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Review Article

Microbial Degradation of Hydrocarbons in the Marine Environment, with a Focus on the Microbial Response to the Deepwater Horizon Oil Spill

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Abstract

The release of petroleum hydrocarbons into marine environments can occur naturally as well as through anthropogenic activities. The entry of hydrocarbons in the environment can lead to changes in microbial communities, with some microorganisms positively responding to and playing an important role in the degradation of the hydrocarbons. Deepwater Horizon (DwH) is recognised as the United State's worst oil spill disaster, having had significant detrimental consequences to marine life in the Gulf of Mexico and the economies that depend on them. The application of sophisticated microbial and molecular techniques, such as stable isotope probing (SIP), next-generation sequencing methods and single-cell genomics, has provided an in-depth and holistic picture on the microbial response, its evolution over the course of the spill, and the role these organisms played in the fate of the oil during the DwH spill. In this review, an overview of evidence-based research on this is presented. Some of the typical hydrocarbon-degrading bacterial suspects were found enriched during the spill, whereas the enrichment of other unexpected species, such as *Colwellia*, became a focus of many studies, including having a role in the degradation of the chemical dispersant, Corexit, that was used to combat the spill. The literature highlights a number of factors that can limit the progress of hydrocarbon biodegradation *in situ* and that certain bacteria encode mechanisms to overcome unfavourable conditions. Also discussed are some of the conflicting reports relating to the toxicity and effectiveness of the chemical dispersant used, and the interest to develop bio-based dispersants.

Keywords: *Hydrocarbon-Degrading Bacteria; Crude Oil; Macondo; Deepwater Horizon; Oil Spills; Hydrocarbons; Biodegradation; Marine Environment*

Introduction

Crude oil, formed naturally over millions of years by the accumulation of decaying organic matter, is in high demand globally by many sectors across industry for its energy-rich and combustible properties [1]. The large-scale extraction of crude oil has resulted in accidental oil spills that can be devastatingly damaging to the health of humans, animals and the environment in general. The carcinogenic properties of hydrocarbons make them lethal if they accumulate in food chains, and their toxicity in the environment can leave permanent effects. Considering that hydrocarbons occur naturally, either at oil seeps or in minute concentrations throughout the world's oceans and seas, microorganisms have over millions of years have evolved the ability to utilise them as a sole source of carbon and energy - such organisms are referred to as hydrocarbonoclastic [2].

On April 20 of 2010, the Deepwater Horizon (DwH) oil platform, located in the Gulf of Mexico (GoM) off the coast of Louisiana (USA), exploded and resulted in the release of ~4.9 million barrels of Louisiana MC252 crude oil into the Gulf over a period of almost 3 months. The leaky well was capped in July 2010, but by then considerable damage to Gulf ecosystems had already occurred. The spill affected ~450 miles of the GoM coastline making it one of the worst environmental disasters in US history [3]. The spill caused adverse effects on deep water benthic life and shore vegetation, the health of aquatic mammals, fish and birds, and altered the microbial diversity of the area [4]. In particular, the entry of crude oil into the Gulf effected significant shifts in the microbial community on both the sea surface and at depth, including a decrease to the microbial diversity, and a bloom in the abundance of hydrocarbonoclastic bacteria. These microorganisms are effectively the first responders in the event of a spill at sea and play a pivotal role in the biodegradation and ultimate fate of the oil.

With the recent advancements in the development of molecular and sequencing techniques, the DwH oil spill provided an unprecedented opportunity to investigate and elucidate the physiological, metabolic and ecological characteristics of hydrocarbonoclastic bacteria in the Gulf both *in situ* and under controlled laboratory conditions. Genomic analysis of the bacterial communities revealed that particular species can degrade specific hydrocarbon substrates, and this can influence overall microbial community structure [5]. Beyond this, laboratory-based research does not take full account of all the various environmental constraints that may influence hydrocarbon biodegradation, although some literature suggests that the low temperatures at depth, nutrient depletion and sunlight may have had a negative effect on the rate of biodegradation in the Gulf during the spill [6].

