Bioremediation of Marine Oil Spills

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Abstract

Spills of crude and fuel oils from tankers, freighters, pipelines, wells, and storage facilities into the marine environment capture the public's attention and demand prompt and environmentally sensitive response technologies. Sometimes it is possible to contain the oil with booms and collect it with skimmers or burn it, but in many cases this is impractical, and aiding natural attenuation (largely by microbial biodegradation) is all that can be done without causing further environmental damage. One approach, biostimulation, is to at least partially alleviate those factors slowing the growth of indigenous oil-degrading microbes. While an oil slick is floating or emerging from a

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wellhead, this can be done by spraying dispersants to encourage the oil to disperse as tiny droplets in the water column, dramatically enhancing the surface area for microbial colonization. If oil reaches a shoreline, biodegradation can be stimulated by carefully delivering biologically available nitrogen and phosphorus to at least partially reduce their limitation on microbial growth. These approaches of biostimulation have been successfully employed many times. The other tactic, bioaugmentation, aiming to add exogenous microbes in the hope that they will "jump-start" biodegradation has yet to be shown to be effective in real oil-spill situations and likely is unnecessary given the ubiquitous distribution of oil-degrading bacteria in the sea.

1 Introduction

Much of the world's oil production of almost 93 million barrels per day in 2014 (Energy Information Agency 2016a; 1 barrel = 42 gallons (US), 159 l) is transported by sea. The International Tanker Owners Pollution Federation (ITOPF 2016) reported that seaborne oil trade has averaged 100 trillion barrel-miles per year since 2000. Despite the best efforts of the industry, some does get into the marine environment. Fortunately the amount of oil released from oil transportation is very small as a percentage of the volume transported and is getting smaller (Etkin 2001; ITOPF 2016). Indeed, releases of petroleum from oil tankers in the 1990s amounted to only 2% of the total oil released to North American waters and 8% worldwide (National Research Council 2002) and is less today (ITOPF 2016). In contrast, natural oil seeps, the largest contributors, accounted for 47% of the oil entering the world's oceans (National Research Council 2002). These seeps have likely been active for millions of years and provided the carbon and energy source and selection pressure for the numerous oil-degrading microbial genera (Prince 2010, Prince et al. 2010, and this series), and it is these organisms that are the foundation of bioremediation strategies – and perhaps even of some fisheries (Levy and Lee 1988)!

2 Fate of Oil in the Sea

Crude oils and refined fuels are very complex mixtures that vary widely depending on their source (Tissot and Welte 1984; Robbins and Hsu 1996; USDOE 2008; Speight 2014). They contain thousands of molecular species, which are often grouped into four categories: saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes. The first two are amenable to gas chromatography, and have been intensely studied. The last two contain heteroatoms such as oxygen, sulfur, and nitrogen and are not amenable to gas chromatography. Until recently their identity was unknown, but recent developments in high resolution mass spectrometry (e.g., Kim et al. 2005; Hughey et al. 2007; Cho et al. 2015) are beginning to reveal

their composition. Commercially, crude oils are classified by their density in units of API (American Petroleum Institute) gravity, which is defined as [141.5/(specific gravity)] – 131.5 and expressed as degrees (°). Thus distilled water has an API gravity of 10°, and denser fluids will have lower API gravities. The median API gravity of crude oil processed in the USA in 2015 was 31.68 (Energy Information Agency 2016b), and this is probably typical worldwide. Almost all oils in commerce float on seawater, although some very heavy bitumen and bunker fuels may "float" beneath the surface of freshwater, as happened with the 1989 *Presidente Rivera* spill in the Delaware River (Wiltshire and Corcoran 1991).

When oils enter the oceans from seeps, urban runoff, or a spill, they become subject to a variety of processes collectively termed "weathering" (Prince et al. 2003b). The actual weathering processes are highly dependent on environmental factors (Lee et al. 2015). Almost all oils float, allowing the smallest molecules to evaporate (Betancourt et al. 2005; Fingas 2013). These molecules are either photochemically degraded (e.g., Hamilton et al. 2003; Wu et al. 2014) or washed from the atmosphere in rain and then biodegraded (e.g., Arzayus et al. 2001; Castro-Jiménez et al. 2012; Chen et al. 2016). Evaporation is usually limited to molecules with less than about 15 carbon atoms, so evaporation is the likely fate of most of a gasoline spill at sea, three-quarters or more of a diesel spill, and perhaps 20–40% of a typical crude oil. Heavy fuels such as marine Bunker fuels do not contain a significant volatile fraction.

Aliphatic hydrocarbons are almost insoluble in water, but small aromatics, particularly the notorious BTEX (benzene, toluene, ethylbenzene, and the xylenes) and small polar molecules such as naphthenic acids dissolve and leave the bulk oil (Lafargue and Le Thiez 1996; Wang et al. 2016b) – their eventual fate is biodegradation (Prince et al. 2007).

Two disparate processes compete when water and oil mix; water may become entrained in the oil to form an emulsion or mousse, as eventually happens at most spills (Fingas and Fieldhouse 2012), or oil can disperse into the water column as a suspension of small droplets, as happened during the 1993 *Braer* spill off the Shetland Islands that occurred in a major storm (Thomas and Lunel 1993). Mousses are thought to be the precursors of tarballs, which can last for decades (Goodman 2003; Warnock et al. 2015). As we shall see below, chemical dispersants that break emulsions and stimulate the natural dispersion process are effective tools in the oil spill response "toolkit."

