Biotechnologies for marine oil spill cleanup: indissoluble ties with microorganisms

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Abstract

The ubiquitous exploitation of petroleum hydrocarbons (HCs) has been accompanied by accidental spills and chronic pollution in marine ecosystems, including the deep ocean. Physico-chemical technologies are available for oil spill cleanup, but HCs must ultimately be mineralized by microorganisms. How environmental factors drive the assembly and activity of hydrocarbon-degrading microbial communities remains unknown, limiting our capacity to integrate microorganism-based cleanup strategies with current physico-chemical remediation technologies. Here, we summarize recent findings about microbial physiology, metabolism and ecology and describe how microbes can be exploited to create improved biotechnological solutions to cleanup marine surface and deep waters, sediments and beaches.

Oil Spills in the Oceans and the Role Played by Microorganisms

The first large marine oil spill occurred in 1907 with the sinking of the *Thomas W. Lawson*, which released 7,400 tons of paraffin oil off the coast of the United Kingdom. Since then, estimates indicate that more than seven million tons of oil have been released into the environment from over 140 large spills [1], with the Deepwater Horizon (DWH) disaster releasing more than 700,000 tons of crude oil in the Gulf of Mexico [2]. While accidents like DWH are widely publicized, more than 90% of oil pollution comes from non-accidental natural and anthropogenic sources [3], including run-off from land-based sources and routine ship operations, such as deballasting and tank washing [4].

Oil spills can have extensive environmental and economic impact. For instance, estimates suggest that more than 250,000 seabirds were killed during the Exxon-Valdez oil spill in 1989 and that the DWH disaster cost more than $61 billion [1]. Crude oil that is released into the environment undergoes chemical, physical and biological modifications known as weathering. The fate of weathered crude oil hydrocarbons (HCs) depends on microorganisms that have developed strategies to increase their bioavailability and degradation pathways that allow them to use HCs as carbon and energy sources. Hazen et al. [5] observed that the half-lives of n-alkanes that leaked into the deep sea during the DHW oil spill were shorter than expected (1-6 days). Even so, slicks of oil heavily enriched with polycyclic aromatic hydrocarbons (PAHs) reached shores and were detected for at least a year [6], requiring cleanup interventions.

Current Response Techniques to Oil Spills in Marine Environments

Current emergency responses include mechanical containment and recovery of spilled oil, addition of chemical dispersants, and physical cleanup of shorelines. When an oil spill occurs in a marine environment, the magnitude of the spill is assessed to determine the most suitable response action. Figure 1 highlights the different cleanup approaches currently used for waters, shorelines and sediments. The primary combat strategy is mechanically containing and recovering oil by using different types of booms, barriers and skimmers, as well as the application of natural or synthetic sorbent materials. Containment booms are typically used in surface waters as barriers to control the oil from spreading and impacting shorelines or other marine resources like aquaculture facilities. With the aid of different types of booms (fences, curtains or inflatable booms), oil is concentrated into thicker layers, facilitating subsequent removal with skimmers. Different types of skimmers (weir, oleophilic and suction skimmers) have been developed that are suitable for specific oil types and in the presence of ice or debris. Sorbents are either natural or synthetic-like polymeric materials used for very small spills to adsorb the spilled oil either on the surface or internally through swelling. They are also used to remove final traces of oil after collection with skimmers, or in areas that cannot be reached by skimmers.

Dispersants (Box 1) are mixtures of surfactants and solvents that reduce spilled oil into small droplets (smaller than 100 μm in size) by the action of waves and currents; they are mainly used in open and deep waters [7,8] (Figure 1). Oil droplets are spread from the seawater surface into the **water column**, thus preventing the oil slick from reaching the shoreline. Oil droplets have a higher surface-to-volume ratio than oil slicks have,

64 which allows more of the oil's surface to be exposed to microorganisms. Dispersants
65 are effective when applied immediately following a spill, before the lightest HC
66 components volatilize, and their performance depends on water salinity, temperature
67 and wave action. Considering the toxicity of the dispersants' components [7,8], including
68 to microorganisms [9], the next technological goal in oil spill remediation is replacing
69 chemical surfactants with non-toxic **biosurfactants** (see Glossary).

70

71 A series of physical methods have been developed for different types of oil
72 contamination. When oil slicks reach sandy beaches or rocky coastlines, they are
73 manually collected or sprayed with water to return them back to the water, where they
74 are adsorbed by sorbent materials (Figure 1). Another technique for oil removal is *in situ*
75 burning (ISB), the controlled burning of oil at the spill's location to reduce the amount of
76 oil on the surface. ISB, which should be coupled to toxicity assessments of burned
77 residues [10], has been used on several occasions including the DWH accident, where
78 the amount of burned oil was higher than the amount of mechanically collected oil
79 despite estimate uncertainties [11]. In deep-sea spills, capping the head of the leaking
80 oil well is the first action to be taken and is complemented by the other approaches
81 (Figure 1).

82

83 **Bioremediation and natural attenuation**

84 Accurate knowledge of the metabolic potential of microorganisms and of the
85 environmental factors that shape their interactions, viability and degradation activity is
86 required to optimize **bioremediation** intervention strategies. HC-degrading marine

87 microorganisms can be divided into specialists and generalists according to their ability
88 to grow respectively on a narrow range of HCs or on a wider set of carbon sources [12].
89 They include strains of *Bacteria*, *Archaea* and *Fungi* (Table 1, Table S1).
90 Hydrocarbonoclastic-bacteria are the most studied in terms of their physiology, ecology
91 and biotechnology because of their ubiquity and prevalence.
92
93 **Natural attenuation** is determined by the complex metabolic networks built up by
94 microorganisms and their interactions with other organisms such as algae [12–14]. In
95 seawater, one of the main factors limiting the rate of HC degradation is low HC solubility
96 [13]. By producing biosurfactants and modifying cell membrane hydrophobicity,
97 microorganisms increase and modulate HC bioavailability according to environmental
98 conditions, such as in the case of *Alcanivorax* spp. [15]. Oxygen availability determines
99 the pathway of HC breakdown. In the water column, under aerobic conditions, microbial
100 enzymes activate HC molecules by incorporating oxygen atoms, generating
101 corresponding alcohols, which are further oxidized to carboxylic acids that are degraded
102 through β-oxidation [16]. The most studied aerobic enzymes are alkane hydroxylases,
103 encoded by the *alkB*, *p450* and *almA* genes [16,17]. Under anaerobic conditions typical
104 in sediments, HC catabolism is activated by the addition of fumarate to the secondary
105 carbon and catalysed by alkyl-succinate synthases [16].
106
107 The entire complexity of the metabolic pathways involved in HC catabolism under *in situ*
108 conditions is not yet understood, especially in relation to the environmental conditions,
109 although the increasing availability of **meta-omics** data is clarifying the picture [18–22].