Another consideration that can influence the microbial oil biodegradation process is the use of chemical dispersants, which can potentially increase the rate of hydrocarbon biodegradation - this is one major reason why they are used. These chemicals are formulations that contain surfactants that work by reducing the surface tension of the oil in seawater, thereby increasing the surface area of the oil by forming smaller and smaller oil droplets to which bacterial cells adhere to and degrade the hydrocarbons within the oil faster. Chemical dispersants may also provide bioavailable nitrogen and phosphorus to bacterial communities, ensuring nutrient depletion of these nutrients does not inhibit metabolic activity [7], although nutrient depletion at depth during the DwH spill is likely to have resulted in the formation of a massive oil plume (discussed below). Furthermore, controversy still surrounds the toxicity of chemical dispersants on marine organisms and their effective use during the DwH oil spill remains a topic of discussion [8,9].

This review discusses the various aspects relating to the role microorganisms played during the DwH oil spill, including the mechanism of bacterial hydrocarbon biodegradation, and the environmental constraints that can influence this. The use of fertilizers or chemical dispersants to stimulate hydrocarbon biodegradation is also discussed.

Factors affecting the rate of hydrocarbon biodegradation

Temperature

Temperature has an influence on the rate of hydrocarbon biodegradation by affecting the chemical and physical structures of crude oil components, the growth rate of hydrocarbonoclastic bacteria, and the rate of enzyme activity [10]. Low temperatures increase the viscosity and decrease the solubility of crude oil components, increasing resistance to biodegradation [11]. However, high temperatures can increase the toxicity of hydrocarbons leading to a decreased abundance of hydrocarbonoclastic species [12]. For the majority of bacteria, the highest temperature at which hydrocarbon degradation can occur is between 30 - 40°C, with optimum biodegradation rate occurring around 15 - 20°C [10,11,13]. The broad range of hydrocarbonoclastic bacteria that can thrive in extreme environments allows certain species to metabolically degrade hydrocarbons at a range of temperatures. Psychrophilic bacteria are capable of hydrocarbon biodegradation through the production of cold-resistant enzymes. On the other hand, thermophilic species, such as *Bacillus thermoleovorans*, have been reported to degrading hydrocarbons at temperatures as high as 60°C [13,14]. Temperature has more impact on the rate of biodegradation by changing the physio-chemical properties of oil, rather than shaping the structure and functions of the microbial community [14].

Liu., *et al.* [15] investigated the effects of temperature on the biodegradation of oil by the dominant bacterial species of the DwH spill. Incubations of 4°C and 24°C selected for psychrophilic and mesophilic bacteria, respectively. *Cycloclasticus* and *Pseudoalteromonas* spp. were found to have better growth rates at 4°C. Such organisms are likely to be more efficient in degrading crude oil hydrocarbons in deeper waters where temperatures can reach 4°C or lower. Members of the genera *Alcanivorax* and *Oleibacter* have been reported to grow at temperatures of 4°C and 24°C, with highest *n*-alkane degradation rates reported at 24°C [15,16].

Nutrients

Low levels of phosphorus and nitrogen can limit the rate of hydrocarbon biodegradation by bacteria in marine ecosystems. An influx of hydrocarbons into marine waters can disrupt the *in situ* carbon, nitrogen and phosphorus ratio, and spur competition for nitrogen and phosphorus that lead to limiting growth and biodegradation rates [11]. Edwards., *et al.* [17] noted that offshore surface waters near and around the DwH spill site were oligotrophic and especially lacking in inorganic phosphorus, thus limiting metabolism and growth of microbial communities in a pulsed hydrocarbon-enriched environment [17]. Furthermore, phosphorus precipitates as calcium phosphate in seawater pH, limiting inorganic phosphate availability to bacteria [18]. Liu., *et al.* [15] also noted depletion of nutrients in surface waters oil slicks, whereas little depletion of nutrients occurred in the deeper water oil plume. This may have resulted in the observed evolution of different bacterial community responses in surface waters compared with deep plume, with members of *Pseudomonas, Alteromonas* and *Oleibacter* having became more enriched in deeper waters [15].

