Bioremediation of petroleum hydrocarbon contaminants in marine habitats

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Bioremediation is being increasingly seen as an effective, environmentally benign treatment for shorelines contaminated as a result of marine oil spills. Despite a relatively long history of research on oil-spill bioremediation, it remains an essentially empirical technology and many of the factors that control bioremediation have yet to be adequately understood. Nutrient amendment is a widely accepted practice in oil-spill bioremediation but there is scant understanding of the systematic effects of nutrient amendment on biodegradative microbial populations or the progress of bioremediation. Recent laboratory and field research suggests that resource-ratio theory may provide a theoretical framework that explains the effects of nutrient amendment on indigenous microbial populations. In particular, the theory has been invoked to explain recent observations that nutrient levels, and their relative concentration, influence the composition of hydrocarbon-degrading microbial populations. This in turn influences the biodegradation rate of aliphatic and aromatic hydrocarbons. If such results are confirmed in the field, then it may be possible to use this theoretical framework to select bioremediation treatments that specifically encourage the rapid destruction of the most toxic components of complex pollutant mixtures.

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Introduction

The marine environment is subject to contamination by organic pollutants from a variety of sources. Organic contamination results from uncontrolled releases from manufacturing and refining installations, spillages during transportation, direct discharge from effluent treatment plants and run-off from terrestrial sources. In quantitative terms, crude oil is one of the most important organic pollutants in marine environments and it has been estimated that worldwide somewhere between 1.7 and 8.8×10^6 tons of petroleum hydrocarbons impact marine waters and estuaries annually [1]. Large oil spills, such as the *Exxon Valdez* and *Sea Empress* incidents, invariably capture media attention but such events are relatively rare; however, a substantial number of smaller releases of petroleum hydrocarbons occur regularly in coastal waters. Around the coast of the UK alone, between the years of 1986 and 1996, 6,845 oil spills were reported. Of these, 1,497 occurred in environmentally sensitive areas or were of sufficient magnitude to require clean-up [2]. As a consequence of the importance of oil spills relative to other sources of organic contaminants in the marine environment, there is a large body of research on oil-spill bioremediation. Furthermore, studies of oiled shorelines have been far more numerous than open water studies, which have often been equivocal [3,4]. Bioremediation of beached oil will therefore form the basis of this article.

It has been known for many years that the major constituents of most crude oils are biodegradable [4,5•] and that their biodegradation in the marine environment is often limited by the availability of nutrients, such as nitrogen and phosphorus [6]. As a result, application of these mineral nutrients to spilled oil has been used to enhance the biodegradation of crude oil following spills on a number of occasions [3,7–9] and experimental studies have been conducted to establish the efficacy of this approach in the field [10–12]. Nonetheless, many issues remain unresolved with regard to bioremediation of oil spills. For example, can an understanding of the effect of bioremediation strategies on indigenous microbial populations be used to select for microorganisms that most rapidly biodegrade the toxic contaminants in oil and can this knowledge, coupled with a greater understanding of how abiotic factors affect bioremediation, permit the development of a predictive framework for bioremediation? Furthermore, can recent improvements in our understanding of anaerobic hydrocarbon catabolism be used to improve and extend the applicability of bioremediation treatments used in the field?

Of course, there are many other issues surrounding the application of oil-spill bioremediation but we have chosen to focus on these two recent developments, that are likely to affect how the technology is practised in the future.