Oil also interacts with small mineral particles in a process termed "Oil-Mineral Fines Interactions" (Owens and Lee 2003; Wang et al. 2013). Like dispersion, this dramatically increases the surface area of the oil and stimulates biodegradation (Weise et al. 1999).

Aromatic hydrocarbons, particularly the larger and more alkylated ones, can be photochemically oxidized (Garrett et al. 1998; Aeppli et al. 2012), converting them to polar and probably polymerized species. Since light cannot penetrate very far into a dark oil slick, photooxidation has little effect on the bulk properties of spilled oil, but it may be important in generating a polymerized "skin" that stabilizes tarballs and "pavements" on beaches. Layers of immobile, hardened oil and sediment,

termed pavements, form when oil reaches a shoreline as a heavy, thick slick. Oil becomes trapped in the sediment, and the oil and the sediment become saturated with each other (Owens et al. 2008). Oil incorporated into such pavements is effectively preserved from weathering processes until this heavy, solidified material is physically disrupted, so a major goal of spill cleanup operations is to prevent the formation of pavements.

3 Eventual Fate of Spilled Oil

The weathering processes described above distribute and change the oil in various ways, but they do not actually remove oil from the environment. Only two processes, combustion and biodegradation, actually eliminate oil by converting it to carbon dioxide and water. Some spills do accidentally ignite, as happened in the 1991 *Haven* spill in the Mediterranean (Martinelli et al. 1995) where some 70% of the cargo was destroyed in the fire. Deliberate ignition is an accepted response option in some situations, such as that of the wood chip carrier *New Carissa* off the coast of Oregon in 1999 (Gallagher et al. 2001). Oil on water needs to be several mm thick to sustain combustion and in general that requires corralling in a boom, preferably a fire-proof one. Under optimal conditions, burning may consume >90% of oil so contained, but there is usually only a small window of opportunity for success (Buist 2003). Burning boomed oil was a significant response to the 2010 *Deepwater Horizon* blowout (Allen et al. 2011).

Far more generally, it is biodegradation that removes oil from the environment. As mentioned above, a diverse group of microorganisms (Prince 2010; Prince et al. 2010) has evolved to degrade hydrocarbons, and many are able to grow on hydrocarbons as their sole source of carbon. They are ubiquitous and obviously very effective, since they have been consuming the vast majority of the oil entering the world's oceans from natural seeps for millions of years (600,000 t, 700 million liters per year; National Research Council 2002). Nevertheless, the biodegradability depends on the oil; McMillen et al. (1995) examined the short-term biodegradability of 17 crude oils in soil microcosms and found that >61% of the most degradable oil (46° API) was lost in 4 weeks, while only 10% of the least degradable oil (15° API) was consumed under the same conditions. Nevertheless, further degradation occurred on a longer timescale and the literature reports biodegradation potentials as high as 97% for particularly light oils (Prince 1993). The rate of biodegradation depends on the oil composition, with the saturate fraction typically being more rapidly biodegradable than the aromatic fraction (e.g., Sugiura et al. 1997). In a very neat experiment, Uraizee et al. (1998) showed that the amount of asphaltenes (e.g., Sirota 2005; McKenna et al. 2013) in the oil affected the biodegradation of the hydrocarbons, progressively inhibiting biodegradation as their weight fraction increased. Thus the biodegradation of the complex mixtures that compose crude oils and refined product is complicated and shows clear preferences for some molecules before others (Prince and Walters 2016). Nevertheless it is a robust process that has been active for millions of years. The focus of this chapter is the

different approaches that have been taken to stimulate this biodegradation to clean up oils spilled in the marine environment.

4 Bioremediation by Alleviating Limiting Factors – Biostimulation

As we have seen above, crude oils and refined fuels are very biodegradable, but they are unusual carbon sources for microorganisms for at least two reasons. For one thing, they are essentially insoluble, so the surface area for microbial colonization and consumption is likely to limit biodegradation. For another, they provide energy without providing the nitrogen and phosphorus required for life, so availability of these, and perhaps other nutrients, is likely to limit degradation. Biostimulation protocols have focused on alleviating these two distinct limitations; the former while the spill is floating as a slick and the latter if oil reaches a shoreline.

4.1 Dispersants

Dispersants are mixtures of surfactants in a hydrocarbon solvent (e.g., Nalco 2014) that together aid the dispersion of oil into water as <100 μm neutrally buoyant droplets. They can do this even if the oil has begun to absorb water en route to becoming a mousse (Lessard and DeMarco 2000; National Research Council 2005). Once applied to the oil, the mixing action of waves and currents both generates the droplets and distributes them into the water column. This usually reduces oil concentrations so that droplets do not coalesce to floating sheens, and allows dilution to the sub ppm level within a day (Cormack and Nichols 1977; McAuliffe et al. 1980; Lichtenthaler and Daling 1983; Prince 2015). Such levels are below levels of acute concern for marine life (Gardiner et al. 2013; Incardona et al. 2013; Adams et al. 2014) and provide a very large surface area for microbial colonization and biodegradation (Prince and Butler 2014; Prince 2015). The rate and effectiveness of the dispersion process depends on the nature of the spilled oil (its API gravity and viscosity, degree of evaporation, extent of emulsification, pour point, etc.), the ability of the dispersant formulation to mix with the oil, and the sea conditions (Lessard and DeMarco 2000), but most oils can be dispersed with enough dispersant and enough energy (typical application rates are five gallons per acre – about 50 l per hectare). It is important to recognize that the effectiveness tests for dispersants mandated by the USEPA for listing on the *National Oil and Hazardous Substances Pollution Contingency Plan Product Schedule* (USEPA 2014), while suitable for purpose in discriminating between good and poor dispersants, substantially underestimate efficacy in the field and in wave tanks. To pass the test, dispersants must disperse at least 45% of Prudhoe Bay or South Louisiana crude oil in a standard "swirling flask" test (USEPA 2006). But the swirling energy used in the test is insignificant compared to even mild turbulence at sea. Tests in the OHMSETT facility, a 200 m wave tank in New Jersey that is 20 m wide and 2.5 m deep, routinely measure

dispersant efficiencies >95%, even at low temperatures with ice in the water (Belore et al. 2009), and the wave energy in that facility pales before the energy in even mild sea states.