Marine microbial communities responding to HC inputs are subjected to ecological successions generally starting with aliphatic HC-degraders (*e.g.*, *Alcanivorax* spp.) and the subsequent enrichment of other microorganisms able to metabolize aromatic HCs (*e.g.*, *Cycloclasticus* spp.) as observed in seawater [23], sediments [24] and beaches [21]. The response of the autochthonous microbial community after an oil spill depends upon the community's initial functional diversity. This response is also modulated by the local environmental conditions.

A metagenomic study of chronically polluted sediments across a transect from the Mediterranean Sea to the Red Sea reconstructed the occurring metabolic pathways, demonstrating that biodegradation capabilities expand according to the increasing yearly average sediment temperature across the transect despite a decrease in bacterial richness [18]. Although some common and specialized HC-degrading bacteria were not detected by 16S rRNA gene sequencing, the metabolic networks accounting for both aerobic and anaerobic HC degradation pathways indicated that the chronically polluted marine sediments harbour a higher catabolic diversification than do freshly polluted samples [18]. This suggests that microbial communities exposed to chronic pollution possess a wide repertoire of catabolic abilities that allow them to respond more promptly to oil spills [18]. HC cleanup may occur through transient enrichment of specialized microorganisms that degrade the different HC classes; their taxa succession patterns have been described [23]. The combination of degradation capabilities and pathways of different microbial community members is required for PAH degradation as indicated by genomes assembled from metagenomic datasets following stable isotope probing of the surface and deep plume waters of the DWH spill [14]. This suggests that

key degradative pathways are distributed across the different members of the DWH community that cooperatively and co-ordinately react to initiate PAHs degradation [14].

Biotechnologies for water and coastal pollution

Bioremediation technologies compatible with natural biogeochemical cycles comprise biosurfactant amendments, **biostimulation** and **bioaugmentation**. Biosurfactants favour oil solubilisation and oil droplet formation in water (Figure 2), making HCs available to non-biosurfactant-producing microorganisms. Bacteria, yeast and fungi mainly produce anionic or neutral biosurfactants. Renowned producers include *Acinetobacter*, *Bacillus* and *Pseudomonas*, with *Alcanivorax* predominating in oil-contaminated marine surface waters worldwide, owing to its capacity to produce large amounts of glycolipid biosurfactants [25]. Compared to dispersants, biosurfactants have low to no toxicity, high biodegradability, surface and emulsification activities and high stability under extreme temperature, pH and salinity conditions [26,27]. Biosurfactants positively impact HC biodegradation [28,29] and have been applied to oil recovery in reservoirs, to oil transportation in pipelines and to production of emulsified fuels [30].

Biostimulation is among the most effective approaches to enhance the degrading activity of indigenous bacterial communities since it improves the C/N/P ratio that is unbalanced after oil spills [27,31–33]. Nutrient delivery represents the main limitation of biostimulation due to rapid leaching along the water column [34]. Nutrient **micro-encapsulation** within slow-release particles (SRPs) eventually combined with biosurfactants and oil-degrading microorganisms may improve biostimulation efficiency

156 (Figure 2). SRPs are produced with low-toxicity polymers, *e.g.,* polyurethane-polyurea
157 copolymers, alginates and chitosans [35,36]. A recent strategy based on microinjecting,
158 cryo-crosslinking and coating with ethyl cellulose films allowed the synthesis of alginate-
159 beads with increased load capacity and slow release of N/P fertilizers [36]. Even though
160 the addition of nutrients can promote the growth of heterotrophs, thus creating
161 competition with HC-degraders [37], increased total petroleum HC removal was
162 reported by using alginate-encapsulated diesel-degrading bacteria as compared to free-
163 cells [38]. Recent metagenomic data indicated that biostimulation with different nitrogen
164 sources favoured different HC catabolic pathways without substantially affecting the
165 taxonomic structure of the bacterial community [39].
166
167 Indigenous microbial populations may not have the full metabolic ability to process the
168 complex mixture of spilled HCs or specialized HC-degraders might be underrepresented
169 in the existing community. Bioaugmentation with site-allochthonous microorganisms
170 might enhance HC degradation [40] although the long-term presence of such non-
171 indigenous microorganisms has been questioned [41]. Autochthonous bioaugmentation
172 (ABA) uses indigenous microorganisms enriched from specific habitats of the
173 contaminated site (*e.g.,* surface or deep-sea water) (Figure 2). This approach has been
174 successfully implemented in a study aimed at simulate an oil spill event, showing that
175 the use of pre-adapted HC degrading bacteria in combination to biostimulation provided
176 the best results in term of HC removal [42]. A further alternative is bioaugmentation with
177 **mobile genetic elements** [43], which aims at horizontal transfer of remediation genes

178 from an exogenous inoculant to indigenous microorganisms by using catabolic plasmids
179 or transposons.
180
181 **Biotechnological approaches to remediate oil-contaminated marine sediments**
182 Marine sediments represent an important sink for petroleum HCs after accidental spills.
183 A number of different chemical, physical, and microbiological processes contribute to
184 the transport of petroleum from a positively buoyant state in the water column down to
185 the seafloor, such as weathering, adsorption onto settling particulate matter (including
186 so-called marine oil snow [44], Figure 2), and the addition of chemical dispersants. In
187 the case of the DWH spill, 1.8-14% of the oil reached the seafloor as estimated using
188 hopanes as a biomarker tracer [45] or 0.5-9% as estimated using radiocarbon
189 distributions [46]. Upon sedimentation, oil penetrates the upper sediment layers
190 (typically 1-30 cm, depending on site conditions). In the sediments it may persist due to
191 the prevailing anoxic conditions (Figure 3A) that drastically limit the occurrence of
192 oxidative biodegradation processes, and the low bioavailability resulting from the strong
193 sorption onto hydrophobic sedimentary materials.
194
195 *In situ* bioremediation is typically regarded as one of the most effective and sustainable
196 strategies to cleanup contaminated sediments. Different approaches have been
197 proposed to stimulate naturally occurring microbial communities that degrade petroleum
198 HCs in marine sediments [47,48]. These typically involve the subsurface addition of
199 degradation rate-limiting nutrients, electron acceptors and (bio)surfactants. The
200 successful stimulation of the indigenous microbial community dwelling in oil polluted

sediment was achieved by adding nitrogen and phosphorous, ultimately resulting in a higher HC removal compared to the non-biostimulated control [31]. Similarly, the activity of indigenous oil-degraders can be boosted by the addition of biosurfactants [42]. The interplay between the bioavailability of electron acceptors (*e.g.*, oxygen, nitrate, sulfate, Fe^{3+}/Mn^{4+}) and HCs is probably the most critical factor affecting the efficacy of sediment bioremediation systems. Under aerobic conditions, petroleum HC biodegradation occurs rapidly and therefore different engineered approaches have been proposed to deliver oxygen to the sediments. Among them, a modular slurry system, which performs *in situ* aeration of the contaminated sediments, while minimizing the risk of spreading the contamination away from the treatment zone, has recently been developed (Figure 3B) [49]. Although the system was highly effective in stimulating the metabolism of aerobic HC-degrading bacteria and in reducing sediment toxicity, its application turned out to be highly labour- and energy-intensive. Other methods include supplying oxygen-releasing compounds (*e.g.*, calcium peroxide-based chemicals) to the contaminated sediment. However, rapid abiotic oxygen consumption by reactions with reduced chemical species (*e.g.*, Fe^{2+}, S^{2-}) and the difficulties in controlling the rate of oxygen release over time are some limitations of these approaches.