Salinity, pH and UV irradiation

According to Varjani., *et al.* [6], there exists a positive relationship between increasing salinity and hydrocarbon biodegradation. Some studies have shown that the addition of sodium chloride to seawater samples can increase hydrocarbon biodegradation rates [6]. However, extremely high salinity has also been shown to prevent enzyme activity and therefore inhibit biodegradation [10].

The pH of a system can also affect the rate of hydrocarbon biodegradation by affecting cell membrane transport systems and enzyme activity [10]. Vyas and Dave [19] investigated the effect of pH on hydrocarbon biodegradation by marine bacteria and found degradation to be at its highest rates at a neutral pH. The pH of most seawater is usually around pH 8.0 - 8.1, therefore it is unlikely pH is a limiting factor for hydrocarbon biodegradation in the GoM [19].

UV irradiation, from sunlight, is genotoxic to bacteria, and has been shown to enhance the toxicity of hydrocarbons towards bacterial communities. To exemplify, a study using pyrosequencing of amplified 16S rRNA genes investigating the composition of microbial communities in samples exposed to natural sunlight compared to samples maintained in the dark showed that hydrocarbon and Corexit 9500 biodegradation rates remained fairly constant in both the light and dark experiments [20]. However, different bacterial species developed in each of the light and dark experiments. This study provided evidence on the effects of UV irradiation that may explain some of the variance in the microbial community response observed in sun-lit surface waters compared to deeper plume waters in the GoM during the DwH spill.

Hydrocarbon bioavailability

Dubinsky., *et al.* [21] discussed that the main factor shaping the dominant microbial communities in the GoM was the bioavailability of certain hydrocarbons. The bioavailability of a hydrocarbon is determined by its structure, composition and the temperature of the environment [13]. The bioavailability of a hydrocarbon decreases as molecular weight increases; thus, PAHs and other HMW hydrocarbons are less bioavailable, limiting biodegradation rates. Furthermore, the longer that HMW hydrocarbons are present in the environment, the less biodegradable they become, limiting the rate of *in situ* biodegradation [22].

The relative aqueous insolubility of hydrocarbons inhibits their uptake by bacterial cells and other microorganisms. Many types of bacteria that degrade hydrocarbons are capable of producing biosurfactants. This increases the dissolution or emulsification of hydrocarbon

species that facilitates their uptake by these and other types of bacterial. Alternatively, bacteria can also produce reactive oxygen species to overcome the low reactivity of hydrocarbons [23]. Biosurfactants are surface-active molecules, usually composed of lipids which are produced by a range of hydrocarbonoclastic species. These types of molecules increase the bioavailability of hydrocarbons by reducing the surface tension between oil and water, making it easier for bacteria cells to uptake hydrocarbon molecules [13]. *Pseudomonas aeruginosa* is a well-characterised hydrocarbon-degrading species that produces biosurfactants, particularly rhamnolipids which are a type of glycolipid biosurfactant [11].

Chemical dispersants

Approximately 2.1 million gallons of the chemical dispersant Corexit 9500 was applied to surface and deep plume waters at the DwH oil spill site. A number of studies have reported that the use of chemical dispersants increased the rate of hydrocarbon biodegradation and had no negative impact on the hydrocarbonoclastic community [7,24]. On the other hand, some studies suggest that chemical dispersants are toxic to microorganisms, including to some hydrocarbonoclastic bacteria [8,25,26]. The effects of Corexit on the DwH spill site are still not fully understood. Kleindienst., *et al.* [8] assessed the impacts of dispersant on microbial communities to find there was in general, no enhancement of bacterial growth, and the biodegradation rate of *n*-hexadecane and naphthalene did not increase in the presence of dispersant. Using oligotyping to hone in more deeply into resolving the community response, the authors found a decrease in the abundance of key hydrocarbonoclastic species, predominantly members of *Marinobacter* and *Acinetobacter* spp. after the addition of the dispersant. It was also suggested this could be due to competition from other species, rather from the toxicity by the Corexit [8].

