THE MICROBIOLOGY OF PERMEABLE PAVEMENTS

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SUMMARY

Pervious pavement systems (meaning permeable pavements within this context and used interchangeably) are known to retain and degrade hydrocarbons within the structure of the pavement. The key to this decontamination has been shown to be the naturally occurring microbial communities living on the pavement building materials. Research by Formpave and Coventry University has shown that once oil is trapped on a suitable strongly oil-retaining geotextile membrane layer, the oil rapidly becomes a food source for the microbial communities. Oil is metabolised by aerobic bacteria and fungi that convert the oil into sugars such as glucose for growth and reproduction. Over time, a network of microbial growth spreads over the surface of Inbitex forming a biofilm, further improving the filtration and purification properties of the system. The presence of a large food source growing on the oil attracts larger predatory organisms such as protozoa and metazoa to the biofilm. These organisms play an important role in maintaining the free-draining characteristics of the geotextile and also the vigorous growth of the oil degrading organisms. An understanding of the processes underpinning the environmental benefits of permeable pavements is essential in order to optimise the operation of the system.

1. INTRODUCTION

Whilst peak run off discharge and total run off volumes have long been important design criteria for pervious pavement systems (PPS) studies on pollution retention were mainly limited to suspended solids and dissolved and particulate metals removal until the mid 1990s (Pratt *et al.*, 1996)). In the middle of that decade the Porous Pavement Research Group at Coventry University started to take an interest in the retention of oils by pervious pavements in car parking situations. Very soon this interest in retention developed into an interest in biodegradation of the trapped oil and this in turn produced two important streams of research, the applied research aimed at optimising the conditions in the pervious pavement for oil biodegradation and more fundamental studies aimed at obtaining an understanding of the microbial ecology of the system. This paper represents an overview of a selection of experiments, which highlight some of the general trends in research within the group since 1995. Little experimental detail is presented but in all cases detailed information is provided in the publications referred to.

Before looking at the experiments carried out it is worthwhile considering what the needs of micro-organisms are when we try to utilise them to biodegade hydrocarbons.

The organism will clearly need a supply of organic carbon both as a basis for building cellular material and to provide energy. In an oil biodegradation system this is clearly provided by the oil. Since the most common (and certainly the fastest) hydrocarbon degrading processes available to microorganisms involve the utilisation of the hydrocarbons by aerobic organisms and since the products of anaerobic

metabolism can be odorous (H_2S) or contribute disproportionately to atmospheric greenhouse processes (N_2O and CH_4) we are required to maintain the system in an aerobic condition. The pervious nature of the sub-surface environment and the provision of pathways for infiltration of water would be expected to supply this. However until the start of our work it was not known whether aerobic conditions would be maintained when significant oil loadings were applied. The system would also require inorganic nutrients (NPK and trace elements) and as our early attempts to encourage biodegradation were performed in lab scale models a suitable means of providing the nutrients in the long term was an important aspect of our work. So important is this subject that it forms the basis of a separate paper submitted for this conference and will only be dealt with briefly here. The system also requires an appropriate water environment, one which will not dry out too quickly between rain events and yet be an environment in which the water continues to percolate through the system. Standing water in the oil contaminated zone may lead to anaerobic conditions and, ultimately to flooding of the pavement in heavy rain. Biodegradation will clearly need a viable population of microorganisms. This will include the bacteria and fungi responsible for the biodegradation process but also higher organisms that are essential both for keeping the drainage properties of the pavements within acceptable parameters and also to recycle nutrients and provide certain complex growth factors which some key degraders require. The final, and oft overlooked, necessity for a biodegradation process is time. This is particularly so for hydrocarbons which degrade relatively slowly and particularly in a situation where water is encouraged to flow through the system thus leading to potential entrainment of the target pollutant. The geotextile is important here both in retaining the pollutant and to provide a suitable basis for rapid biofilm formation and for survival of organisms during periods of stress.