Recently, an innovative bioelectrochemical system termed an “oil-spill snorkel” was proposed to accelerate oil HC biodegradation in marine sediments [50]. The system consists of a conductive graphite rod (the “snorkel”) positioned for electrochemically connecting two spatially segregated redox zones: the anoxic contaminated sediment and the oxic (O_2-containing) overlying water (Figure 3C). The portion of the snorkel

positioned in the anoxic sediment serves as an electron acceptor (*i.e.*, an anode), sinking electrons deriving directly from the microbially catalyzed anaerobic oxidation of HCs and from the chemical and/or biochemical oxidation of reduced species (*i.e.*, S^{2-}, Fe^{2+}) occurring in the bulk of the sediment. Upon transfer to the buried portion of the snorkel, the electrons move to the upper portion (*i.e.*, the cathode), driven by the existing redox gradient, where they combine with oxygen and protons to form water as a by-product. Besides serving as a virtually inexhaustible respiratory electron acceptor in the anaerobic oxidation of petroleum HCs, the snorkel was suggested to indirectly stimulate HC biodegradation by sulfate-reducing bacteria via the scavenging of toxic sulfide diffusing from the bulk of the sediment [51]. This finding has major practical implications as it suggests that the radius of influence of the oil-spill snorkel may extend far from where the rod is positioned. Although the feasibility of the oil-spill snorkel has been demonstrated only at the laboratory scale, the technology has a very low energetic and environmental footprint (*e.g.*, no energy input or maintenance required) and could be ideally applied for long-term remediation of contaminated sediments in remote open sea areas.

Oil spills in the deep sea

The DWH spill (Gulf of Mexico, April 2010) was the first oil spill originating in the deep sea (1544 m below the surface level, bsl). Hydrostatic pressure (HP) and temperature gradients, alongside the large injection of dispersants, fractionated petroleum HCs along the water column, with the slow buoyant migration of light or dispersed HCs forming multiple plumes at 800-1300 m bsl (about 8-13 MPa and 5°C) [52,53]. Bacteria

247 detected in the oil plume were subjected to **microbial succession**, with HC
248 composition and quantity proposed to account for such a change [5,54,55]. While the
249 main physiological drivers for the microbial community shift remain uncertain [56], there
250 is a consensus about the initial enrichment of *Oceanospirillales* and pseudomonads
251 (May 2010) followed by a shift in dominance to *Colwellia*, *Cycloclasticus*,
252 *Pseudoalteromonas* and methylotrophs (until August 2010). Ammonium and dissolved
253 inorganic nitrogen (DIN) were unrelated to plume samples [5,54]. Genes related to the
254 transport of iron or nitrogen- and phosphorous-based compounds [57] or their
255 metabolism (*e.g.*, nitrate and nitrite reduction, [58]) were highly transcribed, but their
256 correlation with plume samples or microbial succession is uncertain [5,20,54,57,58].
257 The gene expression for sulphur cycling and sulphite reduction increased, with sulphate
258 reduction the postulated activity, despite O_2 levels that remained relatively high [5,58].
259
260 Microbial succession was more difficult to assess in the deep-sea sediments because of
261 the patchy oil distribution. Marine snow pulses resulting in oil deposition after June 2010
262 fuelled O_2 respiration on the surface of the seafloor (enriching *Roseobacter*,
263 *Verrucomicrobiaceae* and *Bacteroidetes* until October 2010). Anoxic microniches were
264 favoured and *Deltaproteobacteria* and other anaerobes developed [59]. DIN, ammonium
265 and total petroleum HCs were correlated in surface sediments and denitrification-related
266 genes were highly transcribed [60]. *Deltaproteobacteria* were also enriched in
267 subseafloor samples [61]. One year after the DWH spill, surface sediments around the
268 wellhead area were enriched with *Actinobacteria*, *Firmicutes*, *Chloroflexi* and
269 methylotrophic bacteria [62]. However, a time-dependent survey suggested that the

microbial community of the sediments had returned to pre-spill conditions, with a response to petroleum only individuated at a finer taxonomic level (as with the obligate polycyclic aromatic HC-degrader *Cycloclasticus* [59]). Continued sedimentation imposed anaerobic conditions on the remaining HCs, with total PAHs in 3-cm deep sediments slowly approaching pre-spill levels [63].

Understanding cooperation, competition and succession of microorganisms after an oil spill in the deep sea is key to predicting the success of the oil cleanup. Considering the difficulty of *in situ* studies at extreme depths, testing physiological response of isolates to the specific environmental conditions, such as temperature or HP, is a necessary complementary effort to explain changes in bacterial community composition and activity. However, the impact of HP on the HC degradation physiology after DWH was neglected and recent studies show that HP can affect the metabolism of oil-degrading bacteria. It has been recently proposed that the impaired metabolic response of *Alcanivorax* spp., which are ubiquitous and typically the first microorganisms identified after surface oil spills, to HP explains the lack of their detection in the DWH deep oil plume [64–66]. The DWH deep-sea plume [67] and sediment [61] revealed a low *Alcanivorax* abundance, which was unrelated to hydrocarbon concentrations [61], contrary to the abundance of other *Oceanospirillales* [5]. Consequently, the contribution of *Alcanivorax* to oil degradation in the plume was considered negligible [61]. Meanwhile, results from oiled beach sands [68], oil mousses collected on surface waters [62] and plume samples [14,67] showed a significant *Alcanivorax* abundance after cultivation under atmospheric pressure. *Alcanivorax* isolates were also obtained in