Optimising bioremediation performance

Although there is evidence that bioremediation can be used to treat oil-contaminated shorelines effectively, an important limitation of the technology is the difficulty in formulating treatment strategies that will produce a specified outcome in terms of degradation rate and residual contaminant concentration [13]. This is in part a product of the empirical development of bioremediation. For example, the amount of nutrients (principally N and P) applied to spilled oil may be based on consideration of the amount of N and P required to convert a given amount of hydrocarbon to carbon dioxide, water and microbial biomass under oxic conditions, or from the concentration of nutrients shown to support maximal growth rates of alkane-degraders in culture [12]. It has also been suggested that the amount of slow-release fertilizer applied to a beach should be 'as much as possible without exceeding toxic concentrations of ammonia and/or nitrate' [14] or that nutrient addition should be sufficient to cause a detectable increase in the N and P content of interstitial water, thus ensuring that N and P are not limiting the microbial population [3]. The last two approaches have the advantage that a consideration of the oil concentration, which is often highly variable on oiled beaches, is not required. Nonetheless, this approach almost inevitably results in the addition of more nutrients than is strictly necessary [9]. Clearly, there is little consensus on how to best optimise nutrient amendment but typically values of around 1–5% N by weight of oil have been used with a ratio of N : P between 5 and 10:1 [3].

The dominance of empiricism in bioremediation studies, and biotreatment in general, stems from the fact that the systems are complex and knowledge of how indigenous microbial populations respond to environmental perturbations is somewhat limited. As a result, most treatment strategies are formulated *ad hoc* and rely on data from laboratory or microcosm biodegradation feasibility studies. Consequently a theoretical basis to explain the behaviour of microorganisms in the environment in response to specific stimuli, a prerequisite for objectively 'bioengineering' environmental systems, has not been well developed; however, one study has recently addressed the applicability of resource-ratio theory [15] to hydrocarbon biodegradation [16••].

Resource-ratio theory relates the structure and function of biological communities to competition for growth-limiting resources. When the quantitative requirements for a limiting resource (i.e. the concentration of a limiting resource that supports zero net growth) and the growth and death rate of different competing organisms are known, resource-ratio theory offers the possibility to predict the outcome of such interactions [15,16••]. The manifestation of resource-ratio theory can be seen clearly in chemostat culture experiments where the outcome of competition between two bacterial species for the same growth-limiting resource can be determined from the maximum specific growth rates and half saturation constants of the bacteria growing on a shared substrate [15,17]. Resource-ratio theory is essentially an extension of this.

As it is widely accepted that the underlying principle of hydrocarbon bioremediation under oxic conditions is increasing the supply of limiting nutrients (N and P) to stimulate indigenous hydrocarbon-degrading microorganisms (which will compete for the added nutrients), resource-ratio theory may be applicable to the development of bioremediation beyond its current empirical status. Using the context of resource-ratio theory, it may be possible to devise bioremediation treatments objectively, by imposing conditions that select for the microorganisms most fit to remove the contaminants of greatest environmental concern, at the optimal rate with minimum intervention. For example, because different organisms have different requirements for N and P, provision of these nutrients at different concentrations (both absolute and relative to each other) will select for the organisms most able to utilise the nutrients at the levels provided in the oiled habitat. Thus, addition of N and P at different concentrations should select for different groups of autochthonous organisms. If in addition to differential nutrient requirements the organisms selected degrade contaminants at different rates, it should be possible to supply nutrients at a concentration that selects for the most effective hydrocarbon degraders. A consideration of resource-ratio theory in bioremediation could thus provide a more sophisticated way of manipulating the indigenous microbiota to bring about rapid removal of toxic organic contaminants from many types of polluted habitat.

Although this may be true, is there any evidence that manipulating the supply of N and P results in the selection of different microbial populations? The fit of empirical observations to the predictions of resource-ratio theory, that is, that changes in the ratio of N and P supplied should alter biodegradation rates and concomitant with this changes in the microbial populations should occur as a result of competitive interactions, has recently been examined [16••]. This study demonstrated that the degradation rate of hexadecane and phenanthrene was influenced not only by the absolute amount of nutrient added but also by the relative supply of N and P, which had dramatic effects on biodegradation rate. One of the most interesting observations made was that there were two distinct optima for phenanthrene biodegradation corresponding to a supply ratio of N : P of 5 : 1 and 20 : 1. This implied either that, under these conditions, two different phenanthrene-degrading populations with different nutrient requirements were selected or that different catabolic pathways with different kinetic properties were induced in the same population. Nonetheless, at N and P supply ratios between these two extremes the phenanthrene-degrading activity selected mineralised the contaminant at a reduced rate. Also of note was the fact that the optimum N and P supply ratio was different for phenanthrene and hexadecane, thus a different nutrient supply may be required to achieve optimum degradation of particular components of crude oil. A limited analysis of the bacterial populations selected at different N : P supply ratios indicated that different bacteria were selected depending on the nutrient supply regimen [16••].