2. HISTORICAL DEVELOPMENT OF THE STUDY METHODS

Having looked at the needs of the microoganisms let us now consider the needs of the researchers embarking upon what was a new area of research and which needed to bring together , initially a civil engineer a microbiologist and an analytical chemist and later to bring on board expertise in protozoology, molecular biology and synthetic polymer chemistry. The first item on the wish list was a laboratory scale model system which could be used for oil retention and oil biodegration experiments. For the initial experiments it needed to be fairly large to allow a representative area of pavement to be studied without edge effects. The system needed to be capable of providing realistic rainfall events (fortunately such a facility was already available in our lab, having been developed by Berry (1995) for her hydraulic studies in the early 1990s. It was also necessary to design and build systems to allow sampling of the atmospheres in the sub-pavement environment and to collect effluent samples. It quickly became apparent that it would be necessary to carryout work in smaller model systems. This would allow replication of our experiments and to allow us to use a bioreactor capable of allowing us to attempt to obtain a mass balance for the carbon we were applying as oil. Figure 1 is a schematic of the improved model system used by Bond to produce a mass balance of carbon in the system which also allowed the kinetics of the system to be established. This system also formed the basis of a much simplified version used by Spicer (2006) whilst developing geotextiles capable of supplying inorganic nutrients to the microorganisms without the need for further additions (Spicer *et al* 2005). By the late 1990s these systems had been perfected and we then started to look at the chemical and microbiological study methods that were available to us. This paper is, in many instances, a story of iterative improvements after a number of blind alleys.

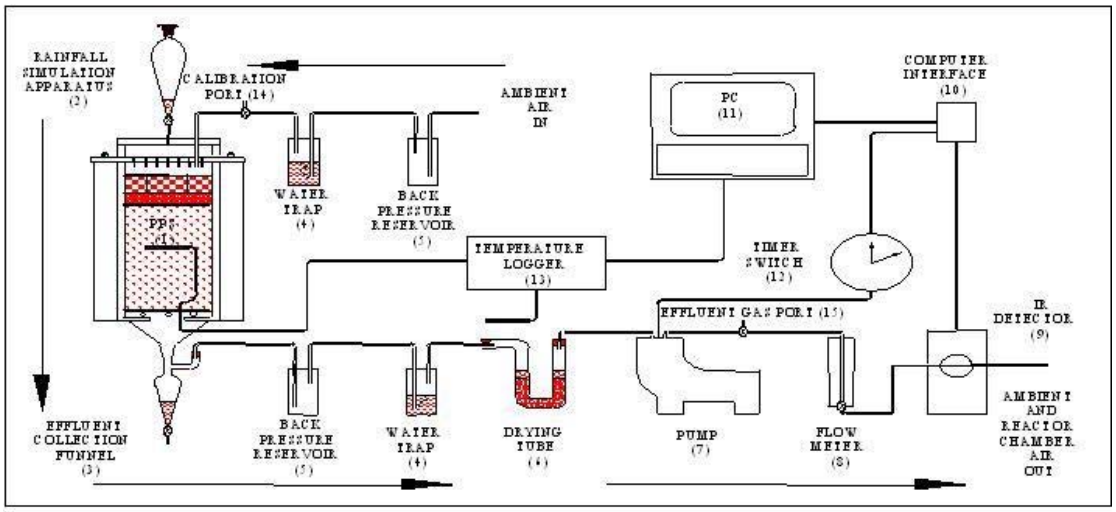


Figure 1. The system used for kinetic and mass balance studies.

One important requirement in our work was the ability to count oil degrading microorganisms in our effluents. Bond (1999) developed a method based on technique previously used for counting oil degrading microorganisms following marine oil spills. This test was adapted for an essentially freshwater environment and served the group reasonably well. However dissatisfaction with the fact that from time to time the microtitre plates used in this test would dry out before the experiment could be considered complete, the tendency to produce false positives and the very long turnaround times for the experiments has lead us in more recent times to produce alternive methods. The two methods finally developed (Puehmeier et al 2005) provided greater reliability both involving a Most Probable Number method requiring cultivation of dilutions of effluent in a minimal salts medium either in sealed vials fitted with septa to extract gas samples (in this case positive vials are identified by analysing the headspace above the medium for elevated carbon dioxide levels) or using the reduction of resasurin in microtitre plates. This work is not described in detail here but the success of the procedure can be seen in Figure 2 which illustrates that it is possible to show a remarkable relationship between the log of the number of oil degrading bacteria and the concentration of carbon dioxide n the sub-surface atmosphere of a pavement structure. It also illustrates a point which will be made later, the value of providing inorganic nutrients to accelerate biodegradation.