An important environmental consideration is assessing the trade-off between intentionally exposing water column organisms to dispersed oil and the often significant effects of unrecovered oil left to drift to potentially strand on a shoreline. In most cases, these considerations suggest a net environmental benefit to the use of dispersants because the short-term, transient exposure of water column communities has much less ecological effect than the prolonged, and often widespread, contamination of oil reaching shorelines (McCay and Graham 2014; Prince 2015; Bejarano and Mearns 2015).

Dispersed oil droplets have only a transient lifetime because their large surface to volume ratio provides a much increased colonizing substrate for oil-degrading bacteria. Furthermore, their dispersion in the water column allows the low levels of biologically available nitrogen and phosphorus in the ocean to support bacterial degradation and maintain a viable degrader community (Swannell and Daniel 1999; Venosa and Holder 2007; Prince et al. 2013; McFarlin et al. 2014). Laboratory studies have shown that oil-degrading microbes colonize the droplets within 2–4 days, and a full heterotrophic community of oil, bacteria, protozoa, and even nematodes soon follows (MacNaughton et al. 2003). Furthermore, the chemical composition of some dispersants may enhance the initial rate of oil degradation because they serve as initial substrates for nascent bacterial growth (Varadaraj et al. 1995; Swannell and Daniel 1999). Dispersant formulations have been proposed that combine dispersion with the delivery of nutrients for microbial growth (Gatellier et al. 1983; Lepain and Bronchart 1986), but these are not currently commercially available and are probably unnecessary given the enormous dilution that occurs with effective dispersion. A persistent myth is that dispersants are unusually toxic, and that nontoxic formulations could be developed. In fact, dispersants such as Corexit 9500 are made from materials "generally regarded as safe for food contact" (Nalco 2014), and their inherent toxicity to marine organisms is lower than that of common dishwashing products (Word et al. 2013): the toxicity of dispersant-enhanced oil dispersions is due to the dispersed oil, not the dispersant (Hemmer et al. 2011).

Dispersants are stockpiled around the world (e.g., Marine Spill Response Corporation 2016), and many regulatory agencies recognize their use as an effective tool (Chapman et al. 2007). The largest use of dispersants to date occurred following the BP *Deepwater Horizon* accident. On April 20, 2010, high-pressure oil and gas escaped from BP's *Deepwater Horizon* exploratory well in Mississippi Canyon Block 252 located 77 km offshore. In the subsequent fire and explosions, 11 men tragically lost their lives. The *Deepwater Horizon* drilling rig burned and 2 days later sank in 1500 m. The blowout prevention device (BOP) at the wellhead and all the emergency shut-off equipment failed.

Upon sinking, the 21 in. (53 cm) riser pipe from the wellhead to the drilling platform collapsed onto the sea floor. Oil leaked from multiple locations along the riser pipe and the top of the BOP. In all, it took 84 days to stop the flow of oil. The oil

from this well is typical of light Louisiana crude from petroleum reservoirs more than 5000 m deep; it has an API gravity of 35.2 (The Federal Interagency Solutions Group: Oil Budget Calculator Science and Engineering Team 2010).

The BP *Deepwater Horizon* oil release was much larger and vastly different from the *Exxon Valdez* spill (Atlas and Hazen 2011). The actual volume of oil and gas released from the Deepwater Horizon well is difficult to determine, but a US Federal Judge (Barbier 2015) adjudicated that 3.19 million barrels of oil entered the water (134 million US gallons, 507 million liters). This is slightly smaller than (admittedly uncertain) assessments of the 1979 spill from the IXTOC-I well blowout in the Bay of Campeche (Jernelöv and Lindén 1981), estimated at 147 million gallons (556 million liters). Both are dwarfed by the deliberate release of oil into the Arabian Gulf in 1991 (250–450 million gallons, Gupta et al. 1993), which in turn was only a small part of the estimated >50 billion gallons of oil that spewed out of Kuwaiti oil wells destroyed by Iraqi troops as they withdrew (Husain 1995).

One of the strategies employed to defray the environmental and safety impact of the oil from the *Deepwater Horizon* was to inject the dispersant COREXIT 9500 directly at the wellhead (and end of the riser pipe before that was disconnected) at a water depth of 1500 m. The goal was to disperse the oil at depth, thereby preventing large slicks from forming at the surface directly above the wellhead where many ships were gathered to stop the leak, and to prevent the oil from impacting the shoreline (Rorick et al. 2012). The EPA established a rigorous, daily water sampling program, once it was demonstrated in early May that less oil was coming to the surface immediately above the wellhead within 4 h of injecting COREXIT 9500 at the wellhead, making it safer for leak operations.

