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Article *in* Water Science & Technology · February 2002

DOI: 10.2166/wst.2002.0116 · Source: PubMed

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Oil bio-degradation in permeable pavements by microbial communities

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Abstract This paper reports on continuing research at Coventry University into the improvement of highway water quality following flow through a permeable pavement. Such pavements have been shown elsewhere to be efficient in-situ bio-reactors, capable of degrading large quantities of clean motor oil. Further laboratory research, reported here, demonstrates that a commercially obtained oil degrading, microbial mixture was not significantly better at degrading clean motor oil than the indigenous microbial biomass established within the pavement over a 4-year period, when provided with an adequate nutrient supply. Scanning electron microscopy has been used to monitor biofilm development, which has also identified that the pavement has developed a complex community structure with high bio-diversity.

Keywords Bio-degradation; biofilm; microbial diversity; permeable pavement; scanning electron microscopy

Water Science and Technology **Vol 45 No 7 pp 51–56** © IWA Publishing 2002

Introduction

Research into water quality improvements achieved by bio-degradation, when oil contaminated inflow passes through a permeable pavement structure (PPS), has been previously reported (Pratt *et al.*, 1996; Pratt *et al.*, 1999; Bond *et al.*, 1999). This paper reports continuing research at Coventry University, now in its fourth year, based upon a laboratory, full-scale model of a PPS.

An essential component of the bio-degradation process is the microbial community. Micro-organisms convert hydrocarbons to CO_2 and water in the presence of moisture, given sufficient nutrients, oxygen and time (Atlas, 1981). During previous research, a commercially obtained oil-degrading microbial mixture, Biotreat HD, obtained from Biologix Ltd UK, had been used to degrade clean motor oil washed through with artificial rain.

The research described in this paper had as one of its aims to assess the nature and biodiversity of the microbial fauna found within the PPS rig, after four years of near-continuous oil and rain inputs. This population was then compared with the commercial, oil-degrading inoculum originally applied to the rig. If an alternative, stable and successful oil indigenous culture was established, this might remove the need for inoculation in the field.

Apparatus and methods

Experimental arrangement

The research was carried out on the same PPS structure as previously described (Pratt *et al.*, 1996; Bond *et al.*, 1999). Three sizes of laboratory rig were used, with proportional oil and rainfall regimes (see Table 1). Ten of the medium sized rigs were either inoculated with 22.4ml of Biotreat HD, oil-degrading microbial inoculum, after diluting 1:20 (rigs no. 1,3,5,7 and 9); or were not inoculated (rigs no. 2,4,6,8 and 10); with rig no.11 functioning as a control (no inoculum, oil or nutrients). Four of the small rigs received 3.1 ml of Biotreat HD, with the remaining four rigs not inoculated. The large rig had been originally

Table 1 Rig types and experimental regimes

Rig type	Number of rigs	Dimensions ($w \times d \times h$,mm)	Rainfall (ml per week)	Oil application (g per week)
Large	1	600×600×780	4242	6.6
Medium	11	220×220×520	380	0.59
Small	8	113×113×270	52	0.08

inoculated with this microbial mixture four years previously and no further application was made: the slow-release nutrients were renewed on Day 1180.

The large rig, 10 of the medium rigs and all the small rigs received an application of 18 g, 1.6 g and 0.22 g, respectively, of slow release fertiliser (Osmocote Plus, made by Scotts Europe B.V., The Netherlands). Artificial rainfall in the form of distilled water droplets was applied to the PPS by means of a purpose-built, rain-maker (Andersen *et al.*, 1999). The rainfall regime for the large, medium and small rigs comprised of two rainfall events per week, at an intensity of 1.6 mm/h, each delivering 2121 ml, 190 ml and 26 ml, respectively. Both the oil and rain were applied in proportion to the rig volume as a means of establishing similar environmental conditions in the small, medium and large rigs. Oil was applied to the pavement surfaces by the use of an oil dripper, which simulated the leakage of a vehicle engine. Glass funnels at the base of the rigs, with taps and bungs, allowed the collection of rig effluent following rain and oil application.

Chemical and microbiological tests

Oil and grease analysis was performed to the American Society for Testing and Materials method D 3921-85 (ASTM, 1985). The method involved liquid-liquid extraction of the effluent in carbon tetrachloride and analysis of the CH_2 and CH_3 bond absorbance on an infra-red spectrometer. Average recovery was found to be 85% and results have been recovery-corrected.