It was proposed that more complex microbial communities in the porous pavements would be both more stable under conditions of stress and capable of the most effective utilisation of resources. If this is the case, then a quantitative measure of microbial biodiversity would be a useful factor in optimising the system. The idea was that a relatively simple tool could be produced that could indicate the "microbiological health" of the pavement system both in laboratory and field situations.

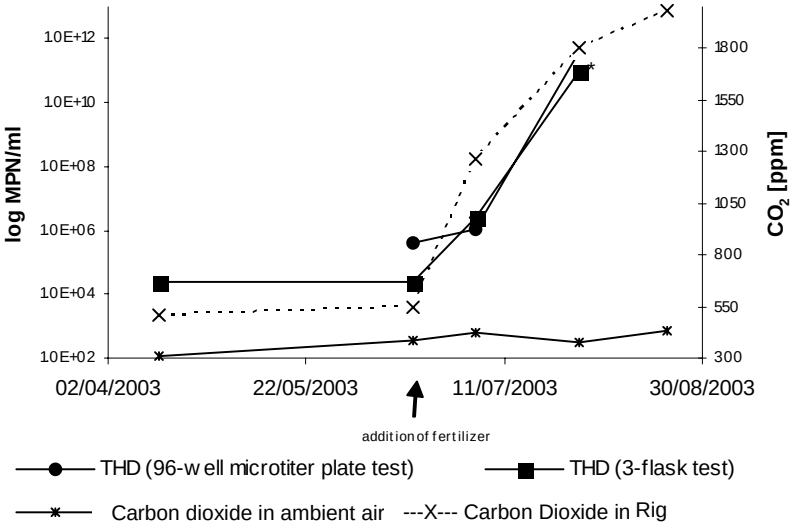


Figure 2. Relationship between log number of oil degrading bacteria and CO_2 concentration

Classical techniques for study of the bacterial diversity in such structures are problematic because the great majority of organisms found in natural systems are not culturable (Amann *et al* 1995, Puehmeier *et al* 2005). In order that this area of research was to progress, considerable efforts have been put into developing a suitable methods of quantifying the microbial diversity which takes into account the non-culturable element. However before the group embarked upon his line of research an approach to this using molecular biology methods was given a great deal of attention, particularly by Puehmeier (2006). This approach involved the use of PCR reaction to amplify the bacterial DNA in our effluents samples and in samples extracted when experimental models were dismantled was .Whilst this technique did not turn out to be the panacea for the measurement of microbial diversity it did produce another important part of the jigsaw in terms of our knowledge of the role of exogenous inoculation of pervious pavements in the long term.

As indicated above bacteria are not the only inhabitants of an oil degrading pervious pavement and investigations within the group have demonstrated both the diversity of the eukaryotic (protozoan and metazoan) component (Newman *et al.* 2001) and their importance. Unlike the bacteria, these eukaryotic organisms are relatively large and have characteristics that often allow them to be identified under an optical microscope. They have often been used as bioindicators (Foissner and Berger 1996, Smith 1996) in rivers, lakes and wastewaters and in our opinion have been established as a good vehicle to reflect overall microbial diversity or system health in porous pavement studies. They can be identified without the need for culturing and advantage has been taken of this to make an initial estimate of the importance of the eukaryotic fraction of the biofilm to system performance.

A study was carried out (e.g. Newman *et al.* 2001) to investigate the development of protozoan colonists after a fixed period of time. Six months after setting up the model structures, a complex community had been produced. Bacteria, fungi, all the major protozoan groups and metazoa were observed to inhabit the PPS system. This was a clear indicator of the relative abundance and diversity of nutrients within the system, the majority of which come originally from oil via the decomposer pathway. It also provided evidence that ordinary pavement construction materials provide the necessary “seed” for a highly diverse oil degrading community. It is probable that protozoa prevent

over-proliferation of the biofilm bacteria and prevent clogging of the geotextile by cells and extracellular polysaccharides. It is also possible that, where the protozoan and metazoan communities are species rich and a dense population, they may stimulate the bio-degradation process. (Darbyshire, 1994)

3. BIOREMEDIATION STUDIES

The aim of this experiment was to study the long term behaviour of a model PPS system. A representative section of the pavement was constructed as described above (except that the depth of sub base was 600mm and the plan area was 600mm x 600mm) in a steel/glass box.

