THE ANALYSIS OF PERMEABLE PAVEMENT MICROBIAL BIOFILMS BY ELECTRON MICROSCOPY

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SUMMARY

Recent research has revealed the structure of microbial biofilms growing within pervious paving systems. Growing predominantly due to the presence of hydrocarbons, these oil-degrading biofilms grow on the geotextile, Inbitex™ on which the majority of oil is immobilised. The properties of Inbitex encourage early biofilm growth and a covering of the surface proceeds rapidly. When mature, the biofilm displays many morphological types of bacteria and fungi, clearly visible by scanning electron microscopy (SEM).

The presence of oil droplets within vacuoles in bacterial cells was confirmed by transmission electron microscopy (TEM), bacteria not grown in oil were shown not to possess the same sized cellular inclusions and no oil was observed within the bacterial cell structures. Electron microscopy is a useful tool with which to analyse the effect of treatments on the microbial biofilm and allows the visualisation of microscopic structures. The relevance of such investigations to pervious pavement systems (PPS) water quality results are discussed as are biofilm structure and function.

1. INTRODUCTION

1.1 Early biofilm investigations

The earliest investigations into pervious pavement system (PPS) meaning the same as permeable pavements and used interchangeably in this paper, oil biodegradation showed that biological activity in PPS simulations gave rise to visible microbial growth which was in the form of detached biofilm (Brownstein, 1998).

Further investigation showed that the biofilm was growing primarily at the site of maximum oil immobilisation, the geotextile. This random non-woven polypropylene mat has excellent oil retaining properties and also became known to be the non-living platform on which the biofilm could grow and biodegrade oil. Indirect measurements of oil biodegradation such as CO_2 evolution, gave some idea of the extent of microbial processes ongoing, but little direct evidence was available as to the composition of the microbial aggregates and their interdependence. It was clear that more thorough analysis of the organisms responsible was required to more fully investigate some of these outstanding issues.

1.2 Microbial food webs and energy flows

Biodegradation processes are always influenced by a mixture of microbial species representing a number of organisms, bacteria, fungi, protists and animals. The actual transformation of hydrocarbons from carbon substrate, into glucose and waste CO_2 in aerobic conditions via microbial metabolism, is

done by the decomposer community, bacteria and fungi. The protists and small animals feed on the decomposers and release back into the microbial community certain chemicals, particularly nitrogenous compounds and others such as vitamins. In addition to feeding on the decomposers which, in many cases actually stimulates the decomposer community, the release of these limiting chemical factors encourages faster decomposer growth. This phenomenon has been called the 'microbial loop' and an example of one of these associations is shown in Figure 1.

In Figure 1 the organic material, in this case oil gives rise to a community with 4 levels of complexity starting with the decomposers and ending (arbitrarily for the purposes of this paper) with very small insects.