At the end of a test, samples of geotextile, 1 kg of the granite and pea-gravel substrate and a 0.5 kg sample of crushed concrete surfacing block were Soxhlet extracted for total oil and grease in carbon tetrachloride and analysed as stated. Total viable count of bacteria was performed by staining a 1 ml sample with acridine orange fluorecent dye on black, 13 mm diameter, 0.2 µm millipore filters. Living or viable cells were distinguished from dead, non-viable cells by a red /orange rather than green colour after examination under a Nikon fluorescent microscope: the results expressed as colony-forming units (CFU) per ml.

Microbial activity was assessed by measuring the hydrolysis of a non-fluorescent dye, fluorescein diacetate (FDA), to a fluorescent by-product, fluorescein, which is turned fluorescent yellow by the activity of microbial enzymes (Lundgren, 1981, Schnurer and Rosswall, 1982). This colour change is quantifiable from readings on an ordinary spectrophotometer set at 490 nm. By comparing the absorbance values from the rig effluents, it was possible to assess the relative activities of micro-organisms under various PPS rig treatments. A gas analyser was used to monitor the O_2 consumption and CO_2 evolution within the large and medium rigs, which indicated microbial activity and was expressed as delta values.

Samples for analysis by scanning electron microscopy were prepared by embedding SEM stubs into the geotextile layer with geotextile fabric stretched across their surface. The small rigs, in which the stubs were located, were subjected to oil and rainfall application for 8 months and then samples were removed and analysed on a Jeol 9000 Scanning Electron Microscope equipped with variable vacuum. Enumeration of protozoa was performed by Haemocytometer direct counts, and identification to genus level by the use of a

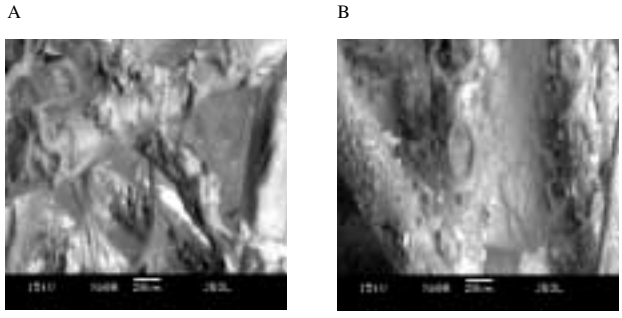

Figure 1 A: Scanning electron micrograph of inoculated small rig geotextile. B: Scanning electron micrograph of non-inoculated small rig geotextile. (Magnification factor × 480.)

colour key (Patterson, 1992). Light micrographs were obtained by the use of a light microscope with camera attachment, onto 25 mm black and white film.

Results

Small rigs. The SEM results revealed a uniform covering of microbial biofilm over the geotextile strands. Clear differences were evident between the inoculated and non-inoculated rigs. The inoculated rigs showed a large quantity of hyphal and/or actinomycete growth, which could be distinguished from the geotextile fibres (which were roughly 50 μm in diameter) in Figure 1. The hyphal growth was much less uniform in size and was stretched between the geotextile fibres. In contrast, the geotextile samples from the non-inoculated rigs had very little in the way of hyphae but did have an almost uniform covering of microbial growth, apparently of bacterial origin.

Medium rigs. Examination of the microbial diversity showed that four of the main functional groups of protozoa were present, namely, microflagellates, naked amoebae, testate amoebae, and ciliates (see Figure 2). Higher organisms were also found, such as multicellular organisms, including nematode worms and rotifers. Protozoan density at the end of the experiment was typically 1.5×10^4 cells per ml in both treatments, as compared with 2×10^2 in the control rig with no oil, inoculum or nutrients.

Reviewing the results from the medium rigs for the trapping efficiency of the clean motor oil showed that both the inoculated and the non-inoculated rigs achieved over 99%. After soxhlet extraction the amount of oil left on the geotextiles, which are the main sites for oil accumulation, was less in the non-inoculated rigs (8.3% of added oil) than in the inoculated ones (9.9%).

Total viable count of rig effluent for both inoculated and non-inoculated rigs showed maxima around 1.0×10^4 viable cells per ml (see Figure 3): microbial activity was also similar with both treatment types showing absorbance values well in excess of the blank and control values (see Figure 4). Mean increases in CO_2 evolution for rig treatments were 0.04% above ambient for inoculated rigs and 0.03% for non-inoculated, with the control unchanged.

