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Direct Wet Surface Imaging of an Anaerobic Biofilm by Environmental Scanning Electron Microscopy: Application to Landfill Clay Liner Barriers

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Summary: To contain domestic waste and its associated pollution within a landfill, engineered mineral (clay) barriers are used and are designed to have a permeability of 1×10^{-9} m/s (Westlake 1995). The rate of permeability of various porous media has shown to be influenced by the clogging of flow paths (media pores) due to biofilm formation (Charckalis and Marshall 1990, Cunningham *et al.* 1991). The term biofilm is given to describe the colonies of surface adherent microorganisms (Donlan *et al*. 1994). In this study, permeability experiments were built and modified to act as microcosms to investigate the influence of biofilm formation on the permeability of clay barriers. Traditional scanning electron microscopy methods disrupt or destroy the biofilm and previous anaerobic studies have involved building closed cells (such as miniature continuous culture chambers) that utilise light microscopes (Robin Jones *et al*. 1997). This paper examines the application of the environmental scanning electron microscope (ESEM) to the direct examination of the clay interface and biofilm formation in situ within the microcosm.

Key words: biofilm, anaerobic, environmental scanning electron microscope, landfill, clay barriers

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Introduction

Landfills are extremely complex environments in which bacteria are both controlled by and exert control over the local ecosphere. Simple laboratory experiments have not been able to replicate the full extent of these interactions (Young 1995). Within a landfill site, the effects of bacterial biofilm formation have been observed in the partial or full clogging of leachate collection drainage pipes (Paksy *et al.* 1998).

To investigate whether bacterial biofilm formation would clog the clay barrier medium (pores) at the waste / clay interface and influence permeability, a number of permeability cells, as described by Head (1994), were modified to incorporate a larger surface area and be utilised as microcosms. This would model the environmental conditions as found within a landfill cell. To represent a typical organic constituent of leachate, phenol was added to distilled water to form a permeant. This served as a model of an organic pollutant. Phenol was chosen as phenolic compounds are among the most common contaminants in landfill leachate (Wang and Barlaz 1998). In addition, very little attention has been paid to phenol effects on the microbial communities of landfills (Tibbles and Baecker 1989).

Half of the microcosms were seeded (with landfill leachate at 5% vol/vol and permeants modified to include a bacterial nutrient source) and shall be defined as phenol + BOD nutrient. The remaining microcosms were non-seeded, permeated with a phenol amended permeant. In addition, a microcosm permeated with distilled water was used as a control**.** These experiments were in operation for over 300 days. Upon decommissioning of the microcosm-modified permeameters, a rapid examination method was required (to prevent re-aeration of the anaerobic surface) to investigate the wet biological biofilm formation on the clay surface.

The environmental scanning electron microscope (ESEM), originally developed by Danilatos (1991, 1993b), was chosen for this purpose. The ESEM is becoming increasingly used in biological studies since it is able to visualise specimens in the wet or partially hydrated state (Gilpin & Sigee 1995). Applications of ESEM in various research areas are given by Danilatos (1993), and an extensive bibliography is available from FEIC Philips web page (accessed 15/02/00).

The ESEM minimises sample damage and changes in biological morphology that the scanning electron microscope (SEM) sample preparation procedures would induce. Specimens do not require extensive manipulation, fixation, dehydration, and air drying or critical point drying and metal coating for viewing purposes that a high vac-

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uum SEM would require. Nonconducting samples such as biofilms must be coated with a conductive film of metal such as gold palladium before the specimen can be viewed in the SEM. Uncoated nonconductors build up local concentrations, referred to as "charging," that prevent the formation of useable images (Little *et al.* 1991). In addition, any energy dispersive x-ray spectroscopy (EDS) analysis must be completed prior to the deposition of the thin metal coating.

The main advantage of the ESEM is the ability to work under a few mbar of pressure and without high vacuum as in a standard electron microscope. This allows the observation of liquid-containing samples without any special preparation or metal coating (Combes *et al.* 1998). Specimen charging is dissipated into the gaseous environment of the specimen chamber (water vapour) and, by adjusting temperature or pressure, controls the hydrated state of the specimen. The ability to visualise specimens in the wet state also raises the possibility of carrying out x-ray microanalysis (XRMA) on wet material, including living specimens (Gilpin and Sigee 1995), such as biofilms.

This paper presents results obtained from direct wet analysis of the biofilm obtained by ESEM and XRMA.

Materials and Methods

Specimen Preparation

Each sample was prepared individually prior to analysis. Upon decommissioning of the permeameters, after 300 days, the microcosm was drained of its liquid phase component, leaving a thin depositional coating over the clay surface. The thin liquid phase coating was required to maintain the wet anaerobic biofilm in its natural state. Reaeration of the anaerobic liquid phase can take place within a number of hours, which would destabilise the biofilm. Hence, a rapid sampling procedure and analysis methodology was adopted.