In this study the oxygen and carbon dioxide in the air spaces within the model were measured at the pea gravel bed and sub base levels, respectively. Elevated carbon dioxide concentrations and reduced oxygen concentrations indicated that increased biodegradation was occurring. It is important to note that the oxygen concentrations never fell below levels at which they will become limiting. Separate gas samples were extracted from this rig to investigate possible local anaerobic zones. Methane was measured by gas chromatography but only trace concentrations could be detected. Concentrations were measured on a cyclic basis as previously described (Bond *et al.* 1999). Lubricating oil additions of approximately 3.3g per week were applied except during periods of holidays and staff changeover. Rainfall events at 1.6mm/hr were applied on average once every 3.5 days (approximating to the mean for London) except during deliberate drought periods. An important question was whether it was possible to maintain the biofilm within the structure for an extended period and whether, over the long term, the oil retaining properties of the structure would be maintained. This is discussed in detail in a separate paper submitted to this conference.

The oil retention performance of the pavement model is illustrated well in Figure 3 which shows the percentage of total applied oil retained by the model system over the first 750 days of the experiment. It can be seen that in the initial days of the experiment the total retained falls rapidly but only to a minimum value of just over 97%. By 600 days the percentage retained is around 98.5% This level of retention maintained for a period of around 5 years.

The process of biodegradation of hydrocarbons involves both shortening of chain length and addition of oxygen to the terminal groups. One would expect then that the nature of the organic matter released would change both because chain lengths are reducing and because partly oxidised hydrocarbons will be more soluble (but given the method of analysis for oil and grease determination would be counted within the TPH figure. A retrospective study by Bond (1999) of the Infrared spectra used to calculate TPH showed that over time the ratio of mid chain CH in CH2 groups to total CH content was gradually falling.

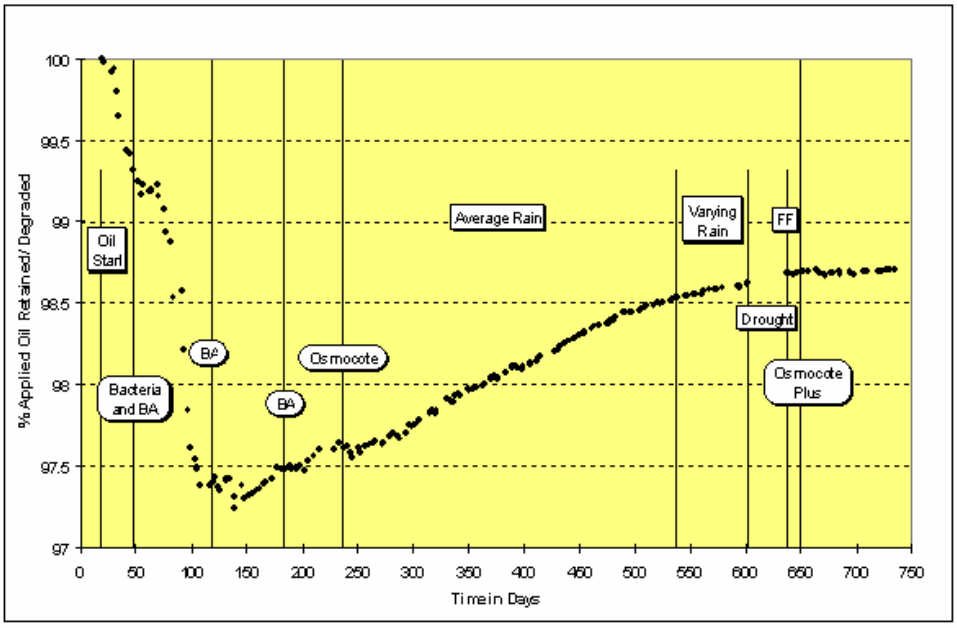


Figure 3. Plot of Percentage of total oil retained against time for the 600mm x 600mm model - First 750 days of experiment.