293 enrichment cultures from HC-free, deep-sea water samples collected from depths up to
294 5000 m below sea level [69].
295 We call these observations the '*Alcanivorax* paradox', i.e., the lack of response of
296 ubiquitous HC-degrading bacteria to HCs in the deep sea. All of the isolation studies on
297 the deep sea neglected to consider HP as a contributing variable despite it being a
298 unique feature of deep-water environments. Recent data showed that a HP of 10 MPa
299 (comparable to that in the DWH plume) inhibits growth of three *Alcanivorax* species (*A.*
300 *borkumensis*, *A. dieselolei* and *A. jadensis*) on dodecane as a sole carbon source and
301 that even a HP of 5 MPa causes a significant reduction in cell replication [64–66]. Under
302 HP transcription, multimeric protein complexes such as ATPase and ribosomes are
303 increased as compared to those under surface-water-resembling conditions. The
304 respiratory chain shifts from cytochrome oxidases to reductases, alongside an
305 enhanced expression of genes of the Na^+-translocating NADH reductase complex
306 (RNF-NQR). Translation, energy generation and electron transport are typical targets of
307 microbial piezoadaptation (Box 2). Under HP, the *A. borkumensis* SK2 strain
308 synthesizes the osmolyte ectoine [65,66], which has been suggested to be a pressure-
309 responsive compound (i.e., piezolyte). Synthesis of ectoine is energy intensive and
310 does not provide apparent advantages to *Alcanivorax* growth under HP [65,66].
311
312 **Concluding remarks and future perspectives**
313 Due to increasing demand for energy worldwide, the exploration of novel oil fields has
314 increased. These efforts increase the risk of exposure of marine life and the marine
315 environment to HCs [8]. Marine microorganisms are the ultimate HC degraders and play

316 a key role in cleanup events [5]. However, they can be exploited more efficiently if the
317 black box of their response to multifactorial environmental and pollution parameters is
318 opened and fundamental questions on their metabolism, physiology and ecology are
319 answered (see Outstanding Questions). Meta-omics data are largely contributing to
320 elucidate how environmental factors drive the assembly and function of HC-degrading
321 communities. More players than those currently known are revealed and they cooperate
322 to add their 'incomplete' metabolic pathways to HC degradation [14]. Metabolic
323 complementation and synthrophy could be as actual strategies for effective oil HC
324 cleanup in marine ecosystems. This suggests that biostimulation and bioaugmentation
325 approaches, including autochthonous bioaugmentation, should be carefully rethought
326 [31]. Furthermore, the recent advancements in understanding metabolism, physiology
327 and adaptation of HC-degrading microorganisms are contributing to explain their
328 successes and failures in various polluted environments. For instance, the impaired
329 response of *Alcanivorax* to HP and its associations to different polluted compartments
330 following the DWH disaster clarifies its absence in the deep-sea oil plume [65].
331 Similarly, recent developments in microbial bioelectrochemistry are contributing to the
332 design of novel solutions for HC cleanup through the channelling of electron flows, such
333 as that exploited by the "oil-spill snorkel," to compensate for the absence of oxygen in
334 contaminated sediments [50]. For certain, novel discoveries in microbial ecology and
335 physiology associated with HC pollution in marine ecosystems will enlighten and lead to
336 more efficient and sustainable microbial biotechnology approaches to ridding the
337 oceans of oil.
338

Figure Legends

Figure 1. Current (Non-Microbial) Technologies for Emergency Responses to Oil Spills in Marine Environments. On the sea surface, the oil spots accidentally released by oil tankers or offshore platforms can be chemically dispersed or physically contained by plastic booms and partially recovered with skimmers or they can undergo *in situ* burning. These processes may enhance accumulation of solid residues (e.g., tar balls) that can sink. The volatile fractions move to the atmosphere where the HCs can be transported far away and fall back to the surface again. HCs can reach the coast and contaminate beaches where they can be removed with oil-sorbent materials. On rocky beaches, combinations of high-pressure water spray and application of sorbent materials are used. A further possibility is the removal of the contaminated sand and *ex-situ* treatment in specialized centres. HC contamination can occur at the seafloor due to losses from oil wells and shipwrecks or during drilling operations. Oil droplets can then move across the water column towards the seafloor, spread horizontally to form HC plumes in the water column or reach the sea surface and eventually the coastal zone. Dispersants are used to decrease the size of oil droplets and increase the surface-to-volume ratio. Oil from sunken ships can be removed through suction from intake platforms positioned on the sea surface, while wellhead capping is necessary to stop oil blowout from deep-sea wells. Natural attenuation and dispersion phenomena in the water column and volatilization in the atmosphere additionally contribute to HC removal.

Figure 2. Biotechnological Approaches to the Remediation of Hydrocarbon (HC)-Polluted Marine Environments. The activity of HC-degrading microorganisms can be enhanced by nutrients and biosurfactants, provided alone or in combination. Besides the HC-degraders, microbes playing key roles such as biosurfactant producers are pivotal to set up a successful intervention. Bioaugmentation (BA) of HC-degrading microbes selected under laboratory conditions can be used to enhance HC degradation rates. A strategy to overcome the reported lack of adaptability of allochthonous microbes consists of autochthonous bioaugmentation (ABA) using isolates obtained from the matrix to be treated. Biostimulation is also used to enhance the activity of the native and/or the augmented microbial communities and biosurfactants can be added to increase HC bioavailability. By exploiting suitable bio-carriers, nutrients, biosurfactants and degrading microbes can be mixed to formulate slow-release particles (SRP) that allow continuous and homogeneous release of the active ingredients in the target environments. SRP applied to the sediments may contain alternative oxidants (e.g., NO_3^-). Microalgae are involved in the water column cleanup following an oil spill. Supporting their growth would enhance marine snow formation, which results in HC precipitation to the seafloor. This might limit the oil-impacted seafloor area before deep-sea currents drive the oil plume far from the HC-spill origin and provide nutrients to microbial HC-degrading populations on the seafloor. All biotechnological solutions must take into account the environmental conditions of the system, including temperature and hydrostatic pressure.

Figure 3. Natural and Engineered Biodegradation Processes in Marine Sediments Contaminated by Petroleum Hydrocarbons. (A) Steady-state stratification of hydrocarbon-fuelled respiratory metabolisms based on the availability of terminal electron acceptors. In the most superficial sediment layer (ranging from a few millimetres to several centimetres depending on site characteristics), microorganisms respire the oxygen that diffuses from the overlaying seawater. Below the oxic zone, nitrate, Mn^{4+} and Fe^{3+}, if present, are used for anaerobic respiration. In the lower sedimentary layer, sulfate, which is not a limiting factor in marine environments, becomes the dominant respiratory electron acceptor. (B) Schematic representation of the "oil-spill snorkel", a conductive graphite rod half-buried in the contaminated sediment that creates an electrochemical connection between the anoxic sediment and the oxic overlaying seawater. In principle, the "snorkel" may accelerate HC oxidation by both serving as a direct electron acceptor in the respiratory metabolism of electro-active bacteria growing at its surface and by stimulating, in the bulk of the sediment, the metabolism of sulphate-reducing bacteria via sulphide scavenging. (C) Schematic representation of a modular slurry system (MSS) designed for stimulating aerobic biodegradation processes in otherwise anoxic sediments. The system allows *in situ* aeration of the sediment, temperature control, and also possibly the delivery of nutrients and/or other biostimulating agents to increase, for instance, the bioavailability of sediment-bound contaminants.

