Rosenberg, E., 2003. Petroleum Pollution Bioremediation Using
537 Water-Insoluble Uric Acid as the Nitrogen Source. Appl. Environ. Microbiol. 69, 6337–6339.
538 <https://doi.org/10.1128/AEM.69.10.6337-6339.2003>

539 L'Haridon, S., Markx, G.H., Ingham, C.J., Paterson, L., Duthoit, F., Le Blay, G., 2016. New Approaches
540 for Bringing the Uncultured into Culture. Chapter 15. In "The marine microbiome. An untapped

541 source of biodiversity and biotechnological potential". Eds. Stal. L., J., Cretoiu, M., S. Springer. Pag
542 401-434.

543 Liu, C., Shao, Z., 2005. *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated
544 from sea water and deep-sea sediment. Int. J. Syst. Evol. Microbiol. 55, 1181–6.
545 <https://doi.org/10.1099/ijs.0.63443-0>

546 Mahjoubi, M., Jaouani, A., Guesmi, A., Ben Amor, S., Jouini, A., Cherif, H., Najjari, A., Boudabous, A.,
547 Koubaa, N., Cherif, A., 2013. Hydrocarbonoclastic bacteria isolated from petroleum contaminated
548 sites in Tunisia: isolation, identification and characterization of the biotechnological potential. N.
549 Biotechnol. 30, 723–33. <https://doi.org/10.1016/j.nbt.2013.03.004>

550 Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D., Borin,
551 S., 2013. Potential for plant growth promotion of rhizobacteria associated with *Salicornia* growing in
552 Tunisian hypersaline soils. Biomed Res. Int. 2013, 248078. <https://doi.org/10.1155/2013/248078>

553 Mapelli, F., Scoma, A., Michoud, G., Aulenta, F., Boon, N., Borin, S., Kalogerakis, N., Daffonchio, D.,
554 2017. Biotechnologies for Marine Oil Spill Cleanup: Indissoluble Ties with Microorganisms. Trends
555 Biotechnol. 35, 860–870. <https://doi.org/10.1016/j.tibtech.2017.04.003>

556 Margesin, R., Gander, S., Zacke, G., Gounot, A.M., Schinner, F., 2003. Hydrocarbon degradation and
557 enzyme activities of cold-adapted bacteria and yeasts. Extremophiles 7, 451–458.
558 <https://doi.org/10.1007/s00792-003-0347-2>

559 McGenity, T.J., Folwell, B.D., McKew, B. a, Sanni, G.O., 2012. Marine crude-oil biodegradation: a
560 central role for interspecies interactions. Aquat. Biosyst. 8, 10. [https://doi.org/10.1186/2046-9063-](https://doi.org/10.1186/2046-9063-8-10)
561 8-10

562 Mi, Z.H., Yu, Z.C., Su, H.N., Wang, L., Chen, X.L., Pang, X., Qin, Q.L., Xie, B. Bin, Zhang, X.Y., Zhou, B.C.,
563 Zhang, Y.Z., 2015. Physiological and genetic analyses reveal a mechanistic insight into the
564 multifaceted lifestyles of *Pseudoalteromonas* sp. SM9913 adapted to the deep-sea sediment. Environ.
565 Microbiol. 17, 3795–3806. <https://doi.org/10.1111/1462-2920.12823>

566 Morales-Jiménez, J., Vera-Ponce de León, A., García-Domínguez, A., Martínez-Romero, E., Zúñiga, G.,
567 Hernández-Rodríguez, C., 2013. Nitrogen-Fixing and Uricolytic Bacteria Associated with the Gut of
568 *Dendroctonus rhizophagus* and *Dendroctonus valens* (Curculionidae: Scolytinae). Microb. Ecol. 66,
569 200–210. <https://doi.org/10.1007/s00248-013-0206-3>

570 Moreira, J.M.R., Gomes, L.C., Whitehead, K.A., Lynch, S., Tetlow, L.A., Mergulhão, F.J., 2017. Effect of
 571 surface conditioning with cellular extracts on *Escherichia coli* adhesion and initial biofilm formation.
 572 Food Bioprod. Process. 104, 1–12. <https://doi.org/10.1016/j.fbp.2017.03.008>

