Bioremediation: a tool for the management of oil pollution in marine ecosystems

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ABSTRACT

During the last decades the course of the anthropogenic contamination of marine ecosystems has paralleled that of the oil industry. Spills of crude oil and its derivatives can have short, medium and long-term negative consequences, and the elimination of pollutants by natural means may take years or even longer. Bioremediation is an emergent branch of environmental biotechnology that is often used to accelerate this process and guarantees the reparation of damaged ecosystems, based on harnessing the metabolic capabilities of bacteria, fungi, yeast, algae and microbial mats to degrade oil hydrocarbons. Bioremediation follows two main strategies: the stimulation of indigenous microbial populations, known as biostimulation, and bioaugmentation, the introduction of viable microbial populations. Choosing one or another depends on the analysis of abiotic and biotic factors influencing the biodegradation process; the former refers to factors related with the pollutant and environmental conditions, while the latter encompasses all factors that have to do with microbial populations. The development of bioremediation has led to the appearance of commercially available products for spill cleanup: fertilizers containing bio-stimulating nutrients, bioproducts based on microorganisms, and chemical products to stimulate the growth of the microbial populations involved in the process of biodegradation.

Keywords: bioremediation, marine ecosystem, biodegradation of hydrocarbons, bioproducts

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RESUMEN

Biorremediación: una herramienta para el saneamiento de ecosistemas marinos contaminados con petróleo. En las últimas décadas, paralela al desarrollo de la industria petrolera, ha aumentado la contaminación en los ecosistemas marinos. El vertimiento de petróleo crudo y sus derivados provocan efectos negativos a corto, mediano y largo plazo. La eliminación natural de los contaminantes puede tardar años, e incluso no ocurrir. Para acelerar este proceso y garantizar la reparación del ecosistema dañado, se emplean técnicas de biorremediación. Esta variante emergente de la biotecnología ambiental, se basa en el empleo de la actividad metabólica microbiana (bacterias, hongos, levaduras, algas y tapetes microbianos) para degradar los hidrocarburos del petróleo. Su aplicación tiene dos propósitos esenciales: la bioestimulación de la población autóctona viable, y la bioaumentación (introducción de poblaciones microbianas viables). Su selección requiere el análisis de factores abióticos y bióticos, que influyen en el proceso de biodegradación. Los primeros incluyen los relacionados con el contaminante y las condiciones medioambientales; y los bióticos, lo referente a la población microbiana. En el desarrollo de esta tecnología, se han formulado varios productos comercializables para la limpieza de desastres: fertilizantes construidos por nutrientes con funciones bioestimuladoras; bioproductos conformados por microorganismos; y productos químicos con la función de aumentar o estimular la población microbiana que interviene en el proceso de biodegradación.

Palabras clave: biorremediación, ecosistemas marinos, biodegradación de hidrocarburos, bioproductos

Introduction

Maritime transportation currently moves close to two billion tons of crude oil. Not surprisingly, accidental large-scale oil spills have become a major source of marine pollution. Most of the responsibility for oil spills falls on large oil tankers (22% of the total); although spills from other vessels and offshore drilling rigs and platforms also account for a significant portion. It is estimated that one out of every million tons of oil transported in ships ends up in the ocean; of those, only 10% are due to maritime accidents. The atmosphere, natural leaks, rivers, urban runoff water, coastal oil refineries and operative discharges from oil tankers also constitute sources of hydrocarbon pollutants, including the spills into the Persian Gulf during the Persian Gulf War of 1991, which dumped as much as 460 000 tons of oil on the sea [1]. Operative discharges are caused by oil tankers washing their tanks while offshore, or by contaminated water pumped out of ballast tanks.

The oil slicks produced by accidental spills take a considerable economic toll, as they interrupt fishing and navigation and, importantly, damage severely the ecosystem by killing birds, fish and sea mammals, among others. The slick caused in 1989 by the collision of the *Exxon Valdez* with Bligh Reef and the consequent spill of 40 to 50 thousand tons of oil into Prince William Sound was considered, until 2010, as the largest ecological catastrophe of its type. Part of the strategy followed in the aftermath of the disaster was the use of an oleophilic fertilizer (Inipol EAP22) to stimulate the biodegradation of crude oil, with the result that only two weeks later a considerable portion of the oil coating the shoreline had been removed. Another large oil slick was caused by the spillage of 227 thousand tons of oil when tanker *Amoco Cádiz* ran aground on the French coast in 1978. Approximately 63 thousand tons of oil were again spilled into the Mediterranean, coating 1900 km of Spanish and

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French shorelines, when the tanker *Prestige* sunk in what is still considered one of the largest ecological disasters caused by oil spills; the largest ornithological tragedy of the European Atlantic was caused by more than 37 thousand tons of heavy fuel spilled by tanker *Erika* on the Breton coast [1]. Bioremediation and the application of bioproducts were among the alternatives considered for the management of the consequences from all these accidents.