Table1. Main cultivated marine hydrocarbon-degrading bacteria and their phylogenetic, physiological and ecological features.

A/AN	Genome	Microorganism	Phylogeny	Target substrate	Habitat/Ecology	Physiology	Refs
A	Available	*Alcanivorax borkumensis*	*γ-proteobacteria, Alcanivoracaceae*	*n*-alkanes	Seawater, sediment, beach sand, coastal salt marsh	Biosurfactant producer, OHCB	[70]
A	Available	*Alcanivorax dieselolei*	*γ-proteobacteria, Alcanivoracaceae*	*n*-alkanes	Seawater, sediment	Resistance to mild pressure increase, OHCB	[69]
A	Available	*Marinobacter hydrocarbonoclasticus:*	*γ-proteobacteria, Alteromonadaceae*	*n*-alkanes, PAHs	Seawater, sediment	Biofilm producer; oil surface colonizers	[71]
A	Available	*Cycloclasticus pugetii*	*γ-proteobacteria, Piscirickettsiaceae*	PAHs	Sediment	Highly efficient transport systems for the capture of nutrients and oligo-elements	[72]
A	Available	*Oleispira antarctica*	*γ-proteobacteria, Oceanospirillaceae*	*n*-alkanes	Seawater	cold-adapted OHCB	[73]

A: aerobic; AN: anaerobic; OHCB: obligate hydrocarbonoclastic bacteria; PAH: polycyclic aromatic hydrocarbons.

Text Boxes

BOX 1. The use and impacts of dispersants following oil spills.

There are consolidated approaches for dispersant administration on oil-polluted surface waters. In general, a dispersant administration protocol takes into account the trade-off between the efficiency of dispersion into fine droplets and the increased toxicity of water-accommodated oil fractions to marine life. It has been recently highlighted that dispersants may alter and steer the diversity and activity of microbial communities with potential, not yet understood effects on oil HC degradation [7]. There are no formulations specifically designed for deep-sea releases. It is believed that in this case no solvents are needed as the light HC oil components are all there, and mixing is favoured by natural gas that is released from the crude oil as it ascends through the water column. During the DWH oil spill, about seven million litres of the Corexit dispersant were injected into the surface of the Gulf of Mexico and deep-sea waters [74]. Key Corexit components such as the anionic surfactant dioctyl sodium sulfosuccinate were not biodegraded *in situ* [75], while addition of Corexit in *ex situ* tests inhibited HC biodegradation and shifted microbial communities towards potential dispersant degraders (e.g., Colwellia; [7]).

BOX 2. Effects of hydrostatic pressure on microbial cells.

Hydrostatic pressure (HP) is an intrinsic feature of the deep sea. HP linearly increases with depth (about 0.1 MPa every 10 m of seawater column) and affects cell structures and functions, with a genome-based response proposed for piezophilic adaptation. Le Châtelier's principle predicts that processes entailing volume reduction are favoured

under HP. HP can affect chemical reactions (both for volume change at equilibrium and for activation) and macromolecule structures, particularly the weak non-covalent bonds and protein complexes with several subunits. This is the case for ribosome assembly or for nucleic acid/protein complexes with volume increases [76–78], where effects are generally observed at sub-lethal pressures. Another recognized HP effect is on cell membranes, whose relative abundance in mono- (and to a lower extent poly-) unsaturated fatty acids, necessary to maintain membrane fluidity and cell homeostasis, can be affected. A high degree of fatty acid unsaturation does not favour fatty acid acyl group packing due to the physical encumbrance of the lateral chains, with positive effects on the cell membrane's homeoviscous properties and curvature elastic stress [79]. Increased unsaturation of fatty acids on the membrane may result from another typical deep-sea condition, *i.e.,* low temperature, whose effects are additive to HP [80]. HP affects membrane transport systems and transmembrane enzymes, such as ATPases and cytochromes, due to direct effects on enzyme folding or the lipid environment on the membrane. Adaptation to HP potentially involves energy generation in the cell, likely to counteract HP-related stressing effects. The model piezophile *Photobacterium profundum* SS9 possesses two complete operons for the F_0F_1 ATPase and multiple cytochrome sets, supporting the pivotal importance of electron and proton transport under high HP [81]. Similarly, *Shewanella piezophila* possesses two respiratory chains, active either under low or high HP [82]. The capacity to offset HP impact on cell turgor pressure has been little studied. This would involve the intracellular accumulation of piezolytes, as occurring for osmolytes and salinity. While their mechanism of action remains unclear, HP increase is consistent with the accumulation

of N-trimethylamine oxide (TMAO) [83], β-hydroxybutyrate [84] and ectoine [65]. In *Alcanivorax borkumensis*, intracellular ectoine accumulation and gene upregulation under HP were correlated with decreased cell damage and higher cell number, but culture activity was not increased. Experiments on the synergistic effects of osmotic and HP increase suggest that ectoine water-reclamation capacity might also explain its function at high HP [66].

Glossary Box

Bioaugmentation: The addition of active microorganisms with specialized metabolic capacities to enhance a metabolic process, such as HC degradation in a polluted site.

Bioremediation: The exploitation of the catabolic abilities of microorganisms that use pollutants (such as HCs) as a source for carbon and energy for their metabolism and growth. Two general approaches can be defined in which the polluted material is left on site (*in-situ* bioremediation) or is removed and treated away from the polluted site (*ex-situ* bioremediation). It can be performed by indigenous microbial communities or by allochthonous microorganisms added to the polluted system through bioaugmentation.

Biostimulation: The modification of the environmental conditions of a polluted matrix to favour the metabolism of microorganisms capable of bioremediation, generally consisting of the addition of nutrients, but also of oxygen or other electron acceptors and eventually electron donors (for instance in the case of reductive dechlorination), or of the addition of substances enhancing HC bioavailability (*e.g.,* biosurfactants).

Biosurfactants: Amphipatic surface-active macromolecules produced by microorganisms to increase the availability of HCs. They can be grouped as low-molecular-weight molecules with good solubilization properties (lipopeptides, phospholipids, but most commonly glycolipids *e.g.*, rhamnolipids, trehalose lipids, sophorolipids) or high molecular weight compounds with coating properties preventing oil-droplet coalescence (*e.g.*, proteins, polysaccharides, lipids and their complexes).