As a result of dispersant addition and the forceful physical injection of the oil in the deepwater of the Gulf of Mexico, fine droplets of dispersed oil formed and moved away from the wellhead in the water column. Droplets between 10 and 60 μm are neutrally buoyant and were picked up by the current between 900 and 1300 m. Fluorescence measurements indicated that these finely dispersed oil droplets moved predominantly to the Southwest at a depth of approximately 1100 m (Camilli et al. 2010). While this was often referred to as a "plume," its concentration of <1 ppm oil (Wade et al. 2016) was invisible to the human eye, although readily detected by sensitive fluorometers. Larger droplets moved to the surface and formed surface slicks, some of which moved toward the shorelines. Surface applications of dispersant were used to break up these slicks to protect ecologically and commercially sensitive shorelines.

Almost immediately, Hazen et al. (2010) reported that there was rapid biodegradation of saturated hydrocarbons in the finely dispersed oil within the deep water even though temperatures were about 5 °C. The average half-life of C_{13}–C_{26} alkanes from four different field samples and two different lab microcosm assays was 3 days (Hazen et al. 2010). During the release (April–July), concentrations of polynuclear aromatic hydrocarbons also decreased rapidly with distance from the release point (the wellhead) and were seen to reach <1.0 ppb within 15–20 mi (24–32 km) in all directions other than to the southwest, where a small number of samples exceeded 1 ppb out to 40 mi (64 km) (Boehm et al. 2016).

The <1 ppm "deep sea plume of oil" was associated with an oxygen dip indicative of ongoing hydrocarbon biodegradation, and substantial enrichment of indigenous oil degrading microorganisms (*Colwelliaceae, Pseudomonas, Cycloclasticus, Oceanospirillales,* and *Pseudoalteromonas*) was detected in association with the deep plume of oil (Hazen et al. 2010; Baelum et al. 2012; Dubinsky et al. 2013). Measured half-lives for $n\mathrm{C}_{13}$–$n\mathrm{C}_{26}$ alkanes ranged from 1.6–9.5 days (Hazen et al. 2010). The available data also shows that bacterial communities evolved sequential capabilities to degrade *n*-alkanes and aromatic hydrocarbons (Dubinsky et al. 2013; Valentine et al. 2012). Valentine et al. (2012) postulated that autoinoculation of oil degrading bacteria occurred as oil circulated around the wellhead before moving to the Southwest.

In a laboratory simulation using dispersed Macondo oil and Norwegian seawater collected below the thermocline in Trondheim fjord, Brakstad et al. (2015b) studied the biodegradation of dispersed oil at two different droplet sizes (10 μm or 30 μm median oil droplets) at a nominal concentration of 2 ppm oil. There was a clear correlation between oil biodegradation and bacterial succession, the latter dominated by *Gammaproteobacteria*. While *Oceanospirillales* and *Colwellia* were associated with *n*-alkane biodegradation, the later abundances of *Cycloclasticus, Marinobacter, Pseudomonas,* and *Pelagibacter* corresponded with degradation of aromatic HCs, and possibly complex alkanes. The enrichment of *Pelagibacter* at the end of the experiment hinted at a return to baseline conditions. The dispersions with the larger oil droplet distribution (30 μm) showed subtly slower biodegradation of some of the oil hydrocarbons than in the smaller droplet dispersion (10 μm), and this was reflected in delayed appearances of some bacterial groups, mainly associated with biodegradation of aromatic HCs. Several of the bacterial groups enriched during biodegradation of the Macondo oil in Norwegian seawater were also associated with oil biodegradation in the deepwater plume from the *Deepwater Horizon* accident and in laboratory studies with Gulf of Mexico deepwater. However, there were subtly different patterns of bacterial successions during biodegradation when compared to Gulf of Mexico water, suggesting differences between different geographical localities, depths, and environmental conditions.

A rather similar study with suspended droplets (Wang et al. 2016a) used water collected at depth near the wellhead and showed that most hydrocarbons were effectively degraded within 64 days. Some compounds exhibited significant lag times, but once this was completed, the half-lives of biodegradation showed good agreement with the alkane ($n\mathrm{C}_{13}$–C_{26}) half-lives of 0.6–9.5 days reported for in situ biodegradation from the *Deepwater Horizon* plume samples and laboratory efforts to recreate in situ conditions (Hazen et al. 2010). The results also matched other chemically dispersed oil studies conducted at low temperatures (−1–8 °C), in which *n*-alkane half-lives ranged from 2 to 10 days while median PAHs half-lives ranged from 2 to 37 days (Siron et al. 1995; Venosa and Holder 2007; McFarlin et al. 2014; Prince et al. 2013).

As the dilute and finely dispersed oil and gas moved away from the *Deepwater Horizon* wellhead, the microbial community exhibited a dynamic successional response (Kimes et al. 2013; King et al. 2015). *Gammaproteobacteria* appear to

have been especially important hydrocarbon degraders that became enriched in the path of the oil and gas. Metagenome analyses showed that bacteria of the order *Oceanospirillales*, which are capable of alkane degradation, were dominant early (Mason et al. 2012). PhyloChip and 16S sequence analysis showed that the *Oceanospirillales* was subsequently replaced by *Cycloclasticus* and *Colwellia* that are capable of degrading aromatic and gaseous saturated hydrocarbons (Hazen et al. 2010; Valentine et al. 2010; Baelum et al. 2012; Redmond and Valentine 2012; Dubinsky et al. 2013), followed by methylotrophic bacteria (Kessler et al. 2011).