The largest ecological disaster caused by oil spills, however, began when the *Deepwater Horizon* drilling rig exploded and sunk in April 2010, uncontrollably pouring oil for more than 60 days into the Gulf of Mexico. According to Richard Harris [2], almost 300 thousand tons of crude had been spilled by May 15, 2010, posing a severe threat to hundreds of marine species and birds. Once the well is sealed and mechanical cleanup concludes, the application of a bioproduct named Oilzapper, produced by The Energy and Resources Institute (TERI) from India, consisting on a consortium of 5 bacterial strains capable of degrading saturated and aromatic hydrocarbons, nitrogenated and sulfur benzene compounds and tar, seems to be a good choice from an ecological point of view.

The geographic characteristics of the continental shelf of the island of Cuba (narrow and elongated) make it highly vulnerable to water pollution accidents. Fortunately, only 5 oil-related accidents have had a measurable impact on marine ecosystems during the last 20 years: three from accidental oil pipeline leaks (Jibacoa beach in 2001, Arroyo Blanco cove in 2002 and Bacunayagua cove in 2008), one in the bay of Matanzas (1998) after the collision of the vessels *Sharadar* and *Bravo*, and another in the bay of Cienfuegos (1992) when tanker *Aida* ruptured its hull against a rock.

Accidental oil spills can have short, medium and long term negative consequences. Oil may undergo natural dispersal and degradation during the course of several years. Research, however, has shown that oil elimination by natural means is a slow process, implying that the recovery of affected ecosystems takes a long time [3]. Recent studies in Alaska [4], where the *Exxon Valdez* spill took place, have measured annual hydrocarbon concentrations of 62258 mg/g (wet weight) in sedimentary samples from the area. Additional proof for the persistence of these pollutants has come from the study of oil concentration profiles in sediment cores, during the course of research on historical anthropogenic discharges [5]. This persistence has to be considered when evaluating the success of recent measures for the control of hydrocarbon pollution, and has triggered the development of several strategies to ameliorate the effects of oil spills and accelerate the recovery of damaged ecosystems. The present review examines the concepts of bioremediation as a tool of environmental biotechnology and its different branches, analyzing the factors that contribute to the biodegradation of oil in marine ecosystems and listing hydrocarbon-degrading microorganisms together with different bioproducts for the bioremediation of this ecosystem.

Environmental biotechnology: bioremediation

Environmental biotechnology first appeared between the 19th and 20th century, prompted by the need to treat urban wastewaters. It was extended, during the second half of the 20th century, to soil remediation, the purification of gases, cleansing of surface and underground water bodies, purification of industrial wastewater, treatment of waste in sanitary landfills and the composting of bio-organic residuals [6].

Environmental biotechnology employs modern biological processes to protect and restore the quality of the environment. It appeared as an attempt to solve many of today's environmental problems, as it can be used to evaluate the status of different ecosystems, transform pollutants into harmless compounds, generate biodegradable material from renewable resources and develop environmentally sound manufacturing and waste management processes [7]. This applied scientific discipline encompasses an application aimed at dealing with pollution that is based on the use of microorganisms to generate fuels and genetically modified plants to absorb toxic compounds.

A growing number of industrial companies are currently pursuing the development of preventive biotechnological processes to reduce their environmental footprint in the current context of international efforts towards a sustainable society. Environmental biotechnology is, doubtless, a welcome aid to manufacture new products with a smaller impact on the ecosystem [7].

Bioremediation, currently regarded as an emergent technology, has become the main application of environmental biotechnology. The term denotes any treatment based on the use of biological systems for the restoration or sanitation of soils, underground waters and atmospheric air contaminated with xenobiotics. It may be defined as a technology for the bio-restoration of natural environments previously polluted with xenobiotics through the elimination, attenuation or transformation of such compounds [8, 9], taking advantage of the metabolic capabilities of microorganisms (bacteria, fungi, yeast and algae) [8, 10] and some plants, together with nutrients and oxygen, to accelerate natural processes of biodegradation. Bioremediation may partially transform the pollutant into a less hazardous form, or may achieve its mineralization, totally degrading it into carbon dioxide aerobically or water and methane under anaerobiosis. It is important to note that bioremediation is restricted to biodegradable pollutants [8].

The first patent for a biological agent to be used in remediation processes was granted in 1974, protecting an oil-degrading strain of *Pseudomonas putida* [11]. By 1991 there were already more than 70 oil-degrading microbial genera in literature; a figure that almost doubled in the two successive decades. These microorganisms belong to at least 11 different prokaryotic divisions [12].

Interest for the use of microorganisms in aquatic ecosystems contaminated with oil pollutants is reflected in recent publications and patents dealing with the study and development of processes for the biodegradation of contaminants. Biological decontamination must be applicable under field conditions; it must respect the natural environment in which it takes place and must be economically competitive. These requirements are met by the microorganisms typically found in polluted areas, which naturally degrade xenobiotics. They can be grown using low-cost substrates [13] and inocula containing them can be man-

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ufactured through conventional industrial techniques, turning them into ideal candidates for application in bioremediation processes.