The next important question is whether or not the PPS can biodegrade the oil in the structure at a sufficiently fast rate to prevent the retention properties of the PPS being overloaded. Bond (1999) used the sealed respirometer system illustrated in Figure 5 in a kinetic study. Log normal plots of the data shown illustrated that pseudo first order kinetics could be used to describe the process. The upper line represents degradation rates with the inoculum only whilst the lower line, with over double the degradation rate, is the situation with slow release fertiliser. Given suitable inorganic fertilisers oil could be broken down sufficiently fast to ensure that at the normal rate at which oil drips onto a typical road surface the system would not saturate within the design life of the structure. A separate paper deals with the important role played by inorganic nutrients.

4. MONITORING BACTERIAL COMMUNITY STRUCTURE

The aim of this initial experiment was to demonstrate whether it is possible to distinguish, if the bacterial community present in the effluent (and thus in the experimental rig itself) from a rig which has been operated for over 1200 days was different from the bacterial community in the commercially available seed which was used to inoculate the rig and initiate biodegradation.

The Polymerase Chain Reaction (PCR) was used to amplify the 16s ribosomal RNA gene (Giovannoni *et al*, 1990) from DNA from cells collected from the effluent from the test rig and from the original inoculum. This technique although simple in principle is very complicated in practice since each different sample type requires optimisation for a number of parameters.

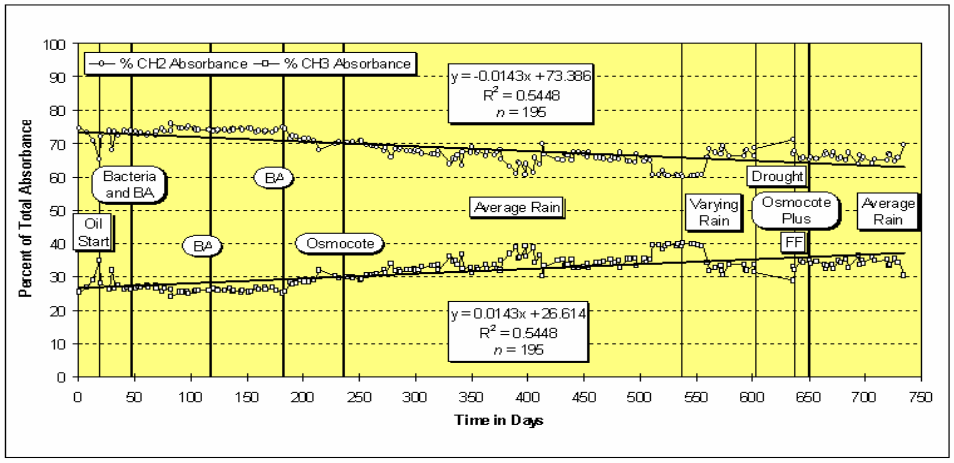


Figure 4. Changes in CH_3:CH_2 ratio in PPS effluents over time (From Bond 1999)