Chemical dispersants have many detrimental effects on ecosystems: their toxicity is fatal for aquatic life causing DNA damage and membrane lysis. The use of such chemicals is controlled by environmental agencies in most countries and only a few dispersants are patented for use [26]. The toxicity of mixed dispersant, seawater and crude oil is difficult to measure as the mixture exists in different states, but it has been shown that a mixture of hydrocarbons and dispersant can be more toxic in aquatic environments than the hydrocarbons alone [25]. A study by McFarlin., *et al.* [27] investigating the biodegradability of Corexit 9500 in seawater identified the biodegradable components of Corexit to be petroleum distillate and non-ionic surfactant fractions. Other fractions were found to be lost to abiotic factors. In this same study, 16S rRNA gene sequencing was used to identify the bacterial species involved in the biodegradation of Corexit and it was found that the abundance of *Colwellia* spp. increased in response to enrichment with both crude oil and Corexit, suggesting members of this genus can biodegrade the dispersant. However, Couto., *et al.* [26] assessed the effect of surfactin (a type of biosurfactant) compared to that by synthetic chemical surfactants on the structure and abundance of hydrocarbonoclastic bacteria. The authors found that surfactin enhanced hydrocarbon biodegradation, as shown by an increase in *alkB* genes, significantly more than synthetic surfactants.

Microbial response to the deepwater horizon oil spill

Microbiology of the deepwater oil plume

Recent advancements in DNA sequencing and molecular biological techniques have made it possible to generate more highly resolved genetic analysis of complex microbial communities. A number of studies used molecular techniques to investigate the abundance and functions of microorganisms in and surrounding the DwH spill site in the GoM [5,28,29]. Construction of 16S rRNA gene clone libraries uncovered the composition and relative abundance of the microbial communities within, above and below a subsurface oil plume that formed at 1200 - 1300m below the surface [30]. Other studies reporting on this were fairly consistent in their findings with respect to the bacteria that became enriched in the plume, with members of the order *Oceanospirillales* as the most abundant taxon [5,28-31].

A phylogenetic shift in species abundance was observed by June 2010, with genome analysis from the deepwater oil plume revealing other members of *Gammaproteobacteria*, *Colwellia* and *Cycloclasticus* to be the dominating species [5]. The first description of this was reported by Hazen., *et al.* [31] and further work on the dominant *Oceanospirillales* showed these organisms can degrade *n*-alkanes such

as butane and pentane, whereas *Cycloclasticus* and to an extent also *Colwellia*, generally favour utilising polycyclic aromatic hydrocarbons (PAHs) as their preferred source of carbon and energy [21]. Single-cell genome sequencing of *Colwellia* from the oil plume provided evidence that this organism was also active in the degradation of gaseous hydrocarbons such as methane [32].

Kessler., *et al.* [33] identified a bloom of methylotrophic bacteria in July 2010 during the spill, though methane in the plume was barely detectable by August 2010. Methane is a major component of crude oil, although loss of methane to the atmosphere was not apparent, suggesting that the majority of the methane was oxidised by a bloom of methylotrophic bacteria in July [33]. Joye., *et al.* [33], however, argued that the methylated waste products of a eukaryotic phytoplankton bloom was responsible for the increase in methylotrophic bacteria. The role of methylotophic bacteria was thus disputed between studies [33]. Since then, further work appears to allude that methylotrophs played a direct role in methane oxidation [34]. Redmond and Valentine [34] document that *Methylococcaceae* are involved in oxidation of methane into methanol through the enzyme methane monooxygenase, whilst *Methylophaga* further degrade methanol. During the spill, the bloom in methylotrophic bacteria coincided with most of the methane becoming oxidised [34].