Large rig. The large rig maintained high water quality in terms of oil in the effluent. After over 4 years almost continuous oil additions (6.6 g per week), the rig has attained a near 99% efficiency in terms of oil retention (see Figure 5). Previously, Bond *et al.* (1999) reported the CO_2 evolution and O_2 consumption for the period up to around Day 750, following which there was a pause in the addition of oil and of monitoring. This paper covers the period of monitoring from Day 830, which has gas concentrations slowly reduce back to

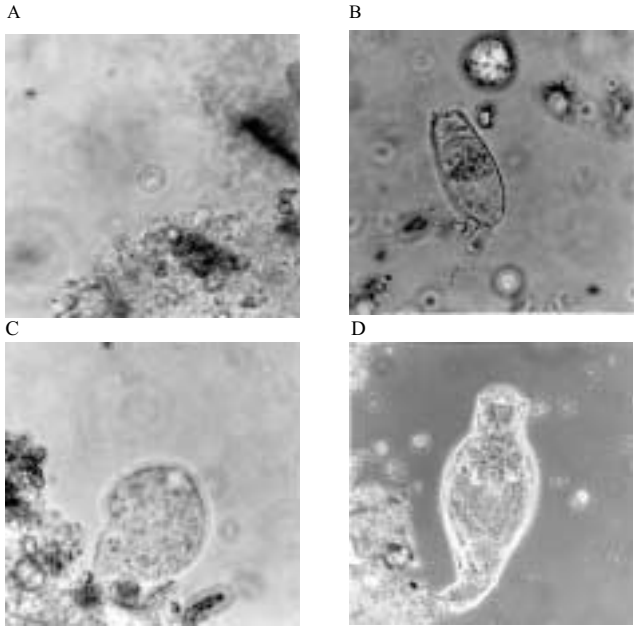

Figure 2 Light micrographs showing microbial diversity: A: choanoflagellate of the genus *Monosiga* ×800. B: testate amoeba of the genus *Euglypha* ×320. C: ciliate of the genus *Colpoda* ×800. D: rotifer ×320

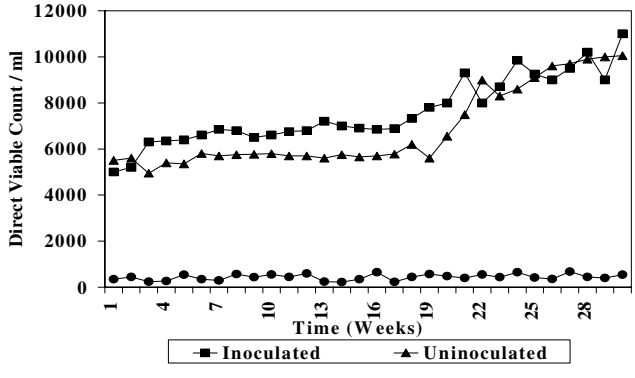

Figure 3 Direct viable count of inoculated, non-inoculated and control medium PPS rigs with Acridine Orange (AO)

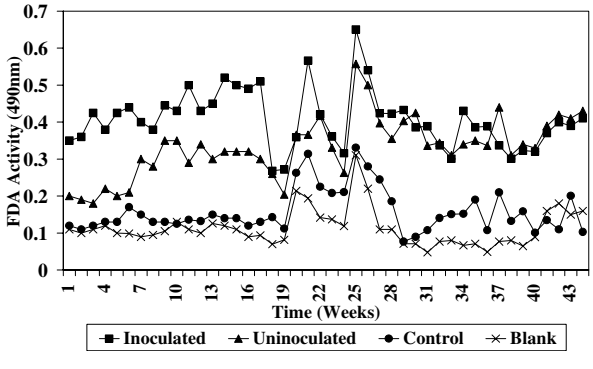

Figure 4 Hydrolysis of Fluorescein Diacetate (FDA) in inoculated, non-inoculated and control medium PPS rigs showing levels of microbial activity