Sterilised razors were used to make incisions into the clay liquid interface at the centre of the microcosm surface. A centimetre square sample was cut into the clay skewed at 45° to the vertical. The specimen was then lifted from the surface using sterilised forceps, and the underlying cut areas were trimmed parallel to the surface to form an undisturbed thin sliver of the microcosm surface, as shown in Figure 1. The specimen was then freely mounted upon the specimen holder stud and immediately placed within the specimen chamber of the ESEM for examination.

Microscopy and Analysis

Specimens were analysed in an ESEM Philips XL30 (Electroscan Corp., Wilmington, Del., USA), fitted with a LINK PENTAFET atmospheric thin-window detector (Oxford Instruments Ltd., Bucks, England). The schematic diagram is shown in Figure 2.

Specimen chamber pressure ranged from 7.9–10.1 mbar for imaging wet samples in a controlled hydrated state, in an environment of water vapour, and 2.7–5.3 mbar for hydrated samples. Specimen temperature was maintained at 279–281 K using a Peltier-cooled stage. Working distance ranged from 11.3–13.1 mm, with the microscope adjusted to the analytical mode for the environmental secondary detector.

X-ray microanalysis was carried out at an accelerating voltage of 10 kV, 100 s livetime, over a full field view (normally 500–2000 μm^2), using a spot size of 0.7 μm diameter. Count rate was typically $1–2\times10^3$ cps.

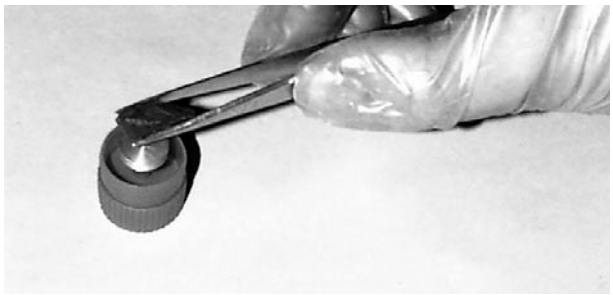

FIG. 1 Sample preparation.

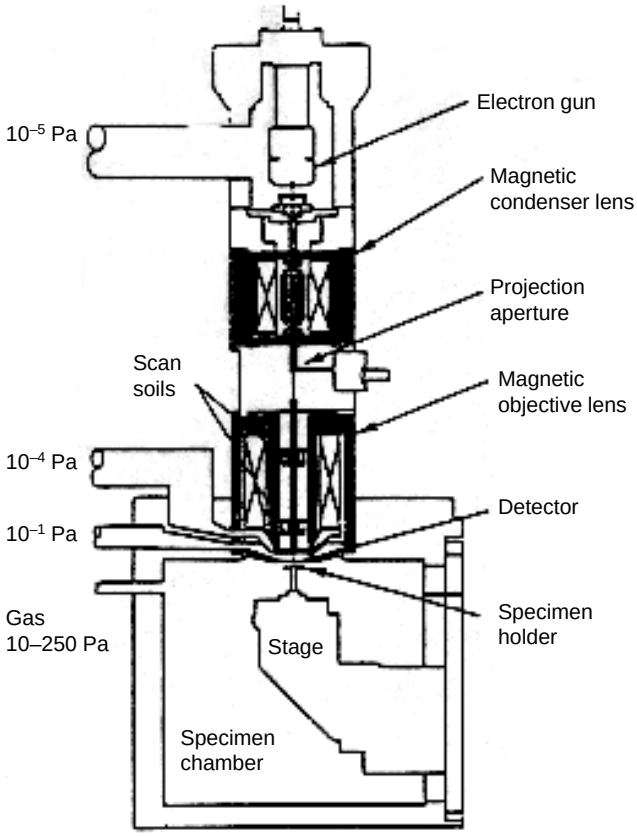

FIG. 2 Environmental scanning electron microscope.

Results

All ESEM images shown are of fully hydrated clay surfaces from the microcosms under anaerobic conditions, with the final ESEM image being that of a specimen that was purposely dehydrated.

Figure 3 illustrates an ESEM surface image of clay from a permeability cell that had only been subjected to a chemical (phenol). There is no evidence of any bacterial formations, and the matrix of compacted clay exhibits the typical macro- and micropore structure of the clay**,** as observed in the control sample (results not presented).

Figure 4a is a replicate of the previously described cell, which had been seeded with landfill leachate containing bacteria. The cell therefore was subject to a pollutant, a nutrient source (phenol + BOD nutrients). Extensive biofilm development can be seen. Macro- and micropores are no longer visible, indicating that these have been "plugged" because of bacteriological action. Figure 4b and c shows the bioweb at a greater magnification. Clumps of a sulphurous iron compound, possibly ferric sulphide, were identified by XRMA emission spectra (see Fig. 5) and are indicative of anaerobic biological activity. This can clearly be identified by the presence of nodules. Encasing the nodules in full or in part is the extracellular polysaccharide "slime" of biofilm.