Microorganisms (mainly bacteria and fungi) can degrade a vast array of structurally dissimilar environmental pollutants. Recalcitrant contaminants, such as the polychlorinated biphenyls produced by oil refineries, polycyclic aromatic hydrocarbons, resins and asphaltenes as well as complex mixtures containing them [14], can be mineralized into carbon dioxide by several ligninolytic fungi and bacteria.

According to the need to transport or not the contaminated matrix, bioremediation techniques can be classified as *in situ* or *ex situ* [15]. *In situ* techniques allow for the on-site treatment of contaminated soil and waters without further transformation. *Ex situ* techniques, as the name implies, require excavating and then transporting contaminated soils or, in the case of contaminated water bodies, pumping-treating-discharging or transferring into reactors.

Selecting a specific technique depends on three basic factors: the biochemistry of the degradation, *i.e.*, the possibility of biologically degrading the pollutant; the bioavailability of the pollutant, meaning how accessible the toxic compound is to the microorganism; and third, bioactivity, the opportunity to optimize the biological activity [15].

The bioremediation of aquifers is performed by a pumping-treating-recharging scheme, consisting of extracting ground water, biodegrading the pollutants in reactors installed on the surface and then pumping the treated water back. Another alternative is the injection of nutrients and bacteria, establishing a recirculation that converts the aquifer itself into a bioreactor [16].

The versatility of bioremediation lies on its ability to be tailored to the needs of each site. Bioremediation is classified into biostimulation (the addition of nutrients to facilitate the metabolic activity of the indigenous degrading microbiota); bioaugmentation (the addition of exogenous degrading microorganisms when the native microbiota is insufficient or overwhelmed) [9]; and bioaugmentation with genetically modified microorganism, a practice almost exclusive to developed countries so far. Recombinant DNA techniques can also be harnessed to obtain products that can potentiate specific degradation pathways for bioremediation purposes; an alternative that is very efficient, but expensive.

Bioremediation, as every technological process, has advantages and disadvantages. Low cost (especially compared to the alternatives) is one of its assets. Although an accurate comparison is difficult due to variations from site to site, bioremediation is, in general terms, at least ten times less costly than incineration, and three times cheaper than some physico-chemical immobilization technologies. Factors such as its low energy consumption, the low price of the nutrients and its operation under environmental conditions combine together to turn bioremediation into a very attractive proposition for developing countries [15]. Bioremediation, in addition, is a clean technology that transforms pollutants into harmless compounds such as carbon dioxide.

By definition, however, bioremediation is circumscribed to biodegradable compounds, and cannot therefore be used in certain cases. It is useless, for example, for the management of radioactive contamination, fails with highly halogenated organic compounds, and is inhibited in the presence of heavy metals or by unfavorable microenvironmental conditions. Every microorganism presents a different tolerance profile, and there can also be cases of inhibition of microbial activity at high concentrations of organic pollutants [16].

Biodegradation of hydrocarbons

Oil biodegradation is a natural process [17-19] that takes months or years, according to prevailing conditions [20], the molecular complexity of oil components and the volume of the pollutant. Biodegradation depends on abiotic, or physico-chemical factors, comprising those related to the structure of the pollutant (chemical structure, concentration and bioavailability) and environmental conditions (oxygen and nutrient availability, pH, temperature, pressure, salinity, presence of heavy metals); as well as biotic factors, dependent on the specific microorganism (microbial population: concentration and interactions).

Physico-chemical processes

When crude oil or distilled oil products are dumped into the marine environment, they undergo immediate physical and chemical changes. Some of the abiotic processes that take place in this situation are evaporation, dissolution, dispersal, photochemical oxidation, oil-water emulsification, adsorption to suspended particulate matter, sinking and sedimentation. These processes take place concurrently, altering the chemical composition and physical properties of the original pollutant and, therefore, significantly influencing the efficacy of biodegradation. Evaporation is generally the most important process during the first 48 hours after a spill, volatilizing low- and medium-molecular weight compounds into the atmosphere. It is estimated that one to two thirds of a spill are thus eliminated during this period (one third of the spill from *Amoco Cádiz* evaporated during the first 3 days, for instance), although evaporation rate decreases with time. Evaporation depends on oil composition, surface area, the physical properties of the slick, wind speed, temperature of both air and water, waves and the intensity of solar radiation. The remaining material is enriched in metals (mainly nickel and vanadium), waxes and asphaltenes rather than the original oil. Specific weight and viscosity also increase with evaporation. From the perspective of mass loss, dissolution into the water column is much less important than evaporation, as the dissolution of a significant portion of the spill into this medium is highly unlikely. Its importance stems, instead, from the fact that some water-soluble fractions (light aromatic compounds) are toxic to marine organisms (including the degrading microorganisms), and their impact on the marine environment thus goes beyond considerations of mass balance [21].

