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Field scale molecular analysis for the monitoring of bacterial community structures during on-site diesel bioremediation

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ABSTRACT

A diesel contaminated groundwater site was surveyed using 16S rRNA gene based analyses to investigate the effect of bioaugmentation on the bacterial communities present. The analyses included the use of denaturing gradient gel electrophoresis (DGGE) to profile microbial community structure and the construction and sequencing of clone libraries in order to identify the organisms present. Community analyses revealed a high degree of similarity in the inoculated compartments during bioaugmentation, not observed once inoculation had ceased. However, it was also shown that there was very little community similarity between the inoculum and the inoculated samples. Instead, the similarity seen during the application of the bioaugmentation treatment was thought to be due to nutrient addition applied along with the inoculum. Furthermore, once the bioaugmentation treatment had ceased the communities around the site became more diverse, suggesting that the hierarchical structure seen during treatment was due to the stimulation of a group of opportunistic indigenous organisms by the nutrients added. The findings not only highlight the importance of monitoring the fate of inocula used in bioaugmentation but also how crucial the process of the selection of species and the culture conditions used in the construction of these consortia.

1. Introduction

The possibility that microorganisms could provide a cheap and efficient solution to the problem of polluted environments has led to the development of so called 'green technologies' for the remediation of waste and the clean-up of contaminated environments. Of the many approaches now available, bioaugmentation used directly at the contaminated site is one of the most widely applied technologies (Chapelle, 1999; Bouchez et al., 2000). The controversial method involves the introduction of pollutant-degrading microorganisms to a site in order to enhance the degradative capabilities of the indigenous microbial populations by increasing the rate and/or extent of pollutant biodegradation (Vogel, 1996; Vogel and Walter, 2001). However, the effectiveness of this approach is still open to question (Bouchez et al., 2000; Thompson et al., 2005). Principally, the survival and efficiency of the pre-cultured inocula, as well as their associated community effects upon the indigenous microbiota, still remain to be established. Many studies have shown that degrading organisms did not successfully colonise the polluted environments to which they were introduced due to biotic (Heynen et al., 1988; Huws et al., 2005) as well as abiotic factors (Evans et al., 1993; Backman et al., 2004). The approach has been further hampered by poor detection strategies for the monitoring of added inocula which tend to identify only a small fraction of the actual community present.

In recent years the use of molecular methods has been increasingly employed in the study of microbial community structure. It is thought that only a tiny fraction, between 0.1% and 10%, of the microbial community is culturable in the laboratory (Amann et al., 1995; Head et al., 1998). Molecular profiling methods, many of which exploit the conserved and variable regions of the bacterial 16S rRNA gene, produce rapid phylogenetic surveys of the microbial population present (Head et al., 1998; Griffiths et al., 2000). Techniques such as denaturing gradient gel electrophoresis (DGGE), the analytical separation of DNA fragments of near-identical length based upon their sequence composition (Myers et al., 1987; Muyzer et al., 1993) have now become widely used in investigations of microbial community structure and function. These methods are now being applied to the investigation of community composition across geographic locations, over time, down the pollution gradient and under various treatments (Whiteley and Bailey, 2000; Griffiths et al., 2003).

In the present study, a field scale analysis of a recirculating pump system incorporating bioaugmentation and processing

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600,000 l of groundwater daily was carried out. In order to analyse the influence of the treatment on bacterial communities, 16S rRNA gene based community analyses were performed on samples taken from around the site during the treatment and once it had ceased. The aim of the study was to use molecular methods to detect microbial community shifts which may have occurred as a result of the application of the remediation strategy and to ascertain whether the bioaugmentation treatment had an impact upon the diversity of the site community. The initial hypothesis was that successful bioaugmentation would lead to the colonisation of the field treatment system and then be detected by community profiling techniques. The site community was profiled spatially during the application of the remediation treatment and after it had ceased using DGGE followed by the construction and subsequent sequence analysis of clone libraries in order to identify the key microbial taxa present at the study site.

2. Methods

2.1. Field site and bioaugmentation treatment system

The study site was situated on an undisclosed oilrig building and maintenance site in the UK where a remediation company, ERS Ltd. (http://www.ersremediation.com/) had set up a recirculating pump system in order to remediate a large scale diesel fuel spill. The system consisted of nine groundwater well pumps from which water was pumped into a number of treatment tanks where any silt and sludge were removed and fuel was skimmed off the surface (Fig. 1). The water was then pumped into the "final tank", where the addition of a diesel degrading inoculum and nutrients took place. Finally, the water was pumped to four "infiltration galleries" situated around the site, reintroducing it into the ground. The inoculum and nutrients were also added at the infiltration galleries. A simplified schematic of the treatment system is shown in Fig. 1. The volume of water pumped around the system was 600,000 l per day. Approximately 500 l of diesel were physically skimmed off and recovered from the contaminated water daily throughout the sampling period.