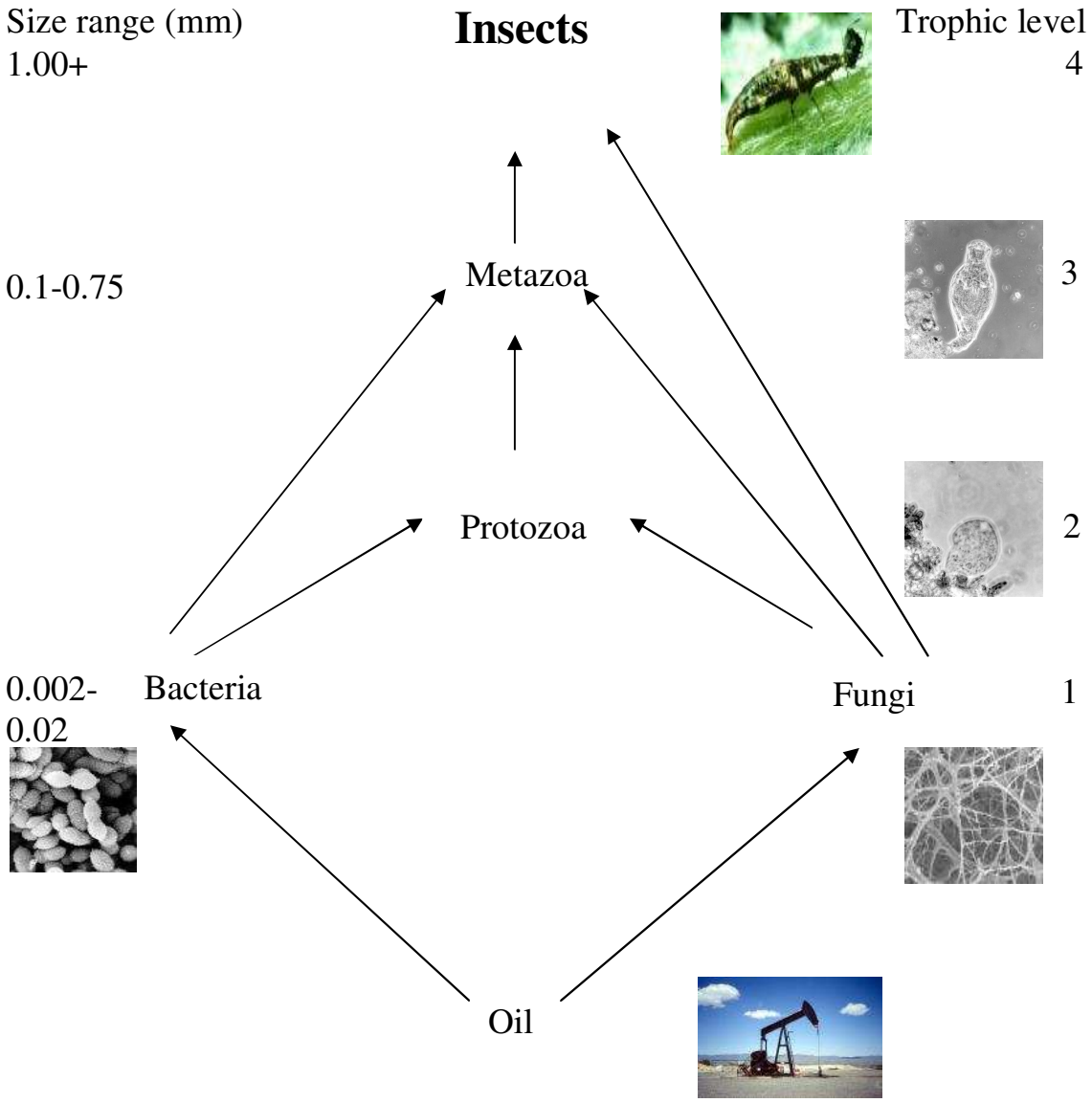

Figure 1. The structure and composition of a simplified microbial food web.

1.3 Biofilm theory.

In most environments microbes are organised into a biofilm. Biofilms have been described as cities built by micro-organisms (Lewandowski, 2000). The most readily available example which is widely known is dental plaque and represents a mass of microbial growth and the organism's secreted products sitting on a tooth surface. The reason for the growth of these organisms is a readily available food source at a concentration conducive for microbial growth and reproduction.

Some biofilms however are capable of performing useful roles, particularly when they facilitate the transformation of polluting compounds such as oil. It is well known that a biofilm is more secure from predators and works faster at biologically transforming materials than the equivalent number of suspended or non-attached organisms (de Beer et al., 1993).

1.4 Electron microscopy

Various methods of microscopy have been used to examine biofilms, with scanning electron microscopy (SEM) the most popular. This technique of observing the surface of materials at high magnification (e.g. x 10000 magnification) lends itself to qualitative analysis of biofilm development. An advantage of SEM over light microscopy is in the resolution. Images appear as 3 dimensional due to the depth of field. Transmission electron microscopy (TEM) is a useful tool for examining the structure of biological samples and has been used to examine biofilm thickness. Sample preparation for analysis by TEM is time-consuming and is best used for production of cross-section images, which has made it less popular than SEM for biofilm analysis.

2. METHODOLOGY

2.1 Biofilm growth conditions.

Samples were prepared for analysis by incubating geotextiles (Terram 1000 and Inbitex) in oil rich samples and also by taking sub-samples from oil degrading PPS geotextiles after varying times within the rig structures. The minimum periods of time before sampling the biofilm were several days to a maximum of six months.

2.2 Extraction and preparation for SEM and TEM.

In order to analyse geotextile samples with no biological materials on them to establish a baseline state, these samples were simply gold coated and placed in the SEM for analysis. This is done to allow an even electrical charge over the sample to obtain good quality images. Where biological material is included, the gold coat also prevents damage of the material by the electron beam.

For both electron microscopy methods with biofilms present, the samples were fixed in glutaraldehyde and dehydrated in steps from 10-100 % alcohol. After this, SEM samples were critically point dried in dry ice under pressure to preserve biological material in a rigid visible form before being gold coated and placed in an SEM for analysis. For TEM after dehydration, the prepared samples were finally fixed using osmium tetroxide, ($Os0_4$) and embedded in araldite before analysis.

Figure 2 below shows clean non-colonised Terram textile with no biological material on it. As can been, a certain amount of pitting or surface roughness is evident in the form of surface depressions. This surface texture had not been previously observed and was probably an artefact coming from the geotextile extrusion process during manufacture. Figure 3 shows microbial colonisation of Terram 1000 after 2 months within a PPS rig. The images show fungal growth with the rounded objects being fungal fruiting bodies showing further proliferation of microbial growth.