Furthermore, a recent field study of oil-spill bioremediation that employed molecular biological methods targeting small subunit ribosomal RNA genes to follow changes in bacterial populations, revealed that the bacterial populations selected in oiled beach plots experiencing ambient levels of N and P were different from those selected in oiled plots amended with additional nutrients [18]. Moreover, aliphatic hydrocarbon degradation was stimulated in the nutrient-amended plots, whereas aromatic hydrocarbon degradation was not [10,18]. A field study conducted on the shoreline contaminated during the *Sea Empress* incident also showed that the addition of N and P significantly stimulated the decomposition of the aliphatic hydrocarbons above controls, but the biodegradation of aromatics was not affected [9]. These field data are also consistent with the predictions of resource-ratio theory, in that specific catabolic activities appear to be preferentially selected in response to amendment with a particular combination of nutrients, rather than an overall stimulation of all catabolic activities. Different nutrient supply optima for the degradation of aliphatic and aromatic hydrocarbons may also explain other data where variations in the predominance of aromatic and aliphatic hydrocarbon degradation have been observed [19–22], particularly as the phenomenon was found to be nutrient concentration-dependent in several cases.

Although these studies provide support for the applicability of resource-ratio theory to oil-spill bioremediation, the evidence is currently at best circumstantial and the relevance of the theory to oil-spill bioremediation remains to be thoroughly tested. Clearly, if resource-ratio theory is to be of practical use in bioremediation it should be able to explain observed patterns of biodegradation and ultimately have utility in predicting the course of biodegradation under specific circumstances.

The fundamental basis of the theory is that the outcome of competition between microbial species will be determined by the steady-state concentration of a growth-limiting resource at which the per capita growth rate of the bacterial population balances the per capita death rate. Thus, to be of use in a predictive manner, such parameters must be known for the different species that comprise the microbial population. In most situations, this requires that the organisms be obtained in pure culture and the resource concentration at which growth balances death must be determined. This is not a trivial undertaking as experimental determination of the substrate concentration at which zero net growth occurs is not straightforward and predictions of this substrate concentration based on kinetic parameters derived from chemostat cultures or resting cell suspensions can deviate considerably from measured values [23]. In principle, if we can determine these values for a large number of cultures that represent the bulk of species selected in response to spilled oil, or if populations with particular kinetic properties (e.g. K_s and μmax) are systematically selected under particular nutrient supply conditions, then it may be possible to use this information coupled with the characterisation of the predominant hydrocarbon degraders present at the site of a spill to inform the optimal nutrient amendment strategy.

Defining the amount of such data required to cover most eventualities, however, is likely to be a difficult task and it may be some time before resource-ratio theory has an impact on operational bioremediation. Furthermore, in oiled beaches, microbial biomass is lost due to predation and physical removal by wave and tidal action; such factors must be considered in addition to the death-rate of cells when extrapolating resource-ratio theory to field conditions. In the interim, it has been suggested that phenomena that can be explained in terms of resource-ratio theory might aid in identifying optimal conditions to promote the degradation of different classes of contaminant [16••]. This is essentially an extension of empirical optimisation methods, with the exception that the response of hydrocarbon degradation to a wider range of nutrient supply combinations would be examined. Although this will involve extensive lab experimentation prior to commencing a bioremediation treatment, it may, with time, reveal systematic response patterns engendered by specific nutrient treatments. Potentially, this information can be used to more objectively inform future bioremediation strategies. For example, it will be possible to determine if the occurrence of multiple hydrocarbon-degradation optima observed with different nutrient supply combinations [16••] is a common phenomenon and whether particular levels and supply ratios of nutrients are routinely associated with degradation maxima.