573 Naether, D.J., Slawtschew, S., Stasik, S., Engel, M., Olzog, M., Wick, L.Y., Timmis, K.N., Heipieper, H.J.,
 574 2013. Adaptation of the hydrocarbonoclastic bacterium *Alcanivorax borkumensis* SK2 to alkanes and
 575 toxic organic compounds: a physiological and transcriptomic approach. Appl. Environ. Microbiol. 79,
 576 4282–93. <https://doi.org/10.1128/AEM.00694-13>

577 Nikolopoulou, M., Kalogerakis, N., 2008. Enhanced bioremediation of crude oil utilizing lipophilic
 578 fertilizers combined with biosurfactants and molasses. Mar. Pollut. Bull. 56, 1855–1861.
 579 <https://doi.org/10.1016/j.marpolbul.2008.07.021>

580 Nikolopoulou, M., Pasadakis, N., Kalogerakis, N., 2013a. Evaluation of autochthonous
 581 bioaugmentation and biostimulation during microcosm-simulated oil spills. Mar. Pollut. Bull. 72,
 582 165–173. <https://doi.org/10.1016/j.marpolbul.2013.04.007>

583 Nikolopoulou, M., Pasadakis, N., Norf, H., Kalogerakis, N., 2013b. Enhanced ex situ bioremediation of
 584 crude oil contaminated beach sand by supplementation with nutrients and rhamnolipids. Mar. Pollut.
 585 Bull. 77, 37–44. <https://doi.org/10.1016/j.marpolbul.2013.10.038>

586 Overmann, J., Lepleux, C., 2016. Marine Bacteria and archaea: diversity, adaptations, and
 587 culturability. Chapter 2. In "The marine microbiome. An untapped source of biodiversity and
 588 biotechnological potential". Eds. Stal, L., J., Cretoiu, M., S. Springer. Pag 21-55.

589 Partridge, J.D., Harshey, R.M., 2013. Swarming: Flexible roaming plans. J. Bacteriol. 195, 909–918.
 590 <https://doi.org/10.1128/JB.02063-12>

591 Prakash, O., Shouche, Y., Jangid, K., Kostka, J.E., 2013. Microbial cultivation and the role of microbial
 592 resource centers in the omics era. Appl Microbiol Biotechnol 97, 51–62.
 593 <https://doi.org/10.1007/s00253-012-4533-y>

594 Raeid M.M. Abed, Jamal Al-Sabahi, Fatema Al-Maqrashi, Amal Al-Habsi, Manal Al-Hinai. 2014.
 595 Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the
 596 Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm
 597 experiments. International Biodeterioration & Biodegradation. 89:58-66

598 Rappe, M.S., Connon, S.A., Vergin, K.L., Giovannoni, S.J. 2002. Cultivation of the ubiquitous SAR11
 599 marine bacterioplankton clade. *Nature* 418, 630–633. <https://doi.org/10.1038/nature00917>.

600 Ron, E.Z., Rosenberg, E., 2014. Enhanced bioremediation of oil spills in the sea. *Curr. Opin. Biotechnol.*
 601 27, 191–194. <https://doi.org/10.1016/j.copbio.2014.02.004>

602 Sabirova, J.S., Chernikova, T.N., Timmis, K.N., Golyshin, P.N., 2008. Niche-specificity factors of a
 603 marine oil-degrading bacterium *Alcanivorax borkumensis* SK2. *FEMS Microbiol. Lett.* 285, 89–96.
 604 <https://doi.org/10.1111/j.1574-6968.2008.01222.x>

605 Sanabria, J., 2014. Environmental Biotechnology Research: Challenges and Opportunities in Latin
 606 America. *J. Agric. Environ. Ethics* 27, 681–694. <https://doi.org/10.1007/s10806-014-9502-2>