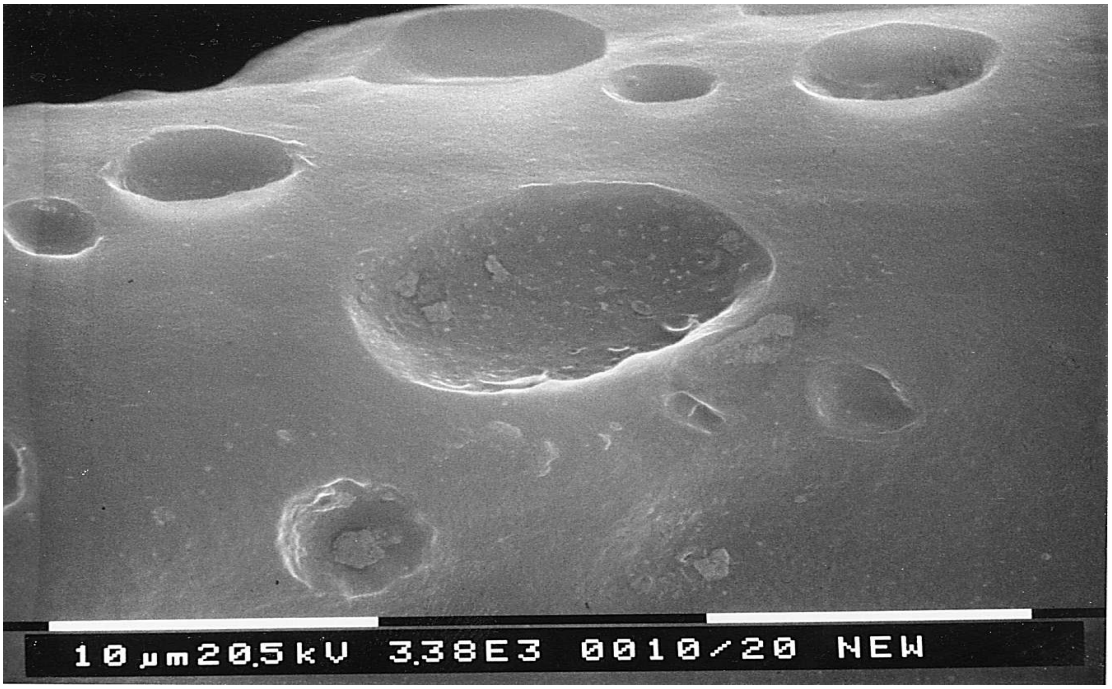

Figure 2. Clean geotextile showing the pitted surface.

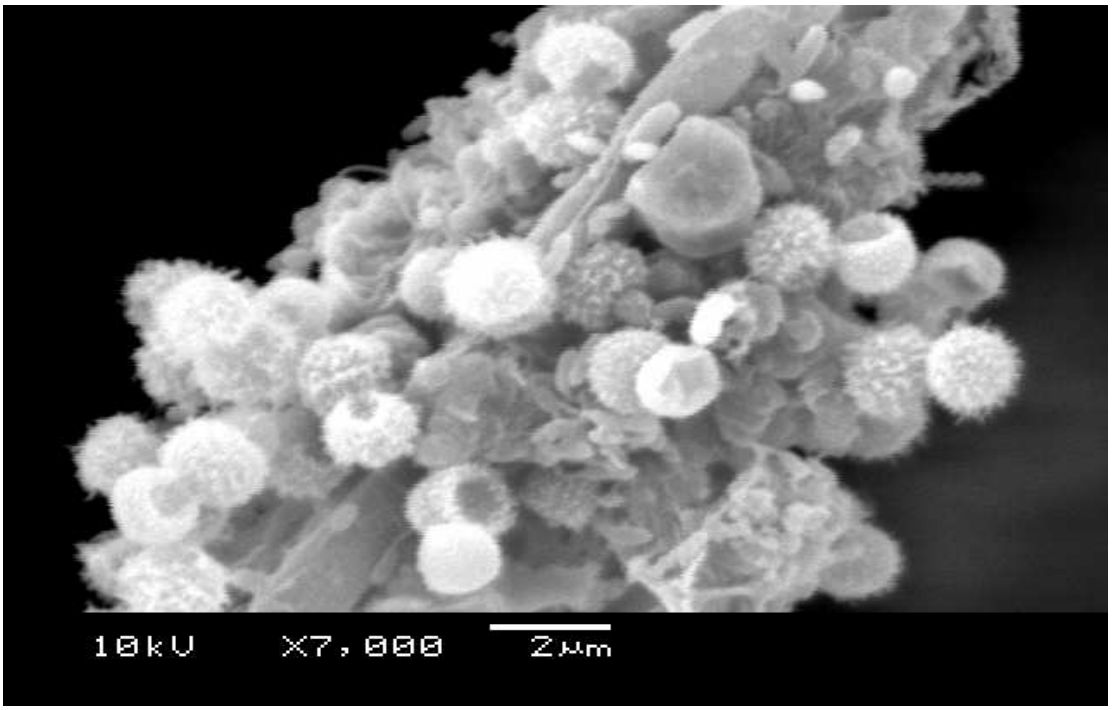

Figure 3. Fungal spores and mycelium (strands) on geotextile.