There are also rather fundamental practical issues to consider. Although it may be possible to identify the nutrient supply combination required to give a desired outcome, it may be less simple to implement these requirements in heterogeneous field settings where indigenous nutrient levels and hydrocarbon loading may be highly variable. Furthermore, factors other than nutrient supply affect biodegradation [22]. Any model developed to predict the course of a bioremediation experiment must consider these factors. A model incorporating the effects of temperature, water availability and gas diffusion on hydrocarbon degradation has been developed [24]. Laboratory incubations with simple respirometric measures of hydrocarbon degradation based on CO_2 production and O_2 consumption were used to determine the key parameters in the model. Not only did the output of the model agree well with real data obtained from larger scale experiments conducted in lysimeters, but the differences in lab incubations relative to field experiments could be explained in terms of the environmental parameters used in the model. Such an approach, linked to optimisation of nutrient delivery based on the principles of resource-ratio theory, may offer the possibility of improving the efficiency of bioremediation treatments in the field, and allow far better prediction of the duration of treatment required to attain a specific outcome.

Another important factor in determining if nutrients must be added to stimulate biodegradation is identification of the nutrient limitation status of the indigenous microbial population. Nutrient concentrations measured using wet chemical or instrumental methods may not necessarily reflect the bioavailability of nutrients; however, it is possible to identify nutrient limitation by the analysis of genes expressed in response to nutrient starvation. For example, specific proteins expressed in response to phosphate limitation have been identified in *Pseudomonas fluorescens*, cyanobacteria and *Thiobacillus ferrooxidans,* and immunological approaches have been used to detect their expression in individual cells [25–27]. Detection of such proteins or their mRNA [28] in natural populations of bacteria at the site of oil spills could provide a means to monitor changes in nutrient limitation status in response to bioremediation treatments. To be most effective, a marker of nutrient stress common to the majority of bacteria would need to be identified. The immunological targets used to date have been somewhat specific [27] and, thus, may be of limited value in determining the nutrient limitation status of diverse microbial assemblages. Identification of more universal genetic markers would also offer the possibility that reverse transcriptase-polymerase chain reaction, cloning and sequencing of nutrient limitation-induced mRNA species could allow identification of the particular components of the microbial population that were subject to nutrient limitation at any particular point in time and space.

Significance of anaerobic hydrocarbon degradation

The majority of bioremediation strategies aimed at ameliorating marine oil spills assume that the principal mechanism of hydrocarbon removal is aerobic respiration. Whereas this may be valid for oil spills on coarse pebble or cobble shorelines oxygen availability is likely to assume greater importance in beaches with fine-grained sediments, such as mudflats or saltmarshes. Furthermore, addition of urea and ammonia-based fertilisers sometimes used for oil-spill bioremediation can potentially exert an oxygen demand due to biological ammonia oxidation. On fine sediment beaches, mass transfer of oxygen may not be sufficient to replenish oxygen consumed by microbial metabolism, though penetration of oil into deeper sediment layers is also likely to be reduced in fine sediments. Under such conditions anaerobic hydrocarbon degradation may be of relevance.

Recent years have seen a growing interest in anaerobic hydrocarbon metabolism [29] and there is a growing realisation that anaerobic hydrocarbon metabolism may be an important process in contaminated anoxic environments [30••,31•]. Not only have bacteria been isolated that appear to be better adapted to growth on low molecular weight aromatic hydrocarbons at low oxygen tensions [32] but also denitrifying, iron-, manganese- and sulfate-reducing bacteria have been isolated that have the ability to degrade simple aromatic or aliphatic hydrocarbons under anoxic conditions [33–37]. Degradation of more complex aromatic compounds, such as polycyclic aromatic hydrocarbons, has been confirmed only recently but no pure cultures of bacteria have been isolated with this ability [30••].