Characteristics of contaminating hydrocarbons

The characteristics of the pollutant are one of the main factors to consider when choosing a course of action for decontamination. Hydrocarbons are composed of hydrophobic, poorly water soluble compounds

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where the number of carbons in aliphatic chains or the presence of conjugations, in the case of aromatic molecules, are inversely correlated with solubility in water; substitutions with polar groups (alcohols, acids, etc.), on the other hand, tend to have the opposite effect [22]. Solubility is an important property from the perspective of bioremediation, as it affects the bioavailability of hydrocarbons. Bioavailability may also be limited by physical or chemical barriers obstructing the passage of the molecule into the cell to undergo metabolic degradation [23], and viscosity also hampers the pollutant-nutrient-bacterium-electron acceptor interaction [24]. The presence of toxic components or microbial growth inhibitors is obviously an additional factor to take into account, while volatility seems to have no effect on biodegradation.

Carbon source

Different bioremediation microorganisms show varying specificities for different oil components. If the binding site of the degrading enzyme is obstructed (due *e.g.* to isoprenoids or molecules substituted with bulky groups such as COOH, or CH_3), the reaction will not take place, and biodegradation will slow down or stop altogether [25]. In general, hydrocarbon biodegradation follows a structural hierarchy, whereby saturated molecules are the easiest to degrade, followed by aromatic hydrocarbons [26-28] and these in turn by resins and asphaltenes [26, 28]. N-alkanes are most prone to oxidation [27, 29], followed by iso-alkanes, although highly branched structures with quaternary carbons are seldom degraded. Olefins are less frequently employed as carbon sources than their corresponding alkanes, followed by aromatic hydrocarbons which, if their molecular weight is small enough, can be metabolized when present at low concentrations. Cycloalkanes are highly toxic, and are used as substrates only under exceptional circumstances [29]. Paraffins are easily degradable. Isomers, cyclic and long-chain hydrocarbons degrade slowly, and therefore require a specific microbiota. Polycyclic aromatic hydrocarbons can also be used as carbon source by different microorganism species [27, 30].

High hydrocarbon concentrations may inhibit microbial activity through a mechanism called carbon source toxicity [23, 31, 32]. There are field reports of effective biodegradation at pollutant concentrations as high as 30%, although the process usually slows down dramatically beyond this point [25]. The specific composition of xenobiotic compounds changes with time due to the action of environmental agents, microbial agents and physico-chemical processes; as a result, the pollutant gets enriched in heavy compounds which are harder to degrade, producing a decrease in biodegradation rate as the pollutant ages [23, 32-34]

Nutrient availability and microorganism growth

Whenever oil spills into an environment poor in inorganic nutrients, the resulting skewed carbon/nitrogen and/or carbon/phosphorous ratios are unfavorable for microbial growth [3]. Nitrogen is important for the synthesis of aminoacids and enzymes. Natural sources of nitrogen for microorganisms include ammonia, nitrate, nitrites and organic nitrogenated compounds, in addition to the amino groups in organic detritus [35]. Almost all microorganisms are able to use ammonia as a nitrogen source, as it can be directly shunted into metabolic pathways [36]; however, even if reduced nitrogen groups (ammonia-like) are preferable from the standpoint of energy balance, oxidized forms (nitrate-like) do play an important part in the degradation of organic compounds despite being unable to substitute for oxygen during respiration. There is, in addition, ample evidence from the field of hydrocarbon-based production of unicellular protein that demonstrates the fundamental role played by the oxygen of nitrogenated compounds in the process of biodegradation, as hydrocarbons often create a highly reducing and hydrophobic environment that may be highly refractory to oxygen transfer [37].

Accordingly, and despite the abundance of ammoniacal supplements in bioremediation studies, the convenience of oxidized nitrogen sources cannot be ignored. Even when using biostimulation on the indigenous microbiota, it is better to favor species that thrive on oxidized nitrogen sources, as they will have an edge in conditions under which oxygen transfer may be severely restricted [37].

Phosphorous is also needed for the formation of cellular enzymatic compounds used in the processes of synthesis and degradation. The most common sources of phosphorous in bacterial sources are K_2HPO_4, KH_2PO_4, NaH_2PO_4, Na_2HPO_4 or mixtures thereof [38].

In general, it is considered that the addition of nitrogen and phosphorous has a positive outcome, increasing microbial populations and pollutant degradation rates [23]. The concentrations of nitrogen and phosphorous directly depend on carbon concentration, with a C:N:P ratio of 100:10:1 [31, 39].