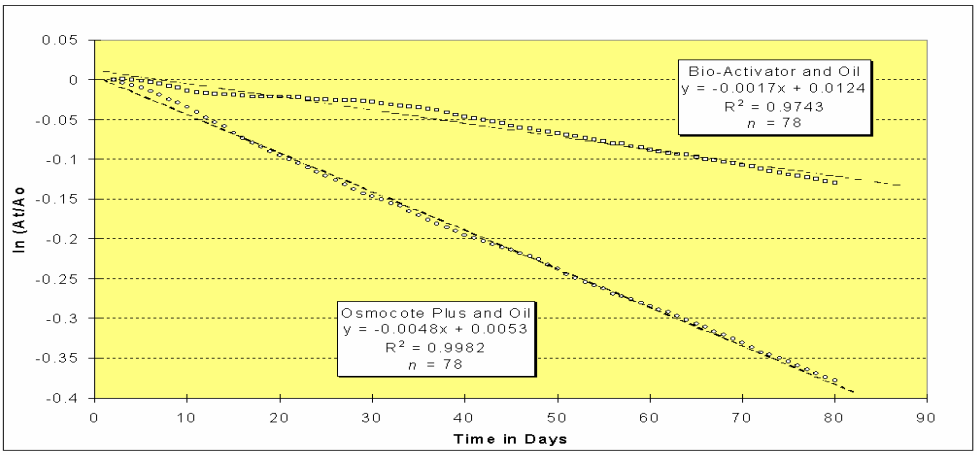
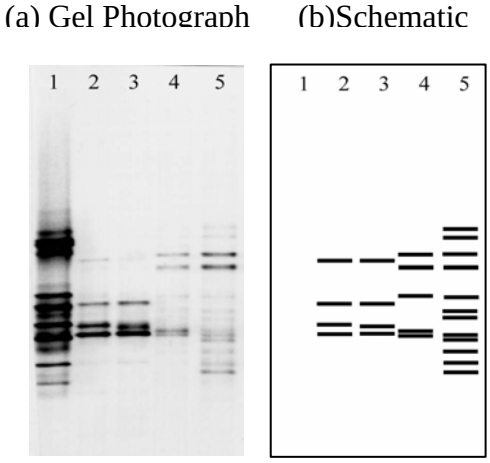


Figure 5. Log normal plots of oil remaining in system against time showing straight line relationship and hence first order kinetics.

Effluent samples were taken from the long term rig, after a period of about three years use, and compared with the initial inoculum itself (see Newman *et al.* 2001). A 200 base pairs region of DNA is amplified by PCR and analysed by differential gradient gel electrophoresis (DGGE). The opportunity was also taken to compare two different methods of extracting DNA from the cells, bead beating and freeze-thaw techniques.

The results of DGGE analysis of bacteria from initial inoculum and long term porous pavement model can be seen in Figure 2 which includes a schematic to identify the band positions observable on the gel but unclear in the photograph. The profile of the initial inoculum in lane 2 (freeze-thaw) and lane 3 (bead beat) indicates four bands, two of which are dominant bands and two of which are fainter bands. The profile of the long term porous pavement model has a greater number of bands which indicates that the sample is much more diverse than the initial inoculum. In the bead beating-extracted sample from the long term rig at least twelve bands can be observed with two bands dominant (clearly not the same dominant bands as the initial inoculum). These results confirmed the earlier conclusion (Puehmeier *et al* 2005) that over time the population within the porous pavement changes and the

initial inoculum appears to be out competed by other bacteria from the environment. Therefore, the initial inoculum does not dominate the established sample, backing up the conclusion (Puehmeier *et al* 2005) that the inoculum is not required for long term use of this technology.



Lane 1, laboratory strain makers; Lane 2 initial inoculum extracted using freeze thaw; Lane 3, initial inoculum extracted using bead beating; Lane 4 long term porous pavement bacterial population extracted using freeze thaw; Lane 5 long term porous pavement bacterial population extracted using bead beating.

Figure 6. DGGE Gel showing changes in bacterial populations over time.

It also indicated that over time the complexity of the bacterial population increases. Sequence analysis of bands from the long term rig have provided information on the bacteria present within the sample.
Coupe (2001) carried out work to backup the contention that inoculation was not necessary for establishing an oil biodegrading community in a PPS. Using inoculated and non-inoculated models he demonstrated that by monitoring the effluent using the Fluorescein diacetate (FDA) method to monitor total biological activity of the effluents the activity of inoculated and non-inocculated rigs became virtually identical within 39 weeks (Fig 7)