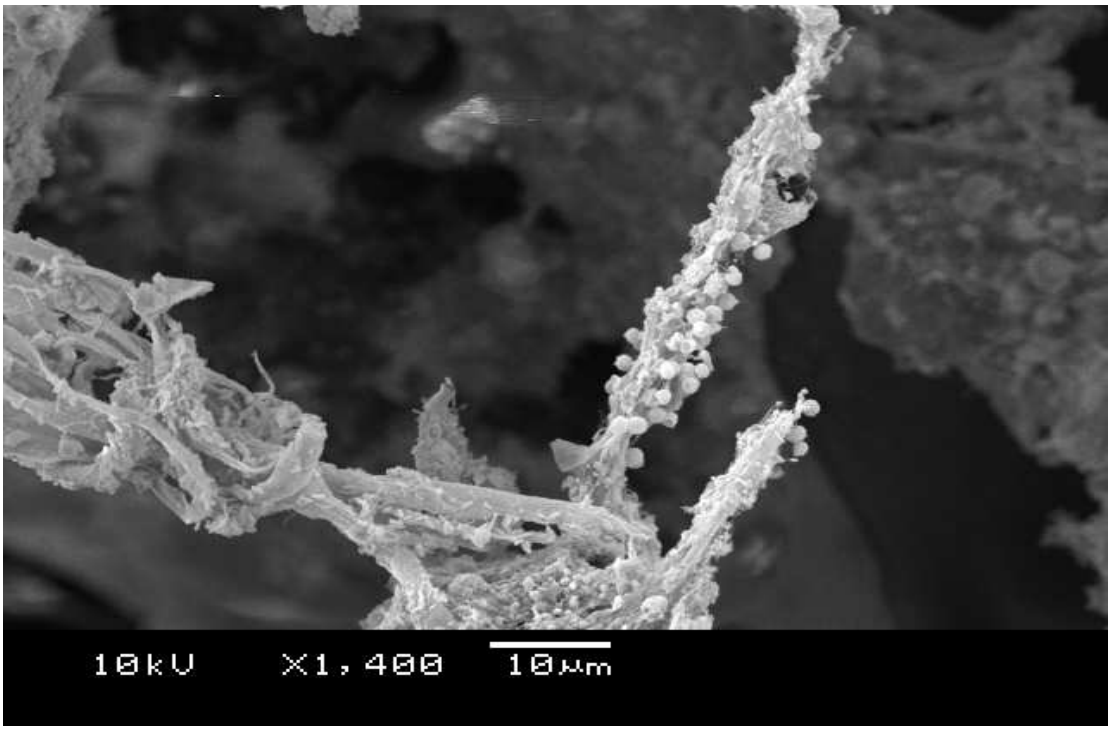

Figure 4. Fungal spores and mycelium (strands) on geotextile.