In marine environments, the most important terminal electron-accepting processes are iron, manganese and sulfate reduction [38], and the limited number of studies to date indicate that the process of anaerobic hydrocarbon degradation in marine environments is associated primarily with sulfate reduction [30••,31•]. In contrast to terrestrial and freshwater environments, nitrate was not observed to stimulate hydrocarbon degradation. These studies reported both alkane and polycyclic aromatic hydrocarbon degradation under anoxic conditions but the rate of anaerobic hydrocarbon degradation was generally lower [30••,31•] than equivalent aerobic degradation rates (e.g. see [14]). Nonetheless, removal of the contaminant hydrocarbons was just as extensive under anoxic conditions [30••,31•] and substantial degradation of high molecular weight *n*-alkanes and the isoprenoid hydrocarbons pristane and phytane was also observed [31•].

Anaerobic catabolism of aliphatic and aromatic hydrocarbons has been demonstrated in a very small number of marine sediments, but it was apparent that previous exposure to high levels of petroleum hydrocarbons was an important factor in determining the rate of anaerobic mineralisation [30••]. Anaerobic hydrocarbon catabolism in relatively pristine environments, therefore, may be of limited significance. Interestingly, it has been noted that inoculation of sediments with low anaerobic hydrocarbon-degrading activity, with high-activity sediment samples stimulated anaerobic hydrocarbon degradation significantly [30••]. This prompted the authors to suggest that such a strategy could be used to initiate anaerobic treatment of hydrocarbon-contaminated anoxic sediments. This could prove valuable in environments where aerobic hydrocarbon degradation is limited due to poor oxygen penetration into contaminated sediments and where enhancement of oxygen mass transfer by mechanical means may be inappropriate.

Bioaugmentation has usually proved ineffective in stimulating degradation of petroleum hydrocarbon contamination [3,4]. If limited anaerobic degradation of hydrocarbons in relatively uncontaminated marine sediments proves to be a common occurrence, and is demonstrated to result from the absence of significant populations of indigenous anaerobic hydrocarbon-degrading microorganisms rather than other abiotic factors, augmentation of freshly contaminated sediments with active sediment may be considered as a viable bioremediation strategy. In contrast, treatment with anaerobic hydrocarbon-degrading isolates is unlikely to be successful for the same reasons that aerobic inoculants for hydrocarbon degradation have had somewhat limited success (e.g. see [12]). As with all bioremediation treatments, the net environmental benefit of this approach would need to be verified in the field.

Conclusions

Despite the growing acceptance of bioremediation as a means to treat spilled oil in marine environments the mechanisms that promote the process under field conditions remain poorly constrained. Although general statements can be made regarding the enhancement of biodegradation by nutrient amendment, there is no consensus on how to best optimise nutrient additions. Subsequently, oil spill treatment strategies are largely developed empirically from previous experience and/or from laboratory feasibility studies. Introduction of a theoretical framework to explain observations from primarily empirical studies of oil-spill bioremediation would be a fundamental step towards the development of more objective spill management practises. Resource-ratio theory has recently been put forward as a theoretical basis to explain some of the effects of bioremediation and many of the observations made in bioremediation studies are consistent with the theory's predictions. Although the introduction of this theory may simply augment current empirical approaches, in the longer term it has the potential to form the basis of more predictable bioremediation strategies, and the introduction of theory to the field of bioremediation is an important progression. To further test the applicability of resource-ratio theory it will be necessary to conduct systematic studies on the effect of different nutrient amendments on bacterial populations and concomitant alterations in biodegradation rates, to identify patterns of microbial diversity associated with optimum contaminant removal. Until recently, such an approach would not have been possible due to the limitations of the methods available to characterise the composition of microbial communities. With the introduction of molecular methods to study indigenous microorganisms, this limitation has been alleviated to some extent. Integrated studies combining careful field evaluation of crude oil biodegradation with molecular approaches to study microbial populations involved in degradation of spilled oil have already begun [18,39] and promise to reveal much regarding the relationship between microbial population structure and the progress of bioremediation.

Anaerobic hydrocarbon degradation in marine environments has only recently been widely accepted and there is a need to determine both how widespread an occurrence this is and in what circumstances it will have a significant impact on the dissipation of crude oil contamination. The environmental factors that promote the process must also be identified if it is to be exploited for the treatment of spilled oil.

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