607 Schneiker, S., Martins dos Santos, V. a P., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., Chernikova,
 608 T.N., Denaro, R., Ferrer, M., Gertler, C., Goesmann, A., Golyshina, O. V, Kaminski, F., Khachane, A.N.,
 609 Lang, S., Linke, B., McHardy, A.C., Meyer, F., Nechitaylo, T., Pühler, A., Regenhardt, D., Rupp, O.,
 610 Sabirova, J.S., Selbitschka, W., Yakimov, M.M., Timmis, K.N., Vorhölter, F.-J., Weidner, S., Kaiser, O.,
 611 Golyshin, P.N., 2006. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium
 612 *Alcanivorax borkumensis*. *Nat. Biotechnol.* 24, 997–1004. <https://doi.org/10.1038/nbt1232>

613 Scoma, A., Barbato, M., Borin, S., Daffonchio, D., Boon, N., 2016. An impaired metabolic response to
 614 hydrostatic pressure explains *Alcanivorax borkumensis* recorded distribution in the deep marine
 615 water column. *Sci. Rep.* 6, 31316. <https://doi.org/10.1038/srep31316>

616 Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremediation. *Trends Microbiol.* 14,
 617 389–397. <https://doi.org/10.1016/j.tim.2006.07.001>

618 Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirković, I., Ruzicka, F., 2007.
 619 Quantification of biofilm in microtiter plates: Overview of testing conditions and practical
 620 recommendations for assessment of biofilm production by staphylococci. *Apmis* 115, 891–899.
 621 https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

622 Takai, K., Moyer, C.L., Miyazaki, M., Nogi, Y., Hirayama, H., Nealson, K.H., Horikoshi, K., 2005.
 623 *Marinobacter alkaliphilus* sp. nov., a novel alkaliphilic bacterium isolated from subseafloor alkaline
 624 serpentine mud from Ocean Drilling Program Site 1200 at South Chamorro Seamount, Mariana
 625 Forearc. *Extremophiles* 9, 17–27. <https://doi.org/10.1007/s00792-004-0416-1>

626 Taktikos, J., Stark, H., Zaburdaev, V., 2013. How the motility pattern of bacteria affects their dispersal
627 and chemotaxis. PLoS One 8. <https://doi.org/10.1371/journal.pone.0081936>

628 Uzoigwe, C., Burgess, J.G., Ennis, C.J., Rahman, P.K.S.M., 2015. Bioemulsifiers are not biosurfactants
629 and require different screening approaches. Front. Microbiol. 6, 1–6.
630 <https://doi.org/10.3389/fmicb.2015.00245>

631 Vacchini, V., Gonella, E., Crotti, E., Prosdociimi, E.M., Mazzetto, F., Chouaia, B., Callegari, M., Mapelli,
632 F., Mandrioli, M., Alma, A., Daffonchio, D., 2017. Bacterial diversity shift determined by different
633 diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria.
634 Environ. Microbiol. Rep. 9, 91–103. <https://doi.org/10.1111/1758-2229.12505>

635 Vigg, C.C., Presta, E., Bellagamba, M., Kaciulis, S., Balijepalli, S.K., Zanaroli, G., Papini, M.P., Rossetti,
636 S., Aulenta, F., 2015. The “Oil-Spill Snorkel”: An innovative bioelectrochemical approach to accelerate
637 hydrocarbons biodegradation in marine sediments. Front. Microbiol. 6, 1–11.
638 <https://doi.org/10.3389/fmicb.2015.00881>

639 Wang, W., Shao, Z., 2014. The long-chain alkane metabolism network of *Alcanivorax dieselolei*. Nat.
640 Commun. 5, 5755. <https://doi.org/10.1038/ncomms6755>

641 Wang, W., Shao, Z., 2012. Diversity of flavin-binding monooxygenase genes (*almA*) in marine bacteria
642 capable of degradation long-chain alkanes. FEMS Microbiol. Ecol. 80, 523–533.
643 <https://doi.org/10.1111/j.1574-6941.2012.01322.x>

644 Xue, J., Yu, Y., Bai, Y., Wang, L., Wu, Y., 2015. Marine Oil-Degrading Microorganisms and
645 Biodegradation Process of Petroleum Hydrocarbon in Marine Environments: A Review. Curr.
646 Microbiol. 71, 220–228. <https://doi.org/10.1007/s00284-015-0825-7>