Bridé and Bos [40] demonstrated how, in specific circumstances, phosphorous may exert a larger influence than nitrogen even though the latter is always required in larger absolute amounts. Additionally, although it is sometimes assumed that nitrogen requirements may be partially met by the nitrogen compounds of crude oil, it is well known that many microorganisms are slow to utilize many of these organic sources of nitrogen, and those present in oil slicks usually fall into the latter case [37]. It must not be forgotten that research into the nutritional demands of microorganisms is often performed with natural substrates, where phosphorous may be the limiting nutrient; therefore, the conclusions from this type of studies, while true under laboratory conditions, are rarely categorical.

pH

Hydrocarbon mineralization is favored at near-neutral pH [3]. There is a pH optimum for each microorganism, also near neutrality in most occasions [41]. Any change in pH will have an effect into the biodegradative activity of microbial populations, as well as on the solubilization and absorption/desorption of ions and pollutants. Cations, such as NH^{4+}, Mg^{2+}, Ca^{2+}, are more soluble at acid pH, whereas anionic forms (NO^{3-}, NO^{2-}, PO_4^{3-}, Cl^-) are better solubilized at alkaline pH [23, 25]. Optimum pH for most bacteria is between 6 and 8, between 4 and 6 for yeast, and between 3 and 7 for filamentous fungi.

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Temperature

Temperature is an important determinant for biodegradation, as each microorganism has an optimal temperature range. Microbial activity slows down at low temperatures due to decreases in the rate of nutrient and waste transport through the membrane [42], with a threshold for the presence of detectable degradation around 0 °C [43]. However, there are microorganisms adapted to cold temperatures that can degrade hydrocarbons [41, 43-47] and on the other extreme, even though microbial activity decreases above 40 °C due to enzyme denaturation [48], some of the thermophile microorganisms found in semiarid shorelines have shown potential for this application [3, 49]. Temperature also affects the viscosity and solubility of hydrocarbons, influencing the volatilization of low molecular weight compounds. For the purposes of biodegradation, temperatures may range from 15 to 45 °C (coinciding with the optimal temperature range for mesophile microorganisms) [50]; however, the optimum is at 30-40 °C. Clearly, daily or seasonal variations make it difficult to stay within this optimum, although the technology to minimize their effect is available [51]. In any case, bioremediation efforts are more effective during spring and summer, and the number of bioremediation projects in low-temperature sites is scarce compared with those targeting mesophilic ecosystems [52].

Electron acceptors. Oxygen availability

The redox reactions of the respiratory chain constitute the biochemical foundation of bioremediation [53]. The electron acceptors most commonly employed by microorganisms are oxygen (aerobic degradation), nitrate, ferric iron, sulphate and carbon dioxide (anaerobic degradation).

In aerobic degradation, oxidation most commonly takes place through oxygenases or intermediates that easily enter the Krebs cycle, such as catechol, protocatechuate and gentisate (aromatic rings) or fatty acids in the case of linear chains. Anaerobic degradation on the other hand is a three-stage process that starts with substrate activation (carboxylations, anaerobic hydroxylations or formation of thioesters of the corresponding aromatic acid with acetyl-CoA), followed by an enzymatic attack (through reductases) on the activated intermediaries and the conversion of non-cyclical compounds (by opening the ring) into central metabolites [23].

The efficiency of aerobic biodegradation depends, to a large extent, on oxygen solubility. The starting steps of the catabolism of aliphatic, cyclical and aromatic hydrocarbons by bacteria and fungi require the oxygenation of the substrate with oxygenases consuming molecular oxygen. Stoichiometrically, approximately 3 pounds of oxygen are required to convert 1 pound of hydrocarbons into carbon dioxide and water. This explains why first approximations to estimate oxygen consumption typically use a 3:1 ratio. Oxygen is seldom limiting in the surface of the water or in superficial layers of water bodies, and marine ecosystems become anoxic only at large depths. Yet, oxygen availability generally is a limiting factor for hydrocarbon biodegradation [54, 55], due to the much lower efficiency of anaerobic degradation [55, 56]. Anaerobic degradation is so slow that it has long been considered ecologically irrelevant, although recent research has evidenced that it is important for catabolic biodegradation pathways [3, 57, 58].

Pressure

If the pollutant is denser than seawater, it will sink to the sea floor where it will be subjected to large hydrostatic pressures. The high pressure/low temperature combination of deep oceanic environments is not favorable for microbial activity, as illustrated by the fact that a biodegrading microbial consortium isolated from the bottom of the sea was tenfold more active at surface than under deep ocean conditions [3].

Salinity

The rate of hydrocarbon degradation is inversely proportional to salinity. Biodegradation studies at sodium chloride concentrations above 2.4% (w/v) have revealed that this inhibition is more pronounced for aromatic and polar than for saturated fractions [59, 60]. Regardless, there are microorganisms that can oxidize hydrocarbons at salt concentrations as high as 30% (w/v) sodium chloride [3].