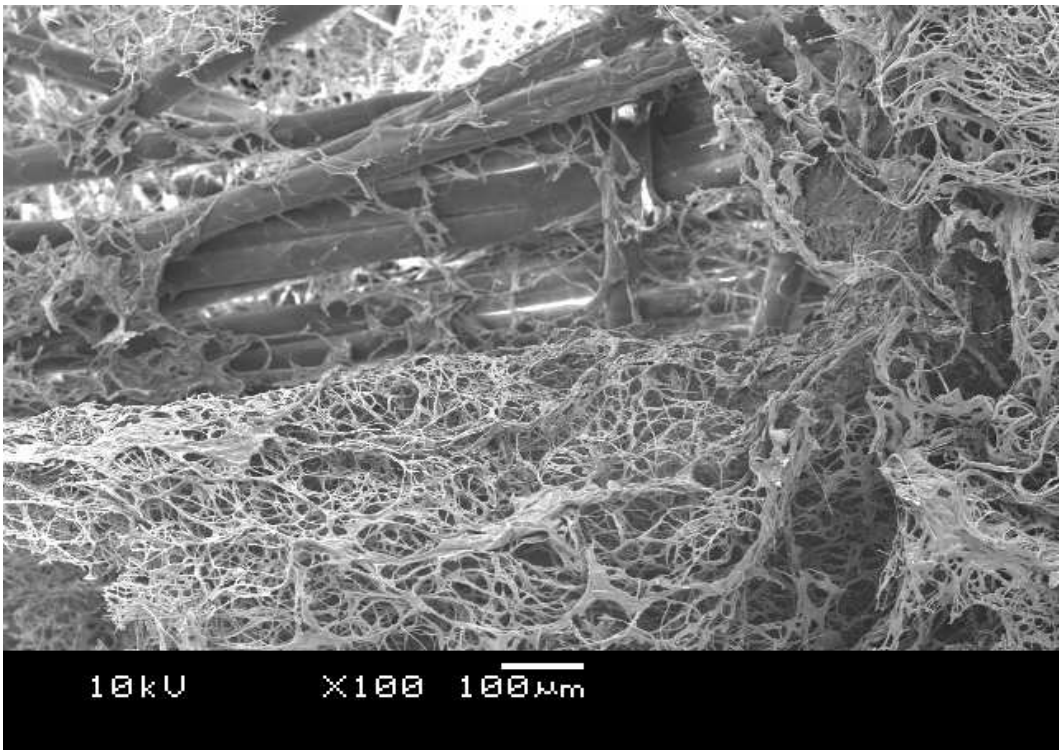

Figure 5. Large microbial aggregates on Inbitex geotextile.

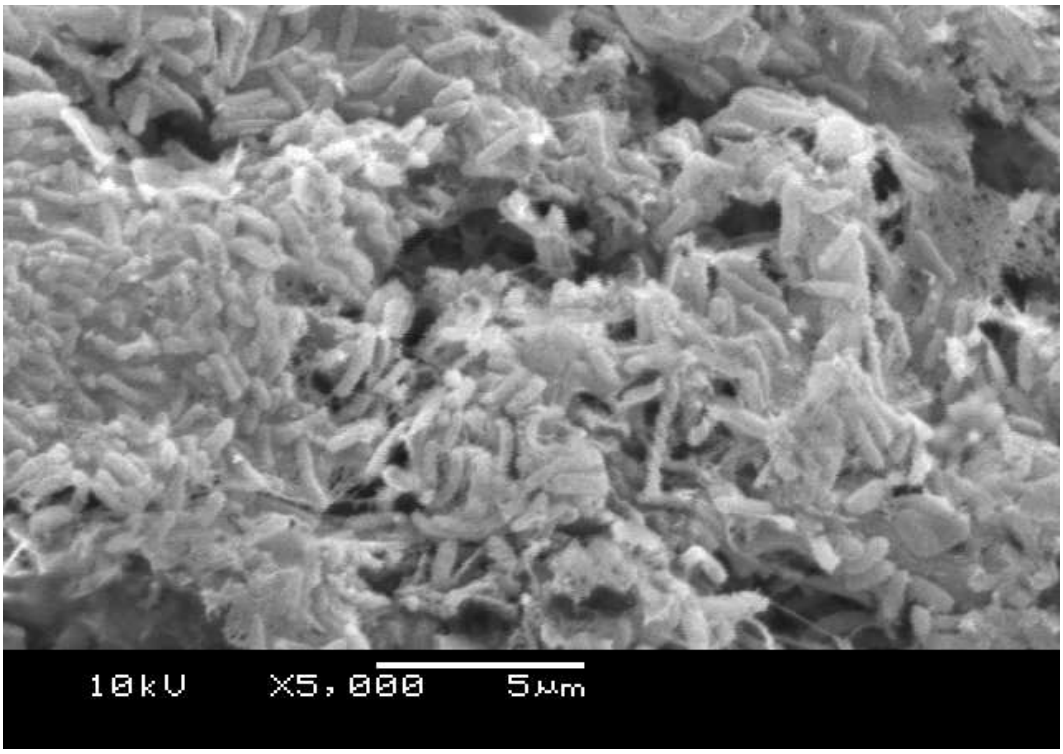

Figure 6. Large microbial aggregates, predominantly fungi, on Inbitex geotextile.