Meta-omics: Molecular analyses based on the comprehensive study of enzymes and proteins (metaproteomics), RNA and DNA sequences (metatransciptomics and metagenomics) or metabolites (metabolomics) in a given environmental sample.

Micro-encapsulation: The incorporation of different chemical components (such as nutrients, enzymes, microorganisms, etc.) within a coating to form small capsules in the size range of micrometers to a few millimetres, to protect the content from early degradation and favour its localized and time-dependent release.

Microbial succession: The temporal change in dominant species within a microbial community in response to the changing environmental conditions, such as the changes in the type and concentrations of carbon sources.

Mobile genetic elements: Genetic elements such as plasmids or transposons that contain genetic modules allowing their movement within the genome of an organism or between genomes of different organisms. They may carry catabolic genes that enable the cells that express them to degrade specific compounds.

Natural attenuation: The process of HC biodegradation following an oil spill, mediated by the indigenous microbial community. This process relies upon the presence of microorganisms with HC-catabolic capacities in the ecosystem prior to the spill. The availability of HC substrates enriches the microorganisms that are capable of exploiting HCs for growth. Natural attenuation is made possible by consortia of microorganisms with complementary functions. These microorganisms include HC degraders, biosurfactant producers, and nutrient providers involved in N and P cycles or siderophore producers.

Water column: the term conceptually represents all the different layers of water present in a water body, such as the oceans, moving from the surface to the sediment on the bottom. Along the water column, different layers can be identified and their boundaries are defined by specific water depth.

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Competing interests

The authors declare no competing financial interests.

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Biotechnologies for marine oil spill cleanup: indissoluble ties with microorganisms

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Trends Box **(Max 900 characters w/spaces. Actual: 898)**

Trends Box

The cleanup of oil spills in marine environments ultimately relies on microbial metabolism of hydrocarbons (HC), which complements the current chemico-physical techniques used in emergency response.

Consolidated biotechnologies include microbial communities biostimulation, biosurfactant supplementation and bioaugmentation HC-degrading microbial cells.

The effectiveness of biotechnologies is limited by our understanding of the microbial ecology of polluted marine systems. We lack knowledge on how environmental factors, such as hydrostatic pressure, temperature and dispersant toxicity, affect microbial successions.

The recent availability of meta-omics data and the improved understanding of microbial metabolism are leading to novel biotechnologies for marine oil spill cleanup, such as slow-release particles for efficient biostimulation and bioelectrochemical approaches for sediment cleanup.

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Outstanding Question Box **(Max 2000 characters w/spaces. Actual: 1664)**

Outstanding Question Box

The continuous increasing understanding of microbial physiology, metabolism and ecology is driving the development of novel, microbially driven biotechnological applications to the oil hydrocarbon (HC) cleanup in marine environments. Among those described in this review, the application of slow-release particles (SRPs), the "oil-spill snorkel" and modular slurry systems are potentially promising biotechnologies for the remediation of polluted water and sediments based on the activity of microorganisms or their products. However, a series of questions and unresolved issues regarding both basic and applied science remain. These questions are:

1) How do we improve the control of component release by SRPs?
2) How do we select novel, improved and versatile biosurfactants or modify existing ones for applications as components of biodispersants in a cost-effective way?
3) What is the fine structure and dynamics of the full functional and metabolic cooperation between microbial components of HC-degrading microbial consortia?
4) How do neglected factors such as hydrostatic pressure affect the response of autochthonous natural microorganisms in polluted marine compartments?
5) How do combinations of environmental stressors act on HC-degraders?
6) Is there a biogeographically driven distribution of marine microbial HC degraders?
7) What is the prevalence and functional importance of unculturable HC degraders that are emerging from recent meta-omics reconstructions?
8) What is the balance and diversification of electron flow in the "oil-spill snorkel"?
9) Is it advantageous to combine different biotechnologies, for instance SRPs and the "oil-spill snorkel"?