Studies that evaluated metabolic potential using protein-coding sequence microarrays (GeoChip) indicated the enrichment of genes involved in both aerobic and anaerobic hydrocarbon degradation in the plume (Lu et al. 2012). Metagenome and metatranscriptome sequence data showed that *Oceanospirillales* were actively involved in aerobic hydrocarbon degradation in May 2010 and that genes for enzymes involved in aerobic alkane degradation were expressed at relatively high levels in the path of the plume (Mason et al. 2012).

4.2 Fertilizers

The necessity of biologically available nitrogen and phosphorus for culturing hydrocarbon degrading microbes has been known for more than 65 years (e.g., Bushnell and Haas 1941), and exploiting the phenomenon to stimulate oil biodegradation dates back more than 40 years (e.g., Davis and Raymond 1964). The use of fertilizers for remediating oil spills at sea was first suggested by Atlas and Bartha (1972), who recognized that the fertilizers would have to stay with the oil for maximal effectiveness. They suggested using paraffinized urea and dioctylpyrophosphate as oleophilic sources of nitrogen and phosphorus, respectively (Bartha and Atlas 1976). Since then there has been a lot of research to test and improve this hypothesis, much of it reviewed in Prince (1993), Swannell et al. (1996), Lee and Merlin (1999), Prince and Atlas (2005), and Head et al. (2006).

Although Atlas and Bartha's initial experiments were aimed at floating oil slicks, the major use of oleophilic and slow release fertilizers has been in stimulating the biodegradation of beached oil. By far the largest use was in the response to the 1989 spill from the *Exxon Valdez*, where about 15% of the 12,600 km shoreline of Prince William Sound and the Gulf of Alaska became oiled to some degree (Galt et al. 1991; Harrison 1991). Most of the oiled beaches consisted of coarse gravel, sometimes protected by boulder armor. The primary treatment of heavily oiled shorelines was washing with cold or warm seawater, without surfactants, and collection of the liberated oil with skimmers (Nauman 1991). Then these shorelines, together with those that were lightly oiled and needed no prior treatment, were treated with fertilizers: an oleophilic liquid product designed to adhere to oil, known as Inipol EAP22 (Ladousse and Tramier 1991) for surface oil, and a slow-release encapsulated agricultural product known as Customblen to deliver nutrients to subsurface oil (Pritchard et al. 1992). Inipol EAP22 was a microemulsion with an internal aqueous solution of urea in an external oil phase of oleic acid and trilaureth-4-phosphate,

co-solubilized by butoxy-ethanol (Gautier et al. 1984). It contained 7.4% nitrogen and 0.7% phosphorus by weight and was applied with airless paint sprayers transported on small pontoon catamarans. Customblen was a high-quality agricultural fertilizer designed to release its nutrients into water percolating the shorelines over several weeks. It consisted primarily of ammonium nitrate, calcium phosphate, and ammonium phosphate, encased in polymerized linseed oil. Customblen contained 28% nitrogen and 3.5% phosphorus by weight and was applied with broadcast spreaders by workers walking the beaches.

More than 250,000 l of Inipol EAP22 was applied in 1989 and about half that in 1990 when there was much less surface oil and bioremediation was the principal cleanup technique. In addition, more than 16 t of Customblen were applied in 1989, and >50 t were applied in 1990 when the residual oil was principally beneath the very surface of the beaches. Small amounts of both fertilizers were applied in 1991 to the patches of residual oil, and the cleanup was declared complete by the Alaska and Federal governments in 1991. Almost 50 t of biologically available nitrogen had been delivered to the oiled shorelines in the 3 years (Prince and Bragg 1997), with no detectable adverse environmental impact.

The bioremediation operations were studied in detail by a team from Exxon, USEPA, and the Alaska Department of Environmental Conservation (ADEC). Careful monitoring of three representative shorelines demonstrated that fertilizer applications were generally successful at delivering nitrogen nutrients throughout the oiled sediment (Prince et al. 1994b). Within days, the indigenous microbial populations responded by increasing their oxygen consumption and their ability to mineralize radiolabeled hydrocarbons in laboratory assays (Lindstrom et al. 1991). And changes in the oil composition on the beaches showed that the rate of biodegradation had been stimulated up to fivefold (Bragg et al. 1994; Prince et al. 1994b). Environmental monitoring and toxicity testing showed that this stimulation was achieved with no detectable adverse environmental impact (Prince et al. 1994b).

The rates of oil biodegradation (determined by loss of hydrocarbons with respect to hopane used as an internal marker within the oil; Prince et al. 1994a), based on field measurements from April 1989–May 1990 at three sites on Knight Island, ranged from ~0.6 to 3.4 g oil/kg sediment per year for surface oil and ~0.3–3.6 g oil/kg sediment per year for subsurface oil (Bragg et al. 1994). This equated to a mean loss in the mass of residual oil of about 31% per year for surface oil and 12% per year for subsurface oil. Oil removal by biodegradation contributed to the total rate of oil removed from shorelines by all factors (physical cleanup, waves, storms, etc.), which amounted to 75–90% of the oil from 1989 through the winter of 1990 (Koons and Jahns 1992; Wolfe et al. 1994).

While much of the oil was biodegraded, some did become sequestered in subsurface sediments where there was little or no water flow (Short et al. 2007). Boufadel and colleagues proposed trying to bioremediate the residual subsurface oil by using high pressure injection of nutrient enriched water (Boufadel and Bobo 2011), and Venosa et al. (2010) performed microcosm studies that showed that additional biodegradation would occur if enriched water did reach the sequestered

oil. However, Pope et al. (2013) argued that water would more likely flow around the sequestered oil and would not deliver the necessary oxygen and nutrients. Therefore, Atlas and Bragg (2009, 2013) argued that this was a case where bioremediation would be ineffective and unnecessary since the oil was sequestered and not in contact with living biota.