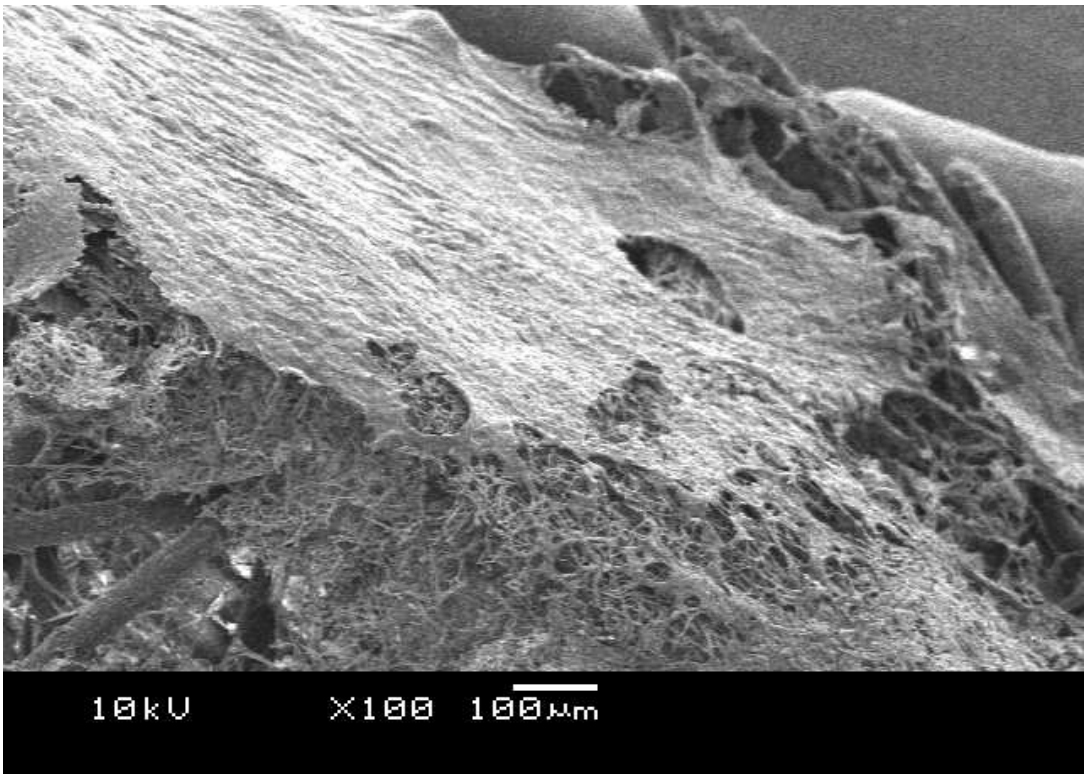

Figure 7. Bacterial growth on Inbitex geotextile.

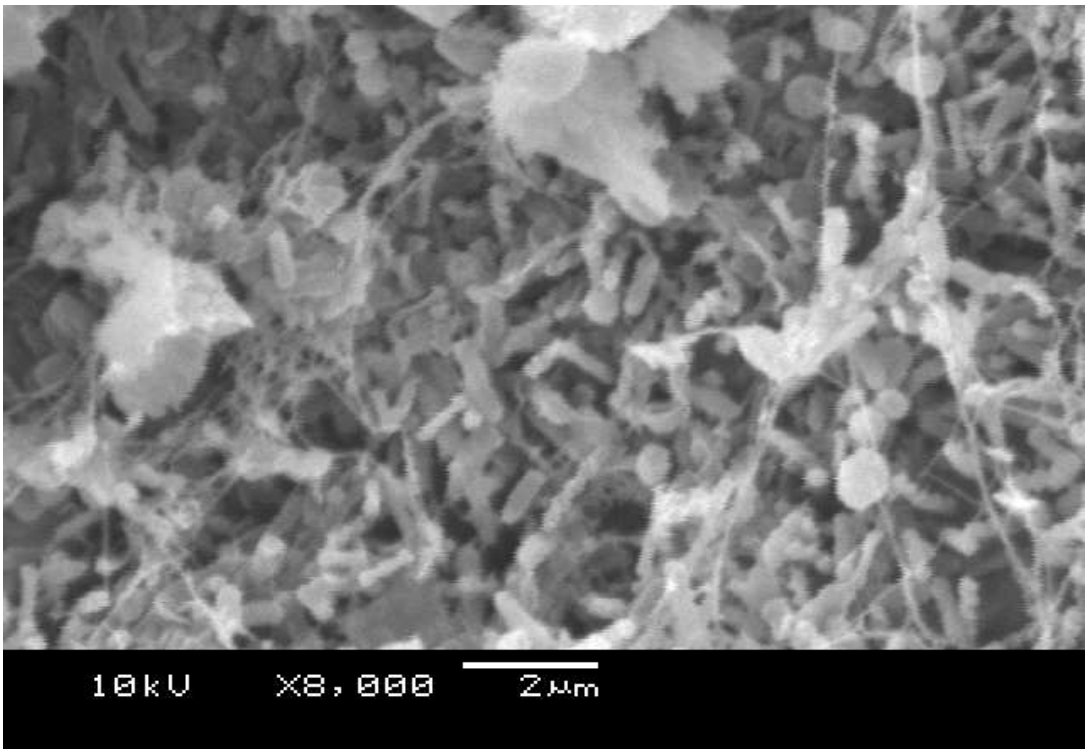

Figure 8. Bacterial growth on Inbitex geotextile.