The bioaugmentation treatment involved the application of a diesel degrading multispecies inoculum following a series of batch culture enrichments performed on indigenous organisms which were designed and prepared by ERS Ltd. Briefly, the enrichments were performed using a minimal medium and site-derived diesel (at 20,000 ppm) and carried out over three weeks. The enrichments were then transferred into a 20 l bioreactor which was used to inoculate a much larger 1000 l site reactor. This site reactor was aerated and diesel fuel concentration was maintained at approximately 20,000 ppm and 25 °C. About one week later, the site reactor was topped up by the addition of a further 1000 l of site-derived groundwater amended with nutrients. This was cultured for a further 2–5 days prior to discharge. A full re-inoculation of the 1000 l site reactor using the initial 20 l culture was undertaken approximately every four to six weeks.

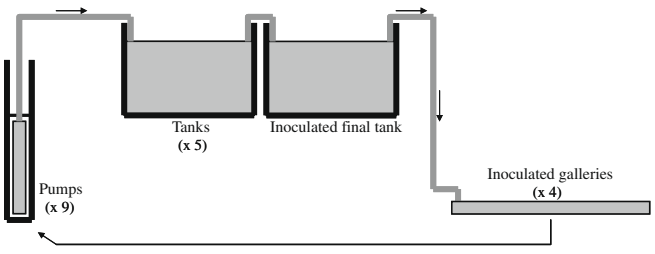

Fig. 1. Schematic of the site groundwater treatment system.

The inoculum was added at a rate of 2000–2500 ml/h. Along with inoculum addition, fertiliser (19:4:4 N:P:K) was added at a rate of 750–1000 ml/h. The inoculum and nutrients were added at two points in the system; the final tank and the infiltration galleries. The augmentation treatment ran for approximately six months. No inoculum or nutrients were added at any point in the remediation system when the bioaugmentation treatment had ceased. Both of the samples were taken from the site during the same season and the recovery of diesel fuel was uniform throughout this period. As a result, the assumption was that abiotic conditions at the site were uniform during the sampling period.

2.2. Sample collection

Two sample sets were taken from the study site: the first during bioaugmentation treatment and the second once the treatment had ceased. The bioaugmentation treatment had been running for over four months when the first sample was taken. The addition of the inoculum to the treatment system had ceased for six weeks when the second sample was taken.

Three sample compartments were identified around the site and within the remediation system as shown in Fig. 1. The three compartments consisted of the pumps, the tanks and the galleries. The pump compartment consisted of nine pump samples. The tank compartment consisted of five treatment tank samples. The galleries compartment consisted of four gallery samples and one final tank sample. The final tank was grouped within the gallery compartment because the inoculum and nutrients were added at each of these sites. The three sample set compartments were used as biological replicates of the conditions/treatments in the pumps, tanks and galleries in the analysis. One pump sample which was taken during the bioaugmentation treatment was not available during the second sampling period. A sample of water was also taken from the nearby dock and included in the community analysis.

The vast majority of samples consisted of contaminated groundwater, but for certain compartments, namely the tanks, the samples also included microbial flocs and some sludge.

2.3. Total nucleic acid extraction

Nucleic acid extractions were performed on water samples by filtering 250 ml of groundwater through 0.22 μm pore nitrocellulose membranes (Millipore) and extracting directly from them. For sludge and floc samples, 0.5 ml were collected in a microfuge tube and used directly in the protocol. Total nucleic acids were extracted from all samples according to the protocol of Griffiths et al. (2000) using a bead beating DNA extraction method.

2.4. 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE) analyses

DGGE analyses were carried out following the methods detailed by Griffiths et al. (2000). The primers used were GC356F 5′-CGC CCG CCG CGC CCC CGC CCC GGC CCG CCG CCC CCG CCC ACT CCT ACG GGA GGC AGC-3′ and 519R (5′-GTA TTA CCG CGG CTG CTG-3′). The reaction conditions consisted of an initial set of 95 °C for 120 s, followed by 33 cycles of 95 °C for 60 s, 60 °C for 45 s, and 72 °C from 90 s. This was followed by a final step of 72 °C for 30 s. DNA was amplified in a final volume of 50 μl using a final concentration of 2.5 U Taq DNA polymerase (Sigma), 1.5 mM Mg^{2+}, 1 pM of each oligonucleotide, and 200 μM of each deoxynucleoside triphosphate. The amplified products were then run onto a 10% (wt./vol.) acrylamide gel containing a denaturant gradient of 30–60%. The gels were visualised and digitised using the VersaDoc imaging system (BioRad, USA). The resultant gel images were analysed for band presence and position using the Phoretix gel