Heavy metals

High concentrations of heavy metals are inhibitory to microbial populations, perturbing metabolism and cell division, denaturing proteins and promoting cell membrane damage. Microorganisms, however, may develop resistance or tolerance to high levels of heavy metals [9]. Removing heavy metals from contaminated ecosystems is a difficult task, as these pollutants are generally not amenable to biological degradation. Zinc, copper, nickel and chromium are essential metals or micronutrients required by microorganisms, plants and animals [60], whereas cadmium, mercury and lead have no known biological or physiological roles [61].

Microbial population: interactions and concentration

The most important factor determining the outcome of biodegradation is the composition of the microbial population itself, which must be adapted for the utilization of the pollutant and be endowed with an enzyme repertoire fitting the required degradation steps [23, 34, 62]. As oil is composed of a wide range of dissimilar chemical compounds, no microorganism can single-handedly degrade all of its fractions, and oil biodegradation in nature is performed instead by mixed populations known as microbial consortia. The biodegradative power of consortia is understandably wider, as their enzyme repertoire is larger and, therefore, better adapted to degrading complex hydrocarbon mixtures [32, 62]. In addition, the division of labor established inside a microbial consortium, where one species performs the initial oxidation steps of a compound while another finishes the process is demonstrably more efficient than the action of pure cultures [24, 29]. The species initiating the process of degradation are known as primary consumers, whereas those degrading intermediate products to completion are denominated secondary consumers. Secondary consumers may also facilitate the growth of primary consumers by secreting metabolic prod-

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ucts (growth factors), eliminating toxic compounds by co-metabolism and exchanging genetic material [23, 63, 64]. A microbial community adapts to the utilization of a specific contaminant by three different mechanisms: the induction or repression of specific enzymes, genetic changes resulting in the appearance of new metabolic capabilities, and the selective enrichment of a specific sub-population with the ability to transform the target compound [23].

Determining the optimal microbial concentration to maximize the rate of degradation depends, therefore, on the type of contaminating hydrocarbon and the specific degradation rate of the microorganism [24].

Competent microorganisms

There is a large number of microorganisms scattered throughout different ecological niches that utilize hydrocarbons as their sole carbon source. They, however, are found only at low concentration in non-contaminated areas, where their populations increase only in response to chronic pollution [65]. Of all living organisms used in bioremediation technology, aerobic heterotrophic bacteria constitute the best-studied group, since anaerobic microorganisms are generally less flexible about the nature of the substrate and much more sensitive to the presence of heavy metals and, therefore, play a smaller role in biodegradation [51]. Bacteria constitute the most important group from a taxonomic standpoint, not only due to the extensive number of existing biodegrading species and genera, but to their metabolic versatility. There is a large number of bacterial species that can use xenobiotic compounds as carbon sources in pure cultures [66].

Starting with the pioneering studies of ZoBell in marine environments [67], numerous bacterial strains capable of degrading different hydrocarbons have been isolated from coastal and oceanic ecosystems [20, 57, 68, 69]. Many of these, such as *Alcalinivorax* or *Planococcus*, use a limited number of carbon sources, preferably oil hydrocarbons; being generally considered as specialists [70-74]. Much less specialized bacteria have been also isolated, however, as exemplified by *Marinobacter*, *Staphylococcus*, *Micrococcus*, *Sphingomonas* or *Geobacillus* [75-78]. Table 1 lists microorganisms reported to be able to degrade different oil hydrocarbons.

Metabolic capabilities are not the only criteria for selecting bioremediating species, however. It is also recommended to select indigenous species from the polluted areas when undertaking marine bioremediation projects, as these are much more resistant to the washing action of tides on coastal surfaces [57].

The available literature on bacterial species goes back to 1946, and far outnumbers that on bioremediating fungi [79]. While this may be taken to imply that direct hydrocarbon assimilation is not frequent in the latter, a number of reports have demonstrated that fungi can also utilize hydrocarbons as their sole carbon and energy source [79, 80].

Stratified benthonic systems

Microbial mats are natural ecosystems where microorganisms belonging to different physiological groups cluster together in a mat a few millimeters thick. Mats constitute complex microbial communities stratified by depth, due to the abrupt gradients of light, oxygen, hydrogen sulfide and redox potential they generate, combined with the metabolic capacities of the microorganisms themselves. These layers can be macroscopically observed as differently colored strata, each having a particular taxonomic composition [81-83]; the most superficial layers are usually composed of oxygenic phototrophic cyanobacterial populations and eukaryotic algae, with heterotrophic bacteria consuming organic matter and oxygen. The layers underneath are most frequently formed by anaerobic phototrophic and heterotrophic organisms, if conditions are adequate [3].