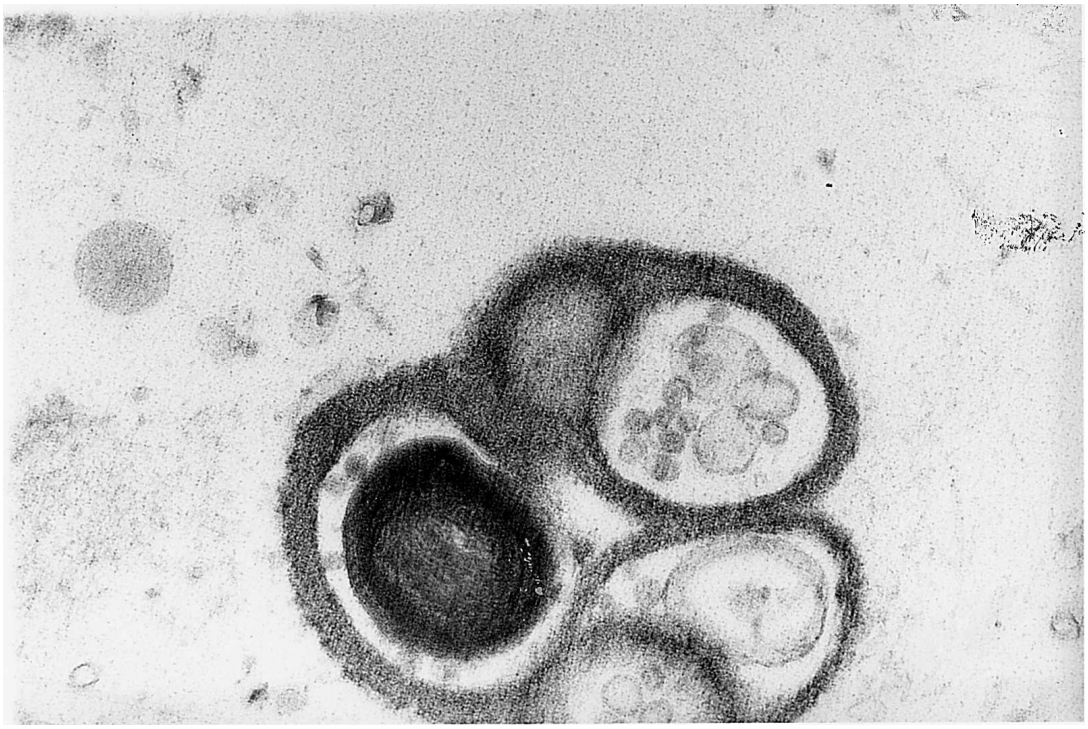

Figure 9. TEM of bacteria with oil droplets inside the cells.

Figures 4 and 5 show the extent of biofilm formation on Inbitex geotextile from PPS rigs with accumulations of well over 500 µm in length and 300 µm depth. These aggregates would be clearly visible to the naked eye should they be dislodged from the geotextile and appear to be composed primarily of fungal biomass. The larger cylindrical structures (about 50 µm in diameter) to the top of Figure 6 are the individual geotextile fibres that are becoming gradually covered by microbial growth.

The images in Figures 7 and 8 are highly magnified to resolve individual bacterial cells of less than 1 µm in length in places growing on Inbitex. Many different bacterial morphologies are visible in these images, including spirilli (spiral shaped), rods and cocci (rounded shape). These accumulations are encased in a mat of extracellular polymeric susbstances (EPS) which protects the bacteria from predatory protozoa and metazoa (small animals). It has been estimated that EPS may account for 80 % of the mass of the biofilm in some cases (Wimpenny, 2000).

Figure 9 shows a TEM image of a PPS grown bacterium from a heavily oiled geotextile. The important detail is the presence of oil droplets within enlarged cell vacuoles. This result was similar to previous results obtained by Kennedy and Finnerty, (1981) who observed hydrocarbons present within the cells of a soil dwelling *Alcaligenes* species by TEM. The presence of seemingly unaltered free product oil within a cell structure indicates considerable tolerance to the toxicity of oil, usually thought to be a serious inhibitor of bacterial cell membrane activity (Sikemma *et al.*, 1995). Bacteria harvested from non-oiled environments have been shown to have smaller vacuoles and no visible oil within their cells.

3. DISCUSSION

The results from the electron microscopy have demonstrated the high levels of biofilm accumulation and also the power of the SEM to reveal microscopic structures, even down to below 1 µm in size. The aim now of this paper is to explain the relevance of biofilm structure and function to the biological decontamination of hydrocarbons. This discussion will draw on results from the history of PPS research to demonstrate the fundamentally important contribution that microbes can make to PPS water quality.

3.1 Long term oil biodegradation.

As shown previously PPS rigs are capable of dealing with and biodegrading very high concentrations of hydrocarbon for a long period of time. For four years a PPS rig retained over98 % oil added at a rate 100 times that expected on an urban surface (Bond, 1999).