647 Yakimov, M.M., Golyshin, P.N., Lang, S., Moore, E.R., Abraham, W.R., Lünsdorf, H., Timmis, K.N., 1998.
648 *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-
649 producing marine bacterium. Int. J. Syst. Bacteriol. 48 Pt 2, 339–348.
650 <https://doi.org/10.1099/00207713-48-2-339>

651

652 **TABLES**

653

654 **Table 1. Summary of the experimental conditions applied for the established enrichments.** The table
655 includes information on the type of matrix used, the site of collection and the geographical area, the
656 incubation time and the provided growth medium and HC source (OSM: Oil Sorbent Material; Vit: vitamin
657 solution; Fert: fertilizing solution; MB: Marine Broth; MW: marine water; DO: diesel oil; CO: crude oil; C15:
658 pentadecane; C12: dodecane; C8: octane; Mix: mix of aromatic HCs).

		Enrichment characteristics				
Sampling site/area (geographical coordinate)	Environmental Matrix	Incubation time	HC delivery	Growth media	HCs	Treatment number
Alboran Sea (36°0'0" N, 3 0'0" W)	seawater	Normal	Liquid	ONR7a	DO	1
ANU7, Adriatic Sea (43°37'22.63" N 13°30'13.64" E)	sediment	Normal	Liquid	ONR7a	CO + C15	2
Augusta, Ionian Sea (37 13'49" N, 15 13'10" E)	seawater	Normal	Liquid	ONR7a	C12	3
	seawater				C8	4
	seawater		OSM	MW + Fert	CO	5
	seawater				Mix	6
	seawater			MW + MB	CO	7
	seawater				Mix	8
Augusta, Ionian Sea (37°13'49" N, 15 13'10" E)	sediment	Long term	Liquid	ONR7a + Vit	CO	9
Gela (37 4'0" N, 14 15'0" E)	seawater	Normal	OSM	MW + Fert	CO	10
Gela	sediment	Long term	Liquid	ONR7a + Vit	CO	11

(37 4'0" N, 14°15'0" E)						
Levantine basin (33°59.945' N 34°25.346' E)	seawater	Normal	Liquid	ONR7a	DO	12
Tyro (33°52.423' N, 26°21.962' E)	sediment	Normal	Liquid	ONR7a	C8	13

659

660

661 **Table 2. Pair-wise PERMANOVA results of the physiological traits characterization of *Alcanivorax* and**
662 ***Marinobacter* strains.** Groups correspond to the phylogenetic species. Asterisk (*) indicates significant
663 differences between samples ($P < 0.05$).

Groups	t	P
<i>A. borkumensis</i> , <i>A. dieselolei</i>	2.2808	0.0098*
<i>A. borkumensis</i> , <i>A. venustensis</i>	4.3381	0.0001*
<i>A. borkumensis</i> , <i>M. adhaerens</i>	7.5111	0.0001*
<i>A. borkumensis</i> , <i>M. alkaliphilus</i>	5.9506	0.0001*
<i>A. borkumensis</i> , <i>M. flavimaris</i>	5.5936	0.0001*
<i>A. borkumensis</i> , <i>M. hydrocarbonoclasticus</i>	3.3565	0.0005*
<i>A. dieselolei</i> , <i>A. venustensis</i>	2.0236	0.0258*
<i>A. dieselolei</i> , <i>M. adhaerens</i>	3.6306	0.0001*
<i>A. dieselolei</i> , <i>M. alkaliphilus</i>	3.8432	0.0005*
<i>A. dieselolei</i> , <i>M. flavimaris</i>	3.6194	0.0004*
<i>A. dieselolei</i> , <i>M. hydrocarbonoclasticus</i>	3.6166	0.0022*
<i>A. venustensis</i> , <i>M. adhaerens</i>	0.93008	0.4504
<i>A. venustensis</i> , <i>M. alkaliphilus</i>	1.4088	0.1612
<i>A. venustensis</i> , <i>M. flavimaris</i>	3.4545	0.0001*
<i>A. venustensis</i> , <i>M. hydrocarbonoclasticus</i>	1.5843	0.1006
<i>M. adhaerens</i> , <i>M. alkaliphilus</i>	2.2533	0.0086*
<i>M. adhaerens</i> , <i>M. flavimaris</i>	6.8931	0.0001*
<i>M. adhaerens</i> , <i>M. hydrocarbonoclasticus</i>	1.7503	0.0608
<i>M. alkaliphilus</i> , <i>M. flavimaris</i>	4.7644	0.0001*
<i>M. alkaliphilus</i> , <i>M. hydrocarbonoclasticus</i>	2.8901	0.0029*
<i>M. flavimaris</i> , <i>M. hydrocarbonoclasticus</i>	4.8385	0.0001*