Figure 1

Click here to download Figure Mapelli et al.2017 FIG1.tif

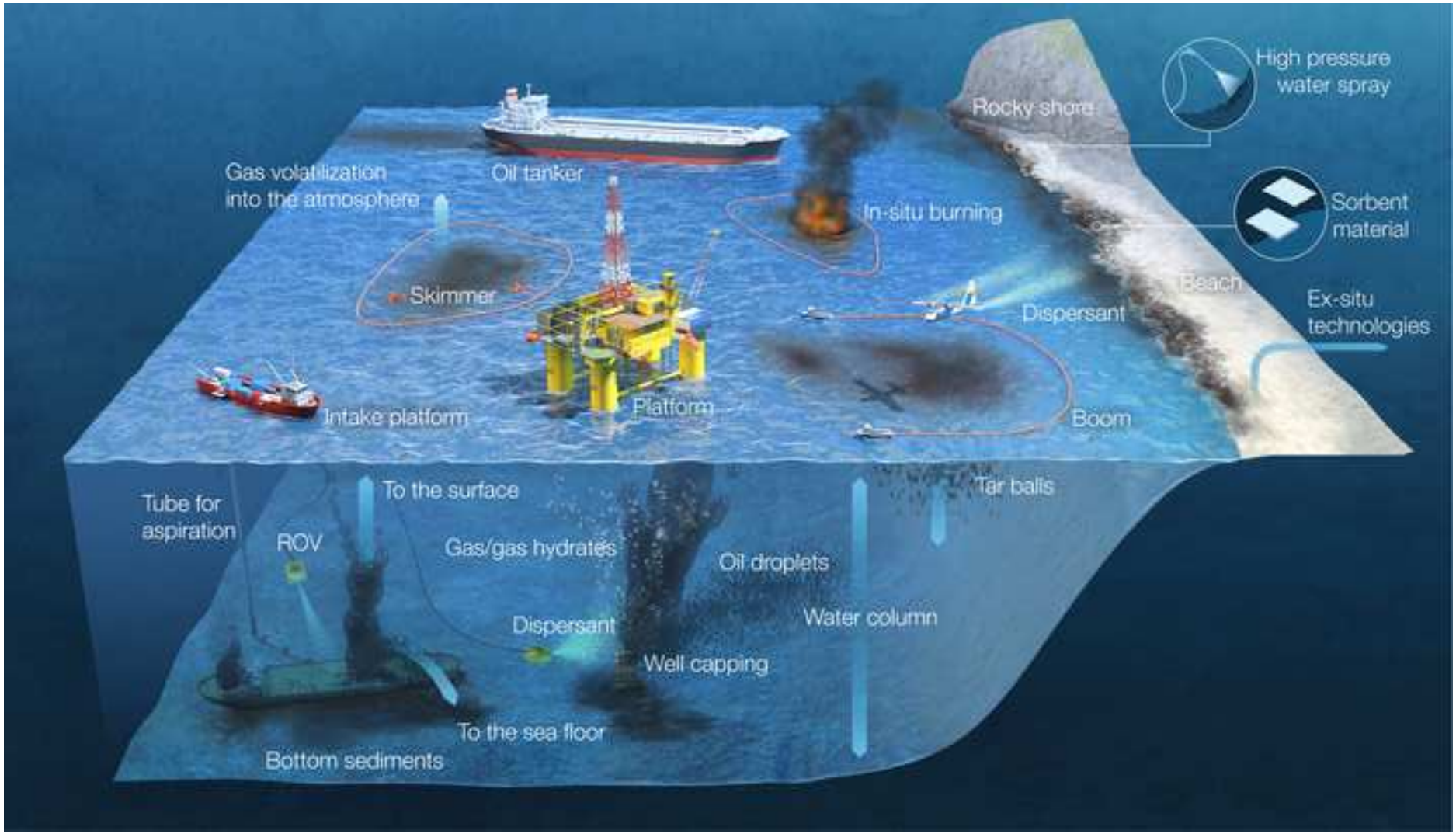

Figure 2

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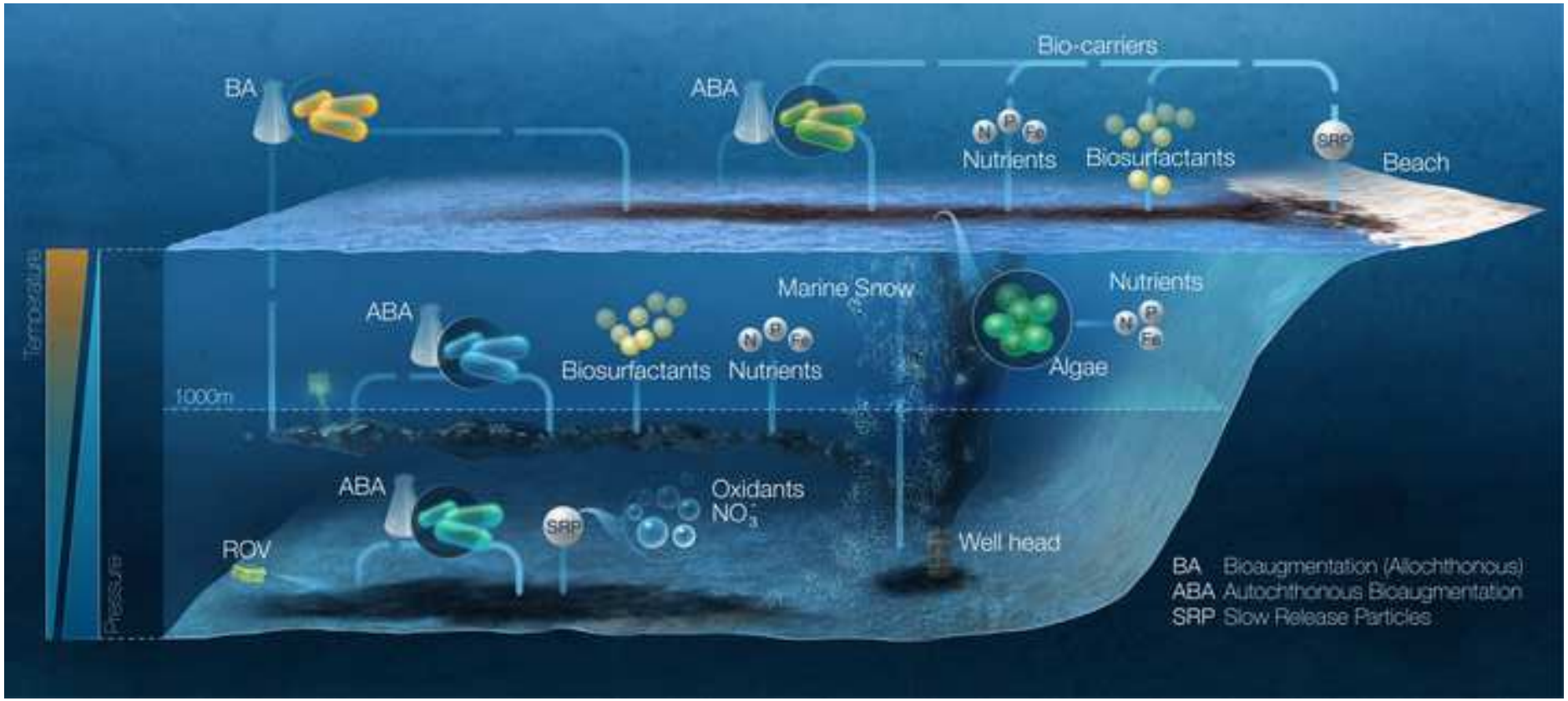
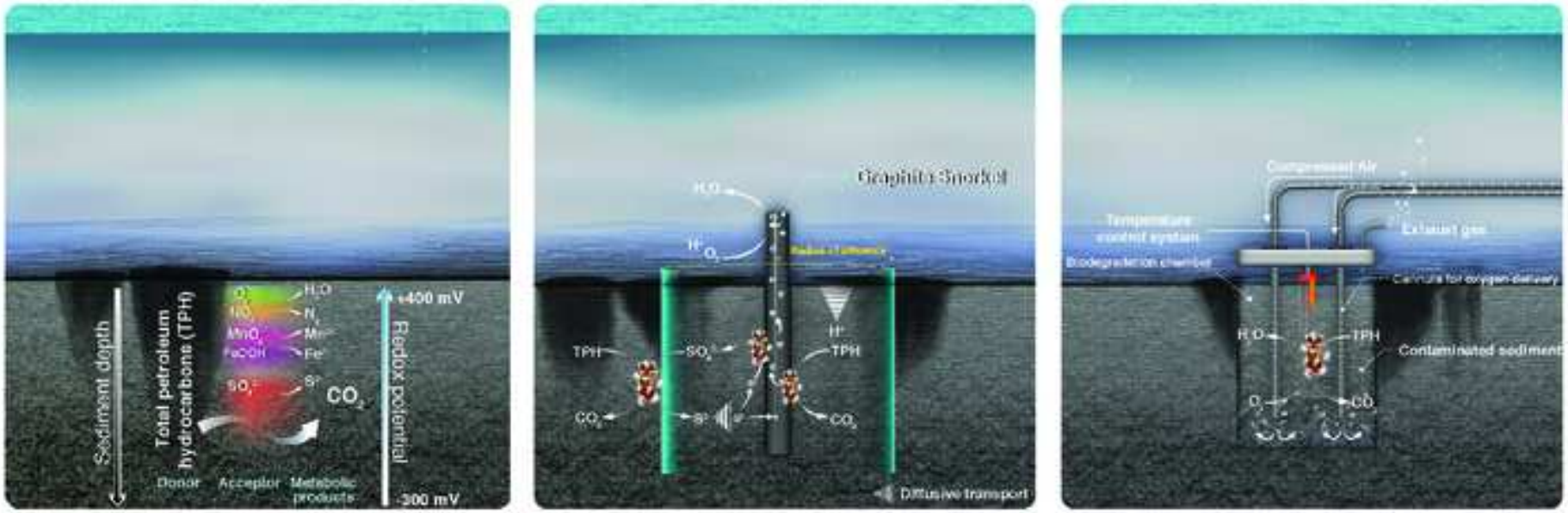


Figure 3

Click here to download Figure Mapelli et al.2017 FIG3.tiff



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Supplementary Table 1 and Supplementary References

Supplementary Table 1. A comprehensive list of main cultivated marine hydrocarbon-degrading microorganisms and their phylogenetic, physiological and ecological features.