As the biodegradation of oils ultimately leads to a residual material similar to a tar mixed in with microbial aggregates, it had been expected that blockage of the geotextile would occur due to the very high oil loadings. However, this has not been observed from measurement of the discharge rates that showed no change in discharge characteristics between day 0 and day 1200.

Once the microbial community became better understood (Newman et al, 2002) it became clear that the complexity of the microbial community was partly responsible for this stability. As decomposers attach to surfaces and begin oil biodegradation they bring with them predators that perforate the biofilm, rip great chunks out of bacterial aggregates or filter feed from the biofilm edges. The sum total of this activity is that the biofilm is constantly undergoing a renewal process and dead material is consumed, solubilised by other microbes and recycled or discharged as ‘plates’ of microbial growth in visible form. This regulation of the PPS biofilm by predators is in fact very similar to the role they play

in soil, increasing fertility and keeping the soil aerated and in a good physical state with sufficient drainage (Kuikman, 1990).

The counting of nematode worms on PPS biofilms by the author has estimated that each gram of geotextile may contain up to 10^8 worms per gram of material. These microscopic animals are 0.2 mm in length but are present in sufficient abundance to strongly affect physical processes at the geotextile surface.

3.2 Recycling and regeneration of nutrients.

As shown in Figure 1 the PPS microbial community is characterised by a great many energy flows. As the predatory eukaryotes feed on the biofilm, they also secrete growth factors such as vitamins and nitrogenous compounds back in to the biofilm. This in turn gives nutrients to the biofilm. This leads to a certain level of recycling of important chemicals (see figure 10 below) and demonstrates that nutrient requirements in a PPS are more complex than simply adding NPK containing fertilisers. As presented by Coupe et al- "The biodegradation of clean and used oils", in these proceedings, many nutrient requirements are met even with oil as a source of N, P, K and C.

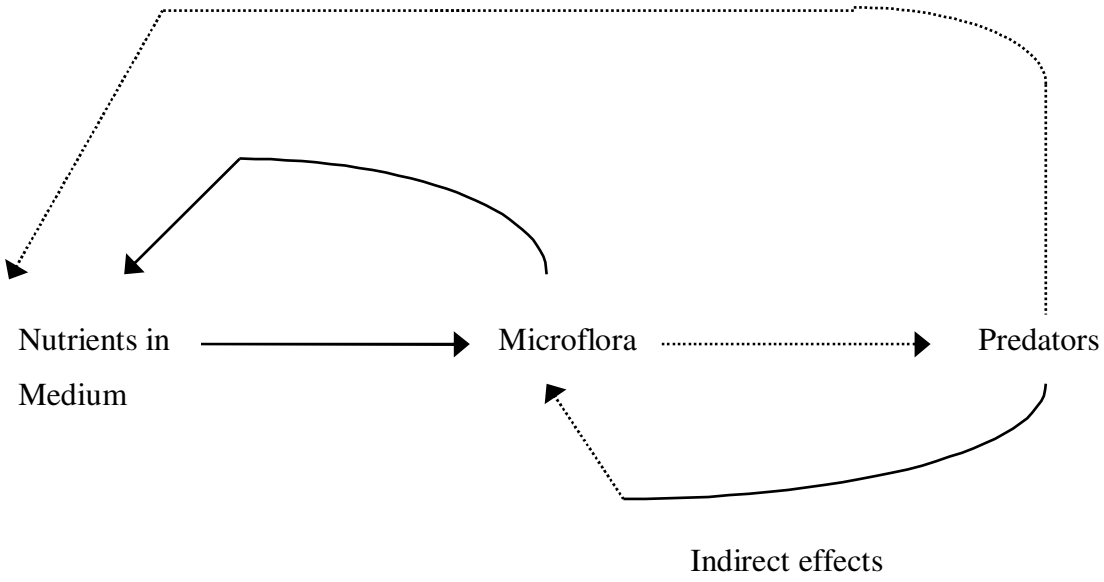

Figure 10. Schematic diagram of the nutrient flow between medium, microflora (decomposers) and predators. The dashed lines indicate effects of predators (Adapted from Griffiths, 1995).

4. CONCLUSIONS

Elucidating the structure of PPS biofilms has given real insight into the type of microbial communities found in such remediation systems. A complex web of interdependencies and processes contributes to the levels of performance observed from PPS rigs and this has furthered the Coventry group's understanding of the inner workings of the 'black box'. Although biofilm has been observed on sub-base materials by the authors and would no doubt give some treatment of oils even without a geotextile, it is the combination of retention and a platform that the geotextile, provides that makes these type of permeable pavement so effective at in situ-clean up.

5. REFERENCES

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