664
665
666
667

668 **Table 3. Pair-wise PERMANOVA results for the physiological traits characterization of *Alcanivorax* and**
669 ***Marinobacter* strains.** Groups correspond to the phylogenetic species, analyzed in a two-factors
670 PERMANOVA within the level “HC delivery system” for the (a) OSM and (b) liquid supplied HCs. Asterisk (*)
671 indicates significant differences between samples ($P < 0.05$).

(a) Groups	t	P
<i>A. borkumensis</i> , <i>A. venustensis</i>	2.7012	0.002*
<i>A. borkumensis</i> , <i>M. adhaerens</i>	5.5277	0.0001*
<i>A. borkumensis</i> , <i>M. flavimaris</i>	5.5936	0.0001*
<i>A. venustensis</i> , <i>M. adhaerens</i>	2.6992	0.01*
<i>A. venustensis</i> , <i>M. flavimaris</i>	2.4925	0.0102*
<i>M. adhaerens</i> , <i>M. flavimaris</i>	5.2009	0.0001*

(b) Groups	t	P
<i>A. dieselolei</i> , <i>A. venustensis</i>	1.9465	0.0349*
<i>A. dieselolei</i> , <i>M. adhaerens</i>	3.6722	0.0001*
<i>A. dieselolei</i> , <i>M. alkaliphilus</i>	3.8432	0.0006*
<i>A. dieselolei</i> , <i>M. hydrocarbonoclasticus</i>	3.6166	0.0017*
<i>A. venustensis</i> , <i>M. adhaerens</i>	0.5753	0.6912
<i>A. venustensis</i> , <i>M. alkaliphilus</i>	1.4334	0.1551
<i>A. venustensis</i> , <i>M. hydrocarbonoclasticus</i>	1.4145	0.1563
<i>M. adhaerens</i> , <i>M. alkaliphilus</i>	2.7174	0.0024*
<i>M. adhaerens</i> , <i>M. hydrocarbonoclasticus</i>	2.0498	0.0304*
<i>M. alkaliphilus</i> , <i>M. hydrocarbonoclasticus</i>	2.8901	0.0029*

673

674

675 **FIGURE CAPTIONS**

676 **Figure 1. Phylogenetic diversity of *Alcanivorax* and *Marinobacter* strains isolated from different samples,**
677 **with different enrichment approaches.** Distribution of the *Alcanivorax* and *Marinobacter* species according
678 to (a) hydrocarbon delivery method applied for cultivation, (b) different carbon sources and (c) the original
679 environmental matrix.

680

681 **Figure 2. Functional diversity of *Alcanivorax* and *Marinobacter* strains.** Non-metric multidimensional scaling
682 (nMDS) analysis based on the physiological strain characterization. The strains are represented according to
683 (a) the hydrocarbon delivery used during the enrichment phase, (b) the environmental matrix, (c) the
684 phylogenetic identification.

685

686

687

688 **SUPPORTING FILES**

689

690 **Table S1. List of the isolated bacteria.** The table reports the taxonomic identification of the strains (according
691 to the 16S rRNA gene sequencing of each ITS-cluster representative strains, indicated in bold) and the
692 adopted enrichment and isolation conditions.

693 **Table S2. Evaluation of the activities of potential interest in bioremediation.** The table includes the results
694 for the isolated strains identified as *Alcanivorax* and *Marinobacter* species (n = 100).

695

a)

b)

c)

(a)

(b)

(c)