Table 1. Microbial genera with potential for the degradation of hydrocarbons

Group	Genus	
Bacterias	*Acinetobacter sp.*	*Lucila sp.*
	Alcaligenes sp.	*Marinobacter sp.*
	Alcanivorax sp.	*Metylophaga sp.*
	Alcanivorax sp.	*Micrococcus sp.*
	Aquaspirillum sp.	*Microscilla sp.*
	Arthrobacter sp.	*Mycobacterium sp.*
	Azospirillum sp.	*Neisseria sp.*
	Bacillus sp.	*Nocardia sp.*
	Beggiatoa sp.	*Novospingobium sp.*
	Cycloclasticus sp.	*Oleiphilus sp.*
	Cytophaga sp.	*Planococcus sp.*
	Corynebacterium sp.	*Porpyrobactes*
	Desulfotalea sp.	*Pseudomonas sp.*
	Desulfovibrio sp.	*Rhodoplanes sp.*
	Erythrobacter sp.	*Rubrivivax sp.*
	Flavobacterium sp.	*Sphingomonas sp.*
	Geobacillus sp.	*Staphylococcus sp.*
	Geobacter sp.	*Sulfitobacter sp.*
	Halochromatium sp.	*Thioalcalovibrio sp.*
	Halomonas sp.	*Xanthomonas sp.*
Fungi	*Alternaria sp.*	*Penicillium sp*
	Aspergillus sp.	*Phanaerocheate sp.*
	Chaetomium sp.	*Pleurotus sp.*
	Cladosporium sp.	*Rhizopus sp.*
	Fusarium sp.	
Yeast	*Candida sp.*	*Saccharomyces sp.*
Algae	*Chlamydomonas sp.*	*Dunaliella sp.*
	Chorella sp.	

The physical structure of these communities allows them to withstand the perturbations represented by oil spills. A number of studies have demonstrated that microbial mats appear and thrive not only in non-contaminated marine environments, but in chronically polluted environments as well.

Microbial mats were first identified as a potential tool for the bioremediation of shorelines with the 1991 Gulf War, when these structures covered, in a surprisingly short time, extensive coastal areas contaminated by oil spills, after which the hydrocarbons were degraded in a few months both aerobically and anaerobically. Different reports have described the degradation of hydrocarbons by cyanobacteria [84], and it has been demonstrated that the mats of coastal areas can degrade oil, although the species responsible for metabolizing oil compounds have not been identified [85, 86]. Undoubtedly, cyanobacteria play a fundamental role in the physiology of mats, as they are responsible for the formation of oxygen gradients and the synthesis of the organic matter consumed by heterotrophic bacteria. It is still unclear, however,

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whether the degradation of hydrocarbons in mats is mediated by cyanobacteria or by heterotrophic species. As noted above, cyanobacteria can oxidize hydrocarbons, as demonstrated by Al-Hasan *et al*. [87] for n-alkanes with non-axenic cultures of *Microcoleus chthonoplastes* and *Phormidium corium,* isolated from polluted sediments in the Gulf of Arabia. Studies with *Oscillatoria* sp. and *Agmenellum quadruplicatum* demonstrated that these strains could oxidize naphthalene [88, 89], and many other reports demonstrate the capacities of other strains for the degradation of diverse oil components [84, 90-92]. However, most of the studies performed with cyanobacteria forget to mention whether the cultures were axenic, and some research suggests that the heterotrophic bacteria associated to cyanobacteria are indeed responsible for the biodegradation [3]. According to the latter authors, cyanobacteria by themselves would not be responsible for directly oxidizing oil components, but would indirectly play an essential role by supporting the growth and activity of degrading microorganisms [93].

Bioproducts and fertilizers

A number of bioproducts and fertilizers of varying origin and composition have been developed for the bioremediation of marine ecosystems. Fertilizers, containing microbial nutrients (nitrogen and phosphorous, essentially) are employed for the bio-stimulation of the indigenous microbiota; their use is recommended only after confirming that local microorganisms possess the necessary enzymes to degrade the pollutant. Bioproducts, on the other hand, are composed of microorganisms and nutrients, and are employed in ecosystems where the endogenous microbiota cannot degrade all hydrocarbons and, therefore, may need a bio-reinforcement. Nutrients are seldom omitted from bioproducts, as the latter must stimulate the growth of both the indigenous microbiota and the introduced microbial population; some bioproducts, in addition, are formulated with a microbial bio-surfactant to decrease the surface tension of the water-oil interface and increase the bioavailability of the pollutant. These products have capabilities for bio-augmentation and/or bio-stimulation. Tables 2 and 3 contain a selection of bioproducts and fertilizers of varied origin and composition.

The application of these products does not introduce additional contaminations, as the microorganisms consume them completely (including the contaminating carbon source) and die later.

The formulation of bioproducts has been pioneered in Cuba by a group from the Institute for Oceanology from CITMA which, starting in October 2004, branched out into the Center for Marine Bioproducts (CEBIMAR). In 1994 they patented a product denominated BIOIL [94] (table 2). BIOIL was first used in 1992 in the bay of Cienfuegos, achieving an oil removal rate of 80%. The product was later applied to a spill in the bay of Matanzas in 1998, where it exhibited an efficacy of 92% [35], and in 2001 and 2002 was used for spills in Jibacoa beach (North of Mayabeque province) and Arroyo Blanco cove (Holguín) respectively, with an efficacy of 99% [95]. BIOIL-FC was used in 2008 at the Bacunayagua cove (North coast, at the Mayabeque-Matanzas border) for a 98% efficacy.