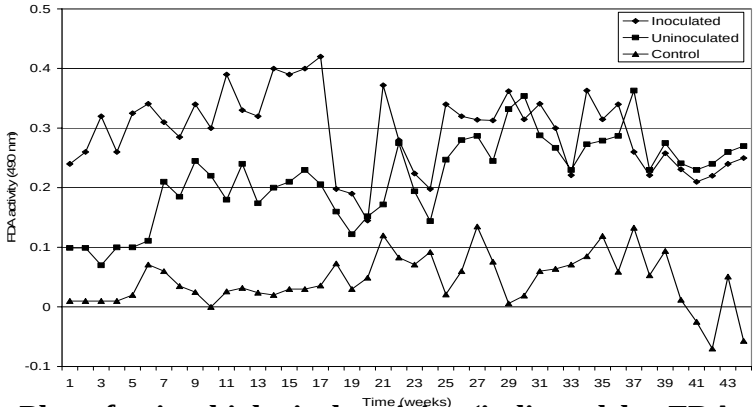


Figure 7. Plot of microbiological activity (indicated by FDA method) of inoculated and non-inoculated rigs over 44 weeks.

5. PROTOZOAN COLONISATION STUDIES

An experiment was also carried out to identify the source of protozoa on the PPS model system and to measure the rates of colonisation experienced under the relatively ideal conditions provided in the

laboratory environment. Rig dimensions were as described previously (Newman *et al.* 2001). The rigs had a one-off application of 0.22 g Osmocote Plus slow release fertiliser and 0.08 g per week of oil; they received 52 ml of artificial rainfall per week in two 26 ml rainfall events using the device previously described (Pratt *et al* 1996).

To compare the contributions of the microbial community already on rig materials with that brought in from the surrounding environment, one of the rig boxes together with the substrates and apparatus contained within was autoclaved at 121 °C for 20 minutes to destroy spores and trophozoites. The other rig and its substrates was washed with warm water to remove particles and loose organic matter before placing them in the laboratory.

The day after setting-up, samples were taken from the effluent; identifications were performed as described previously (Newman *et al* 2001). A diversity survey was carried out weekly. Within the laboratory environment, the range of temperatures during the experimental period was 16-20°C.

Results from the colonisation experiment (which are also shown graphically in Figure 8 show that the ubiquitous microflagellate *Heteromita globosa* was found in both sterile and non-sterile rigs by the end of the first week. In the non-sterile rig *Colpoda cucullus* was also found within this time period. The sterile rig accumulated only *Heteromita* within the duration of the experiment. In the non-sterile rig effluent, flagellates, ciliates, gymnamoebae and testate amoebae were found within 4 weeks of the start of the experiment and metazoa were present after six weeks The results for this time series colonisation study closely reflected the earlier fixed term study (Newman *et al* 2001) with several of the key genera represented, such as *Euglypha, Colpoda* and *Heteromita,* being found in both studies.

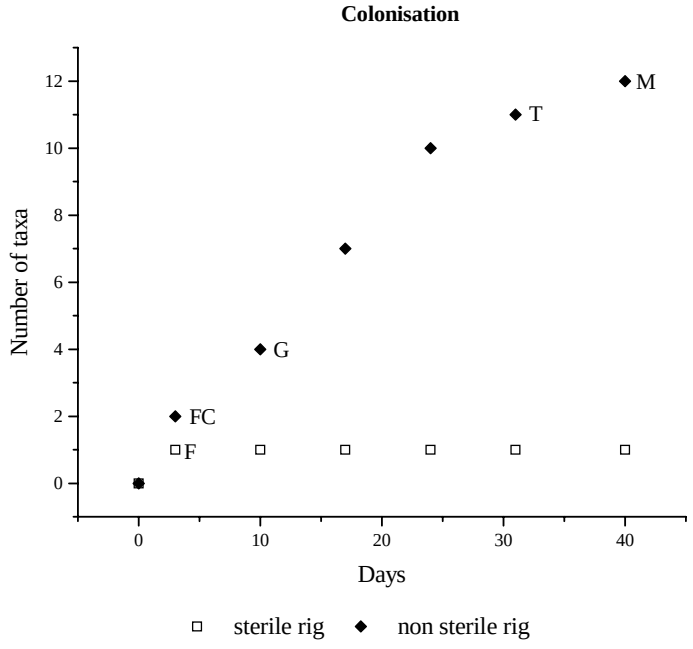


Figure 8. Colonisation of sterile and non-sterile PPS models
Letters denote first appearance of taxonomic groups.
F=Flagellates G=Gymnaoebae C=Cilliates T=Testate Amoebae M=Metazoa