The next use of bioremediation on a real spill seems to have been the work of Rosenberg and colleagues on a 1994 spill of heavy crude oil on a sandy beach north of Haifa, Israel (Rosenberg et al. 1996). They were concerned that approaches such as those used in Alaska did not deliver the nitrogen fertilizer specifically to the oil-degrading microbes, so they developed an enrichment culture of oil-degrading microbes able to get their nitrogen from polymerized formaldehyde-urea resin (Rosenberg et al. 1996; Rosenberg and Ron 1998). The spill of several hundred tons of crude oil contaminated a large amount of sand, and 30,000 m^2 were treated with a mixed bacterial culture plus their polymerized fertilizer and tilled twice a week. Overall, the treatment apparently resulted in the degradation of 88% of the oil after 4 months, during which time there was virtually no change in the oil concentration of a control plot that was tilled in the same way. Unfortunately no chemical analyses were done on the residual oil to confirm that the loss was due to biodegradation rather than physical loss, perhaps due to surfactants produced by the organisms, but the results appeared very promising. Later this group favored the use of uric acid as an insoluble lipophilic fertilizer (Koren et al. 2003), perhaps even in the form of guano (Knezevic et al. 2006), and showed that one of the more abundant hydrocarbon degraders, *Alcanivorax* (e.g., Cappello et al. 2007b; Harayama et al. 2004), is able to use uric acid as a nitrogen source.

Although bioremediation by adding fertilizers played an important role in the *Exxon Valdez* response, this success has not been followed by widespread use. This can be attributed to the fact that many spills get little if any cleanup, while those that do are treated with dispersants that prevent serious shoreline oiling. Furthermore, many high-visibility spills are near beaches that are amenable to rapid physical cleanup. For example, the vast majority of the oil from the 1996 *Sea Empress* spill was dispersed at sea or collected when it landed on sandy beaches (Colcomb et al. 1997; Lunel et al. 1997; Law and Kelly 2004). Swannell et al. (1999) did a small field trial with soluble fertilizer applied once a week, and a slow-release fertilizer held in bags, on a small cobble beach, and showed that both stimulated biodegradation without any detectable adverse environmental impact. Nevertheless, the technique was not used on a larger scale.

Bioremediation was also used on small experimental scales following the 2002 spill of very heavy fuel oil (API gravity of 11°) from the *Prestige*. Jiménez et al. (2006) showed that an oleophilic fertilizer rather similar to Inipol EAP22 significantly enhanced the biodegradation rate of high molecular weight *n*-alkanes, alkylcyclohexanes, and benzenes when applied to oil that had been on a cobble beach for 10 months, even though the experiment was done over the winter of 2003–2004. In contrast, Fernández-Álvarez et al. (2006) found no beneficial effects

of adding soluble fertilizer or bacterial cultures, although their site seems to have contained more weathered oil than that of Jiménez et al. (2006). But in any case, cleanup from this spill relied principally on physical removal because the heavy oil mousse did not penetrate into most beaches (Fernández-Álvarez et al. 2006).

There have, however, been many field trials on experimental oil spills, and the experiments of Lee and colleagues in Canada, Sveum and colleagues in Norway, and Swannell and colleagues in the UK have been reviewed in detail elsewhere (Prince 1993; Lee and Merlin 1999; Swannell et al. 1996; Prince and Atlas 2005; Head et al. 2006). More recent trials include the work of Venosa et al. (1996) on a beach in the Delaware estuary, Prince et al. (2003a) on a shoreline in Spitsbergen, Maki et al. (2003) on a beach in the Sea of Japan, Xu et al. (2005) on a beach in Singapore, and Mills et al. (2004) in a wetland in Texas. All demonstrated that nutrient addition stimulated the rate of biodegradation of at least some components of their oils by severalfold – quite in line with the field data from the *Exxon Valdez* spill (Bragg et al. 1994).

In contrast, Oudot et al. (1998) found no stimulation when they did ostensibly similar experiments in the Bay of Brest, which they attributed to high background levels of nutrients throughout their test site. Indeed, background levels were also rather high in the Delaware Bay (Venosa et al. 1996), and the stimulation of biodegradation by fertilizer was correspondingly small. This demonstrates that there is no point in undertaking a nutrient bioaugmentation program if lack of nutrients is not significantly limiting biodegradation. On the other hand, both these experiments involved small amounts of oil, so the requirement for nutrients at the sites was only a small fraction of that naturally available. It is not unreasonable to imagine that a large spill at those sites would deplete the ecosystem of nutrients, and that nutrient addition might then have a markedly positive effect.

Similarly, Venosa et al. (2002) saw very little stimulation of biodegradation in a nutrient amended freshwater wetland on the St. Lawrence River, Canada, and Tate et al. (2012) saw little stimulation of biodegradation by nutrients in a *Spartina alterniflora* dominated Louisiana salt marsh. Here it seems likely that oxygen rather than nutrients was the major intrinsic limitation on biodegradation.