Using stable isotope probing with ^{13}C-labelled *n*-hexadecane, members of the genus *Methylophaga* were found able to directly utilise *n*-hexadecane as a carbon and energy source [35]. Although, the abundance of these organisms in the water column, and more specifically in the oil plume, was much lower compared that of enriched hydrocarbonoclastic bacteria [34,35], this expanded the known substrate range for these methylotrophic organisms, which been prescribed to C1 carbon substrates. This study also noted that the bloom of methylotrophic bacteria occurred during the initial stages of the spill but remained undetected due to the dominance of other hydrocarbonoclastic bacteria [35]. Members of *Flavobacteria, Alteromonadaceae* and *Rhodobacteraceae* that were also found enriched in the plume were suggested to have been involved in metabolising the dead cellular biomass from the bloom of hydrocarbonoclastic bacteria [5].

Microbiology of oil-impacted surface waters, salt marshes and beaches

In aerobic conditions, BTEX hydrocarbons are oxidised into organic acids and then transformed into fatty acids, which are metabolized to produce methane and carbon dioxide [6]. *Pseudomonas* spp. have shown the potential to aerobically biodegrade benzene and can use it as a sole carbon source, however this process is inefficient under anaerobic conditions [36]. In 2012 a study investigated the rate of aerobic benzene degradation in saltmarshes on the Louisiana coast affected by the DwH oil. Benzene exposure acts as a natural feedback, stimulating an increase in benzene biodegradation where benzene exposure has previously occurred, resulting in a larger microbial community adapted for benzene biodegradation. Iron acts as the electron acceptor in anoxic areas of the saltmarsh, rather than oxygen. However, the presence of citrate or glucose inhibits benzene biodegradation, indicating that BTEX hydrocarbons are not the preferred carbon sources for the microbial communities in saltmarshes of the GoM affected by the DwH spill. It is likely that previous exposure to oil in these areas was rare compared to deep waters, and the bacterial communities lacked hydrocarbonoclastic species [37].

High-molecular-weight (HMW) PAHs have higher toxicity and resistance to biodegradation compared to aliphatic hydrocarbons. Kappell., *et al.* [38] found enrichment of functional genes involved in PAH-degradation after oil exposure in beach sands affected by the DwH spill. However, PAH-degradation genes were still present in non-polluted beach samples, indicating native bacterial communities in the coastal regions of the GoM quickly responded to oil intrusion [38]. PAH-degrading bacteria, particularly *Cycloclasticus*, were isolated from water samples collected in the GoM during the DwH oil spill, and also identified through stable isotope probing of plume and surface oil slicks samples. *Colwellia* spp. were also found abundant in surface oil slicks and plume samples and appeared to respond well to phenanthrene in laboratory culture. A number of other hydrocarbonoclastic taxa became enriched in surface oil slicks, predominantly *Alteromonas, Marinobacter, Pseudomonas, Pseudoalteromonas* and *Halomonas* [16,30]. These organisms were practically undetectable prior to the onset of the spill [16,30].

Response and dynamics of hydrocarbon-degrading genes

Cyclohexane, an alicyclic hydrocarbon, is one of the most recalcitrant constituents of crude oil. Microbial enzymes find it difficult to perforate and incorporate oxygen atoms into the circular structure of cycloalkanes [39]. However, through single-cell genome sequencing, Mason., *et al.* [29] reconstructed the metabolic pathway of cyclohexane degradation in two single *Oceanospirillales* cells, originally obtained from the DwH site. This study managed to map the complete pathway of cyclohexane biodegradation, indicating *Oceanospirillales* were actively biodegrading cyclohexane during the DwH oil spill [29].