Conclusions

No technique for the removal of oil from marine ecosystems is 100% effective. Bioremediation, to be feasible, is best preceded by physical removal methods. With the exception of few scenarios, this technology makes the remediation of contaminated ecosystems possible. Favoring *in situ* over *ex situ* processes is advantageous from both ecological and economical points of view, as the latter requires transferring contaminated matter to the treatment site.

The biodegradation of contaminants depends on abiotic factors, including those related to the pollutant (solubility, viscosity, toxicity, volatility and bioavailability) and environmental conditions (pH, temperature, salinity, pressure, presence of heavy metals, oxygen and nutrient availability), as well as biotic factors, which comprise those related to the microorganism (microbial population: concentration and interactions). Although all these factors must be analyzed and taken into account for a bioremediation process, special attention must be devoted to the availability of nitrogen and phosphorous as well as the enzymatic composition of the microbial population.

More than 100 genera and twice as many species from 11 different prokaryotic divisions have been reported to degrade hydrocarbons, demonstrating the versatility and genetic/enzymatic variability of mi-

Table 2. Selection of commercially available bioproducts for the cleanup of marine ecosystems polluted with crude oil and its derivatives

Bioproduct	Manufacturer	Characteristics
B 350	Bio-System Co., USA	Hydrocarbon-degrading aerobic and facultative anaerobic bacteria and micronutrients
BIOIL	Cebimar, Cuba	Mixed A-5 culture immobilized in citric pectin
BIOIL-FC	Cebimar, Cuba	Five strains with high degradative activity for different oil fractions together with an emulsifying bio-surfactant obtained from *Bacillus licheniformis* CBM-60, one of the strains of the consortium.
Biotemposcreem	Russia	Lyophilizate containing 14 species of hydrocarbonoclastic microorganisms, nutrients and a biodegradable biological catalyst
JE 1058BS	Bio Research Centre, Japan Energy Corporation	Contains a bio-surfactant obtained from broth cultures of Gordonia sp. strain JE-1058; the product is sterilized
L 1800	Bio-System Co., USA	Hydrocarbon-degrading aerobic and facultative anaerobic bacteria, micronutrients and biodegradable surfactant
Micro-Blaze ® Spill Control	México	Formula of surfactants, nutrients and bacteria (non pathogenic). Contains natural bacteria in the form of spores (facultative), non vegetative. Not genetically modified; these bacteria are part of the natural microbiota of the environment (rivers, dams, soil, air, etc.)
Oilzapper	The Energy and Resources Institute (TERI), India	Consortium of five different bacterial strains. Can be used at 8 to 40 °C; the half-life of the product, once employed, is three months
Oppenheimer	Oppenheimer Biotechnology, Russia	Based on archaebacteria.
Putidoil	Russia	Lyophilized product formed by at least three strains of Pseudomonas putida, residual nutrients from the culture medium and conventional cryoprotective agents
Terra WR 100, Terra WR 300, Terra WR 500, Terra WR 600	Nimasco, Multinational Group	Combination of fertilizers and indigenous microorganisms, supplemented with surfactants

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Table 3. Selection of commercially available fertilizers used as biostimulants in processes of remediation of marine ecosystems contaminated with crude oil and its derivatives

Fertilizer	Manufacturer	Characteristics
Customblen	Elf, France	Slow-release encapsulated inorganic fertilizer, composed of ammonium nitrate, calcium phosphate and ammonium phosphate
Inipol EAP 22	Elf, France	Oleophilic organic fertilizer. Microemulsion containing nitrogen as urea, and tri-(Laureth-4) phosphate as source of phosphorous, encapsulated in oleic acid
Nitrophoska® Suprem	Compo, Germany	Fertilizer: 20% nitrogen (9.5% as N-NO3 and 10.5% as N-NH4; 5% P2O5 and 10% of potassium. Contains microelements such as magnesium, boron, sulfur and zinc. NPK + Mg + SO3 +B +Zn (20-5-10 + 3 + 15 + 0.02 + 0.01)
S 200	International Environmental Products, USA	Oleophilic fertilizer containing nitrogen and phosphorous

croorganisms. The use of microbial mats for bioremediation has yet to be exploited to its full potential, a situation arising, in part, from the complexity of their microbial populations.

The commercial availability of more than fifty bioproducts and fertilizers produced by companies and research institutions from around the world signals the existence of an attractive and effective alternative for dealing with the negative effects produced by oil spills on marine and coastal ecosystems. Bioremediation, despite its advantages, cannot be used indiscriminately, requiring previous analyses of ecological and economic feasibility that must take into account the conditions of the damaged ecosystem in order to best select between bioaugmentation, biostimulation, or both.

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