When fertilizers are to be applied, the question arises “How much?” This is not a trivial issue, since excess nutrients washing off a shoreline might stimulate algal growth and even be toxic to invertebrates and fish. Several groups have suggested aiming for a nominal fertilizer nitrogen to oil carbon ratio of about 1:10, but this is a fruitless pursuit since oil concentrations typically follow log-normal distribution on a shoreline (see Limpert et al. 2001). To avoid potentially deleterious effects, it is appropriate to aim for nutrient levels near 100 μM biologically available nitrogen in the interstitial water of the oiled sediment (Prince et al. 1994b; Boufadel et al. 1999; Prince and Atlas 2005), which can be measured with simple hand-held colorimetric tests on site as appropriate (Prince et al. 2003a). No adverse effects have been seen at such levels (see Prince and Atlas 2005). The precise form of nitrogen does not seem to be very important – as discussed above, ammonium, nitrate, urea, uric acid, and polymerized forms have all been used with success.

5 Bioremediation by Adding Oil-Degrading Microbes – Bioaugmentation

The publicity around the first patented genetically engineered organism (Chakrabarty 1981), which could indeed degrade hydrocarbons, has led many to imagine that bioremediation means adding genetically enhanced bacteria to stimulate biodegradation. But in fact there have been very few cases where bacteria have been added in order to stimulate oil spill cleanup, and those few cases have used naturally occurring strains.

The first well-publicized use of added bacteria was on the 1990 spill from the *Mega Borg* in the Gulf of Mexico (Holden 1990, and see Leveille 1991), but nothing beyond anecdotal results were ever reported. It is only fair to note, however, that the logistical problems involved in monitoring the treatment were not settled before the spill dispersed.

A more recent use of added bacteria has been on the 1997 spill of Medium Fuel Oil from the *Nakhodka* in the Sea of Japan (Tsutsumi et al. 2000), but the only analyses reported in the paper are image analyses showing that the very heavy oil left the treated rocks – to what extent this was due to biodegradation is unknown.

Laboratory experiments have not been more optimistic. Indeed the most insightful demonstration was performed by the USEPA when testing potential inoculants for stimulating oil biodegradation in Alaska following the *Exxon Valdez* oil spill (Venosa et al. 1992). Eight microbial inocula were tested in small laboratory reactors that allowed substantial degradation of oil by the indigenous organisms of Prince William Sound; all eight potential inoculants had a greater stimulatory effect on alkane degradation if they were autoclaved prior to addition. This suggests that the indigenous organisms readily out-competed the added products but that autoclaving the products released some trace nutrient that was able to stimulate the growth of the endogenous organisms. Nevertheless, tests continue (e.g., Neralla and Weaver 1997; Tam and Wong 2008; Bao et al. 2012).

Putting aside the huge technological challenge of promptly adding a substantial number of viable and thriving organisms to a significant spill, the fundamental basis of bioaugmentation may rest on erroneous preconceptions. It makes the assumption that hydrocarbon-degrading microbes are relatively rare in the environment and so will be slow to colonize a spill. But there is no evidence this is true. Rather it seems that hydrocarbon-degraders are ubiquitous but typically limited by the availability of substrate. Thus when a spill occurs, they rapidly increase in numbers, indeed they "bloom." Actual quantitation is fraught with experimental biases, not least because it is widely believed that only 1% or less of bacteria seen in environmental samples with a microscope can be cultured. Nevertheless, experiments that use consistent methods can give comparative results even if absolute numbers may be underestimates. Perhaps the most thorough sampling to date was done by Braddock et al. (1995) following the *Exxon Valdez* oil spill in Prince William Sound Alaska. Total bacteria were counted using epifluorescence microscopy (i.e., without culturing) while oil-degrading organisms were counted with a dilution method that detects organisms

by their ability to grow and disrupt a floating sheen of oil (Brown and Braddock 1990). Oil-degrading microorganisms defined in this way made up only about 0.002% of the total bacterial population, detected microscopically, on beaches outside the path of the spill, but 2% of the population on oiled shorelines. Thus the presence of oil was correlated with a thousand-fold increase in the population of culturable oil-degrading microbes. These changes can occur within a few days – Kasai et al. (2002) and Cappello et al. (2007a) showed that well-known hydrocarbon degraders, such as *Cycloclasticus* and *Alcanivorax,* were detected within days in oiled mesocosms flushed with seawater and that these numbers increased, as did their activity, when fertilizers were present. And these organisms do appear to be ubiquitous (Yakimov et al. 2007). Interestingly, blooms of oil-degrading organisms occur even following spills of very heavy oils not usually thought to be prime targets for bioremediation, such as the spill from the *Nakhodka* (Maruyama et al. 2003), indicating that even these heavy oils can lift the carbon limitation of oil-degrading microbes in the sea.

Data from the *Deepwater Horizon* blowout add further support to the notion that oil-degrading microbes are ubiquitous and ready to respond rapidly if oil enters the environment. Molecular data indicated that offshore sediments close to the wellhead became enriched in hydrocarbon degrading bacteria (Kimes et al. 2013). Single-cell sequencing revealed that surficial deep-sea sediments (0–1 cm) had elevated hydrocarbon concentrations and high populations of *Gammaproteobacteria*, including a strain of *Colwellia* sp. that had metabolic potential to degrade a wide range of hydrocarbons (Mason et al. 2014). Metagenomic analysis and qPCR-targeted functional gene assays of subsurface (1.5–3 cm) deep-sea sediment cores from September to October 2010 revealed increased levels of Deltaproteobacteria (which include anaerobic hydrocarbon-degrading sulfate reducers) and genes associated with the anaerobic degradation of aliphatic and aromatic hydrocarbons (e.g., benzoyl-CoA reductase genes and *bssA* and *assA* which encode benzyl- and alkyl succinate synthase, respectively) in the sediments located within 3 km of the wellhead (Kimes et al. 2013). Fully concordant with these microbial populations, the residual oil in these deep-sea sediments was biodegraded (Stout and Payne 2016).