These results show that ordinary, relatively clean, substrates can act to inoculate PPS rigs with a wide diversity of micro-organisms. The consequences for bio-degradation are that there is a considerable reservoir of diversity present within initial PPS substrates, despite the fact that they would not appear particularly rich in spores or organisms. Figure 8 shows that clean non-sterile substrates added to the PPS can, under moist, high nutrient high oil application conditions and with sufficient time develop a high diversity of protozoan strains. However this diversity within the laboratory environment comes almost exclusively from the substrate itself and not the surrounding environment. Results from this study have shown that a diverse eukaryotic community can easily and rapidly establish itself within permeable pavements under defined conditions. There may be specific benefits to the biofilm function from this biodiversity.

6. CONCLUSIONS

This paper has demonstrated some of the wide ranging analyses employed in the attempts of the group to understand the biological processes responsible for the successful biodegradation results gained in earlier work. Further understanding of microbial processes has led to an increased optimisation of the PPS system and epresents a gradual shift away from treating the PPS system as a ‘black box’ whose inner workings could not be discerned.

In particular the work described here will be a foundation for further work. This will allow investigation of the microbiological effects of replacing the virgin stone sub-base with recycled material which will be the next phase of the investigation.

7. ACKNOWLEDGEMENTS

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8. REFERENCES

Amann, R.I., Ludwig, W.,Schleiffer, K.H. (1995), Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143-169.

Berry T (1995) PhD Thesis, Coventry University , UK

Bond P (1999), PhD Thesis, Coventry University, UK

Corliss, J.O, and Esser, S.C. (1974). Comments on the role of the cyst in the life cycle and survival of free-living protozoa. Transcripts of the American microscopy society. 93: 578-593.

Darbyshire, J.F. (1994). Future Prospects. In: Darbyshire, J.F. (ed) Soil Protozoa. CAB international. Guildford.

Foissner, W. and Berger, H. (1996) A User-friendly guide to the ciliates (Protozoa, Ciliophora) commonly used by hydrobiologists as bioindicators in rivers, lakes and wastewaters, with notes on their ecology. Freshwater Biology. 35: 375-482.

Giovannoni SJ, Britschgi TB, Moyer CL and Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345: 60 – 63.

Hughes, J. and Smith, H.G. (1989). Temperature relations of *Heteromita globosa* Stein in Signy island fellfields. Antarctic special topic. 117-122.

Miller, K.M., Ming, T.J., Schulze, A.D., Whitler, R.E**.** (1999), Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. Biotechniques 27: 1016-1030

Newman, A.P., Pratt, C.J., Coupe, S.J. and Cresswell, N. (2001). Oil bio-degradation in permeable pavements by inoculated and indigenous microbial communities. Proc. 4th International conference on innovative technologies in urban drainage (Novatech). Lyon, France, 425-432.

Puehmeier T, De Dreu D, Morgan JAW, Shuttleworth A and Newman AP, (2005) Enhancement of Oil Retention and Biodegradation in Stormwater Infiltration Systems Proc. 10th International Conference on Urban Drainage, Copenhagen/Denmark, 21-26 August 2005,

Puehmeier T (2006) PhD Thesis, Coventry University , UK

Pratt, C.J., Newman, A.P. and Bond, P.C. (1999). Mineral oil bio-degradation within a permeable pavement: Long term observations. Water Science and Technology. 39: No. 2, 103-109.

Pratt C.J., Newman A.P. and Brownstein J.B. (1996), Bio-remediation processes within a permeable pavement: initial observations. Proc. Seventh Int. Conf. on Urban Storm Drainage, F. Sieker and H-R Verworn (Eds.), Hannover, Germany,1455-1460.

Smith, H.G. (1996). Diversity of Antarctic terrestrial protozoa. Biodiversity and conservation. 5: 1379-1394.

Spicer, G.E., Lynch, D.E., Newman, A.P. and Coupe, S.J. (2005). The development of geotextiles incorporating slow release phosphate beads for the maintenance of oil degrading bacteria in permeable pavements. 10th International Conference on Urban Drainage Copenhagen, Denmark, August 21-26, 2005.