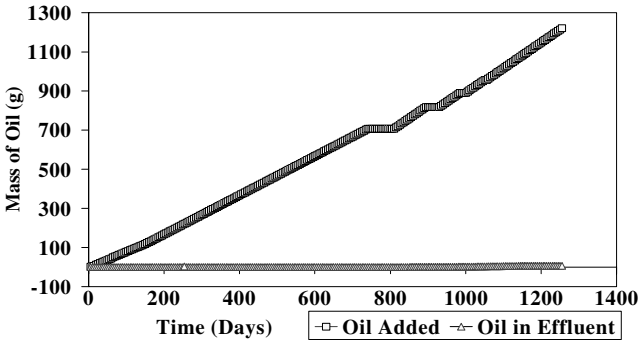

Figure 5 Cumulative applied and effluent oil in large rig PPS

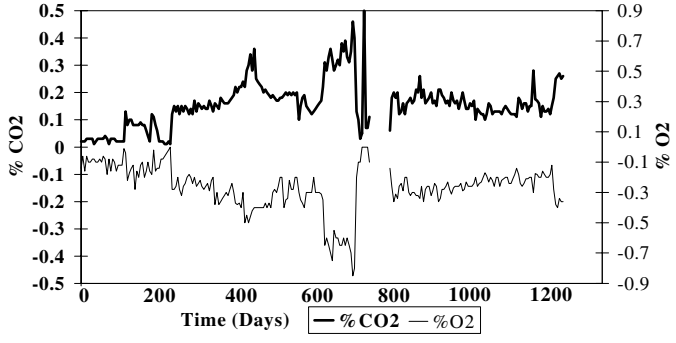

Figure 6 CO_2 evolution and O_2 consumption in large rig

nearer ambient conditions (Δ-values: 0.12% O_2 and 0.12% CO_2). It was assumed that this was due to the depletion of the nutrient pellets. Following their replacement with new Osmocote Plus, slow release fertiliser, on Day 1180 the balance between the two changed to –0.4 O_2 and 0.27 CO_2 within 48 hours (see Figure 6).

Discussion

It is clear that the large and diverse microbial communities found in the rigs are utilising the oil supplied, in conjunction with the added NPK fertiliser, to grow and reproduce. The total viable bacterial count was large at around 1.0×10^4, even in the effluent, and the activity measurements indicated that the oil and nutrients were the distinguishing factor between the high activity in both the inoculated and non-inoculated rigs and the low activity in the control rig. This was supported by CO_2 evolution in these rigs and the absence of CO_2 above ambient in the control rig.

In addition, the activity, total viable count and oil retained on the rig materials were similar in the inoculated and non-inoculated rigs. This suggests that the commercial, oil-degrading inoculum is no more efficient at utilising the oil than indigenous fauna, which have colonised the rigs. Under the highly favourable, laboratory conditions a microbial community may rapidly adapt to become an oil degrading assemblage. This has been reported to occur in as short a time as a few months (Atlas, 1981) and appears to have occurred in this 8-month experiment. A diverse and highly abundant protozoan and metazoan community appears to have been established and, although these organisms are natural predators of the bacterial biofilm, it has been suggested that they are more likely to have a stimulating effect on bio-degradation than an inhibitory one (Huang *et al.*, 1981).

Many of the protozoa, such as the colpodid ciliate and some naked amoebae, are fairly ubiquitous in their distribution, partly due to their ease of dispersal in an encysted state. The

choanoflagellates have been recorded relatively few times in groundwater as compared with in freshwater and marine environments, most genera lacking an encystment capability. The testate amoebae are also thought to be limited in their dispersal abilities due to their relatively large size (Smith, 1996). It is interesting to note the presence of members of the latter two groups in the laboratory rigs. It is possible that wind blown cysts or whole organisms are gradually added to the community in addition to those found initially on rig materials. It is apparent from the SEM micrographs that a complete and complex microbial biofilm has been created in the 8-month experimental run.

The SEM analysis has also shown that differences exist between the geotextile surface in inoculated and non-inoculated PPS rigs. The rig inoculated with Biotreat HD had large quantities of fungal and/ or actinomycete hyphae, whereas the non-inoculated rig had not. The medium rigs were not geographically isolated from one another, but were, in fact, in one line adjacent to one another. It is interesting that significant cross-contamination did not appear to occur: it is possible that competition from indigenous strains prevented widespread cross-rig dispersal.

The large PPS rig has continued to perform well despite the continued and consistent oil application over a 4-year period. The speed at which the microbial biofilm was able to respond to changed conditions demonstrated considerable resilience. An 8-month period of decline in the nutrient status was reversed within 48 hours once an application of fresh Osmocote Plus was provided. A similar response was seen after a 75-day period (Days 755 to 830), when drought conditions were in operation: the CO_2/O_2 balance returned to nearly ten times ambient conditions within 48 hours.

Conclusions

The addition of a commercially available oil degrading microbial mixture does not seem to be required for the establishment of microbial populations within the PPS. Further research is needed to assess the importance of the species composition, particularly of the higher organisms to the bio-degradation process, as is an understanding of the rate and methods of dispersal and colonisation. The ability of such populations within the PPS to resist and be resilient to changes in the environment suggest that, once established, a very robust biofilm is produced, which is capable of withstanding major fluctuations over a long timescale.

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