Just as in the gravel shores of Prince William Sound (Lindstrom et al. 1991; Braddock et al. 1995), the arrival of oil from the *Deepwater Horizon* on sandy shorelines resulted in significant increases in oil-degrading microbes, especially *Gammaproteobacteria* (Kostka et al. 2011; Newton et al. 2013; Lamendella et al. 2014; Kappell et al. 2014; Rodriguez-R et al. 2015) and fungi (Bik et al. 2012). The hydrocarbon-utilizing bacterium *Desulfococcus oleovorans* comprised over 50% of the microbial community in heavily oil-contaminated anaerobic marsh sediments (Atlas et al. 2015).

6 Conclusions

Biodegradation is the ultimate fate of the majority of the hydrocarbon that enters the marine environment, so stimulating this biodegradation is an environmentally responsible approach to minimizing the environmental impact of oil spills if it can

be done with net environmental benefit (API 2013). But it is essential that the intervention address the factors actually limiting biodegradation (Atlas and Bragg 2009).

Dispersants are the optimal tool for this approach, for dispersed oil has a "half-life" by biodegradation of a couple of weeks or so (Hazen et al. 2010; Baelum et al. 2012; Prince et al. 2013, 2016; Prince and Butler 2014; Brakstad et al. 2015a; Wang et al. 2016a). This can be compared to a lifetime of months to years if oil reaches a shoreline and is not completely collected (Bragg et al. 1994; Aeppli et al. 2014). Dispersants must be used promptly, however, for their efficacy decreases as oil evaporates and incorporates water (Lessard and DeMarco 2000). This is principally a function of dispersant incorporation into the oil slick, since penetration becomes more difficult as oil viscosity increases (Canevari 1985). Once dispersants are incorporated, even in calm conditions, they will aid dispersion when rougher weather arrives (Lewis et al. 2010). Insistence that dispersants be seen to be effective on a trial basis before large-scale application can begin may thus hinder effective response.

Timely and effective oil dispersion should minimize, or even eliminate oil stranding. But some accidents occur so close to shore that oil will reach the shoreline and most likely require some physical cleanup, perhaps involving bioremediation. Again it is essential that bioremediation protocols address the factors actually limiting biodegradation (Atlas and Bragg 2009). For example, there is little point in adding nitrogenous nutrients to a site where such nutrients are not the fundamental limitation, either because there is already a sufficient supply (e.g., Oudot et al. 1998; Venosa et al. 1996) or because something else, such as oxygen, is the likely limiting factor (e.g., Venosa et al. 2002; Tate et al. 2012). To date there have been no successful demonstrations of the stimulation of biodegradation of oil under anaerobic conditions (e.g., Mills et al. 2004). Microbial degradation clearly occurs in such environments (Mahmoudi et al. 2013; Looper et al. 2013; Atlas et al. 2015), but no environmentally benign approach to stimulating the process has yet been achieved.

Beyond that, the composition of the oil must be borne in mind. For example, it is generally accepted that bioremediation is not a very useful technology for very heavy oils, such as those from the *Nakhodka* (Tsutsumi et al. 2000), *Erika* (Oudot 2000), or the *Prestige* (Díez et al. 2005), since only a small fraction of such oils will be biodegraded in a reasonably short timeframe. In the *Erika* case, only 11% of the oil was degraded in 80 days under what were thought to be optimal conditions. As discussed above, this is enough to cause a microbial bloom (Maruyama et al. 2003) but not enough to warrant consideration as a remediation approach unless the environmental impact of the most degradable fraction is a particular local concern. Similar considerations apply to the weathered residua of oils that were initially more degradable. For example, Díez et al. (2005) demonstrated that the rate of biodegradation of the three and four ring aromatic compounds in *Prestige* cargo oil decreased substantially after all the *n*-alkanes had been degraded, even in the presence of nutrients, and Venosa et al. (1996) saw a similar effect in their field trial on the Delaware Bay, as did Wrenn et al. (2006) in continuous flow microcosms. This may be a reflection of co-metabolism (Kanaly and Harayama 2000), or perhaps the fact that alkane degraders such as *Alcanivorax* grow much faster than aromatic degraders

such as *Cycloclasticus* (Kasai et al. 2002; Yakimov et al. 2007). In either case, while it does not indicate that biodegradation has ceased, it does suggest that the system is unlikely to be fertilizer limited once the alkanes are degraded (Atlas and Bragg 2009).

The biodegradation of oil pavements (Owens et al. 2008) is also unlikely to be nutrient limited. As discussed above, a photochemically polymerized surface layer likely protects encased hydrocarbons from microbial attack. But a thin coating of oil on sediment in a nutrient poor area, for example, one not subject to anthropogenic run-off, would be a prime target for nutrient assisted bioremediation.

In conclusion, bioremediation by the use of dispersants or fertilizers epitomizes modern environmental thinking, working with natural processes to remedy an accident in an environmentally responsible way. By its very nature, bioremediation addresses the most bioavailable hydrocarbons first, thereby minimizing exposure to higher trophic levels. Neither dispersants nor fertilizers are the panaceas that have sometimes been portrayed, but they are important tools in reducing the ecological impact of many oil spills (Canadian Coast Guard 1995; Owens 1996; NOAA 2016). In some cases, they are essentially the only approach for treating marine oil spills effectively.

7 Research Needs

There are currently no reliable bioremediation treatments for anaerobic environments, such as marsh sediments.

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