A study by Lu., *et al.* [40] highlighted the significant roles of biogeochemical cycling and nutrient availability in structuring of microbial communities. These authors found that in addition to affecting the expression of hydrocarbon degradative genes, the genes involved in nutrient cycling had also increased in abundance in plume samples taken during the DwH spill when compared to samples taken from unaffected or reference waters in the GoM. Genes for iron reduction, carbon and nitrogen cycling and metal resistance were more abundant in oil plume samples. Furthermore, presence of bacteriophages, and genes involved in bacteriophage replication, were also higher in plume samples. Bacteriophages are responsible for a huge proportion of nutrient cycling in the oceans, and a higher abundance of bacteriophages can thus enhance hydrocarbon degradation by preventing nutrient depletion [40].

Hazen., *et al.* [31] used DNA microarray analysis on samples collected from the deepwater oil plume in order to identify the most abundant functional genes. Microarrays are designed with oligonucleotide sequences/probes that hybridise to complementary mRNA, which when they do the complex emits chemoluminescence that is quantified relative to abundance of the targeted gene transcripts [41]. The study found around 1600 functional genes involved in biodegradation of *n*-alkanes, cycloalkanes and aromatic hydrocarbons, confirming a rapid response to hydrocarbon intrusion into the GoM [31]. Mason., *et al.* [29] further built upon these findings, establishing that expression of the alkane-1-monooxygenase enzyme was significantly higher than PAH-degrading enzymes within plume samples. This confirmed that the chemical structure of *n*-alkanes makes them the most biodegradable component of crude oil, and that a wider range of bacterial species are capable of alkane biodegradation [29].

To investigate microbial community function, Rivers., *et al.* [42] collected metatranscriptomic data to analyse whole community transcripts in the plume and found enzymes involved in alkane degradation to be significantly more abundant in plume samples. The alkane-1-monooxygenase enzyme (*AlkB*), which is responsible for degradation of alkanes, was more abundant in plume transcriptomes compared with non-plume transcriptomes [42]. Using GeoChip microarray analysis to identify anaerobic hydrocarbon biodegradative genes within GoM coastal sediments revealed an increase in functional genes active in anaerobic processes, including denitrification, methanogenesis and sulphate reduction, confirming the presence of an active microbial community with the metabolic potential to biodegrade hydrocarbons in the absence of oxygen [43]. Interestingly, genes involved in encoding chemotactic and motility proteins were also enriched in members of the *Oceanospirillales*. As hydrocarbons are a source of energy for hydrocarbonoclastic species, they are chemo-attractants, stimulating expression of chemotaxis and aiding movement towards oil droplets [29,44].

Conclusions and Further Research

The entry of crude oil and its refined petrochemical derivatives into the marine environment can cause disastrous consequences. If it were not for the presence of hydrocarbonoclastic bacteria and other microorganisms, oil in the marine environment is likely to accumulate, and it has been suggested that over time would produce a global sea surface oil slick of several microns thick. The role of such bacteria in the biodegradation of oil hydrocarbons is, thus, fundamental, and the Deepwater Horizon oil spill was testament to this. The mass intrusion of oil into the Gulf of Mexico during this historic spill resulted in the enrichment of specific hydrocarbonoclastic bacterial taxa in sea surface oil slicks, a deepwater oil plume, and in salt marshes and beaches where the oil had reached. The oil also triggered, or enhanced, the expression of genes involved in the hydrocarbon biodegradation process. A number of factors can influence the response of these microorganisms in the event of a spill, and also to their hydrocarbon degradation. Temperature and nutrient limitations can often

have a profound effect on the rate of hydrocarbon biodegradation, leading to inhibition of enzyme expression and/or activity, whereas temperature itself can cause changes to the physicochemical properties of hydrocarbons that consequently affect their biodegradation. There is evidence that pH, salinity and sunlight exposure can influence rates of hydrocarbon biodegradation in marine environments, but further work is required to better understand this, and in particular across the many different environments that exist in the global ocean. Whilst the onset of a hydrocarbon contamination event can disrupt *in situ* nutrient concentrations, the application of fertilizers or dispersant(s) has been shown in some studies to compensate for this. The effects of chemical dispersants to marine life, including to hydrocarbonoclastic communities, remains contentious and requires more research.

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