A/AN	Genome	Microorganism	Phylogeny	Target substrate	Habitat/Ecology	Physiology	Refs
A	Y	*Alcanivorax borkumensis*	*γ-proteobacteria, Alcanivoracaceae*	*n*-alkanes	Seawater, sediment, beach sand, coastal salt marsh	Biosurfactant producer, OHCB	[1-4];
A	Y	*Alcanivorax dieselolei*	*γ-proteobacteria, Alcanivoracaceae*	*n*-alkanes	Seawater, sediment	Resistance to mild pressure increase, OHCB	[5-7]
A	Y	*Marinobacter hydrocarbonoclasticus:*	*γ-proteobacteria, Alteromonadaceae*	*n*-alkanes, PAHs	Seawater, sediment	Biofilm producer; oil surface colonizers	[8-10]
A	Y	*Cycloclasticus pugetii*	*γ-proteobacteria, Piscirickettsiaceae*	PAHs	Sediment	Highly efficient transport systems for the capture of nutrients and oligo-elements	[11, 12]
A	Y	*Oleispira antarctica*	*γ-proteobacteria, Oceanospirillaceae*	*n*-alkanes	Seawater	Cold-adapted OHCB	[13]
A	N	*Oleibacter marinus*	*γ-proteobacteria, Oceanospirillaceae*	*n*-alkanes	Seawater	Adapted to tropical marine environments	[14-15]
A	N	*Oleiphilus messinensis*	*γ-proteobacteria, Oleiphilaceae*	*n*-alkanes	Seawater, sediment	Biofilm producer on oil droplets, OHCB	[16]
A/AN	Y	*Pseudomonas pachastrellae*	*γ-proteobacteria, Pseudomonadaceae*	*n*-alkanes, PAHs	Sediment, beach sand	Bioemulsification activity	[17-19]
A/AN	Y	*Pseudomonas stutzeri*	*γ-proteobacteria, Pseudomonadaceae*	*n*-alkanes, PAHs, BTEX	Seawater, marsh and marine sediments, beach sand	Biofilm producer	[19–21]
A	N	*Halomonas halodurans; Halomonas organivorans*	*γ-proteobacteria, Halomonadaceae*	*n*-alkanes	Seawater, sediment	Key role in N metabolism to sustain degrading consortia	[22,23]

A/AN	Genome	Microorganism	Phylogeny	Target substrate	Habitat/Ecology	Physiology	Refs
A	Y	*Thalassolituus oleivorans*	*γ-proteobacteria, Oceanospirillaceae*	*n*-alkanes	Surface seawaters, sediments, coastal and estuarine areas	OHCB	[24]
A	Y	*Alteromonas naphthalenivorans*	*γ-proteobacteria, Alteromonadaceae*	PAHs	Seawater, tidal flat sediment	*r*-strategist, fast growth in nitrogen-deficient seawater	[25]
A	Y	*Acinetobacter venetianus*	*γ-proteobacteria, Moraxellaceae*	*n*-alkanes	Surface water, sediment.	Biosurfactant producer	[26]
A	Y	*Dietzia maris*	*Actinobacteria, Dietziaceae*	*n*-alkanes, PAHs	Seawater, deep sea hydrothermal field	Biosurfactant producer	[27,28]
A	N	*Rhodobacter sp. SS12.29; Rhodococcus sp. ice-oil-488 s*	*α-proteobacteria, Rhodobacteraceae*	PAHs	Seawater	Key role in reducing the accumulation of metabolites resulting from PAH degradation	[29]
A	N	*Sphingopixis sp.*	*α-proteobacteria, Sphingomonadaceae*	PAHs	Seawater	Key role in reducing the accumulation of metabolites resulting from PAH degradation	[29]
AN	Y	*Desulfatibacillum alkenivorans*	*δ-proteobacteria, Desulfobacteraceae*	*n*-alkanes	Sediment	High metabolic versatility for anaerobic alkane utilization	[30]
AN	N	*Desulfosarcina-Desulfococcus cluster strains*	*δ-proteobacteria Desulfobacteraceae*	Short chain *n*-alkanes	Sediments of marine HC seeps	Propane and butane degraders; sulfate-reducing bacteria	[31,32]
AN	N	*Desulfococcus oleovorans*	*δ-proteobacteria, Desulfobacteraceae*	*n*-alkanes, aromatic HCs	Sediment	Sulfate-reducing bacteria	[33,34]

A/AN	Genome	Microorganism	Phylogeny	Target substrate	Habitat/Ecology	Physiology	Refs
A	Y	*Bacillus pumilus*	*Bacilli, Bacillaceae*	*n*-alkanes, PAHs	Sediment	Resistance to heavy metals	[20,35]
A	N	*Bacillus stratosphericus*	*Bacilli, Bacillaceae*	PAHs, BTEX	Seawater	High metabolic versatility, biosurfactant producer	[36]
AN	Y	*Archaeoglobus fulgidus*	*Euryarchaeota, Archaeoglobaceae*	*n*-alkanes	Shallow marine hydrothermal system	Extremophile	[37]
AN	Y	*Thermococcus sibiricus*	*Euryarchaeota, Thermococcaceae*	*n*-alkanes	Oil reservoir	High metabolic versatility	[38]
AN	Y	*Ferroglobus placidus*	*Euryarchaeota, Archaeoglobaceae*	Aromatic HCs	Shallow marine hydrothermal system	Hyperthermophilic	[39,40]
A	N	*Dothideomycetes-related taxa*	*Fungi*	PAHs	Beach sediment, tarballs, salt marshes	-	[41,42]

A: aerobic; AN: anaerobic; OHCB: obligate hydrocarbonoclastic bacteria, PAH: polycyclic aromatic hydrocarbons; Y/N: Y, genome available for at least one strain/ N, genome not available (Genome availability checked on NCBI database on 27 November 2016).

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