Figure 6 is an ESEM image of the same specimen that was purposely dehydrated. Pressure was reduced slowly from 9.9 to 2.8 mbar to dry the sample out. Desiccation of the specimen surface covering upon dehydration indicates that the surface is covered by a biofilm. In the chemical only

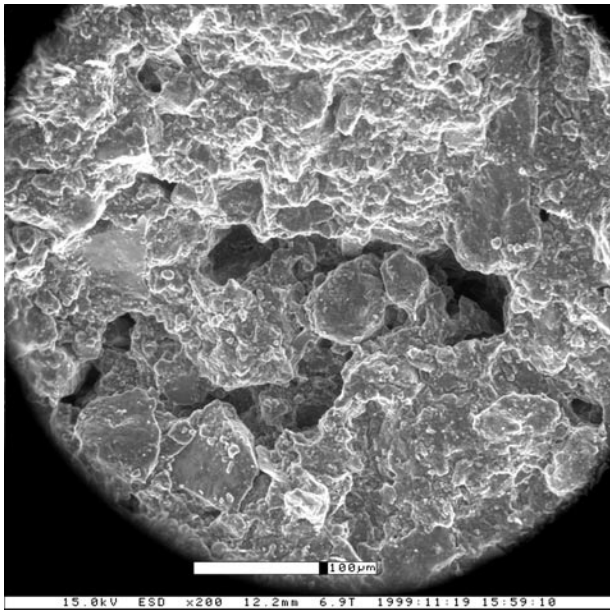

FIG. 3 Environmental scanning electron microscope surface image of the permeability cell containing chemical only (phenol) (marker 100 µm).

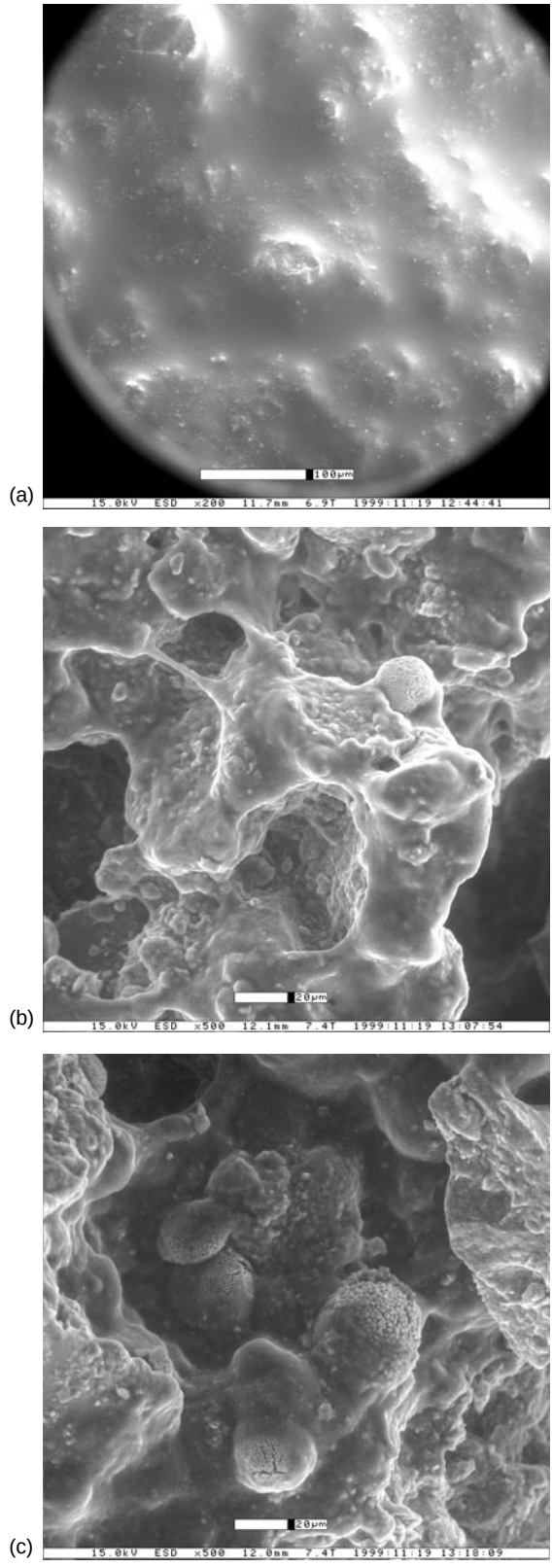

FIG. 4 (a) Environmental scanning electron microscope surface image of the seeded permeability cell (phenol+BOD nutrient) (marker 100 µm). (b) Bioweb at a greater magnification. (phenol+BOD nutrient) (marker 20 µm). (c) Bioweb at a greater magnification (phenol+BOD nutrient) (marker 20 µm).

sample, no cracking was seen when dehydrated. This shows that the cracking observed must be an effect induced by something other than clay and chemicals.

Discussion

Environmental scanning electron microscope imaging of the microcosm surface shows that there is a clear difference between the nonseeded and seeded microcosms. The nonseeded cells with pollutants added indicate that the clay matrix is unchanged and looks identical to that of the control microcosms, which had distilled water as permeant.

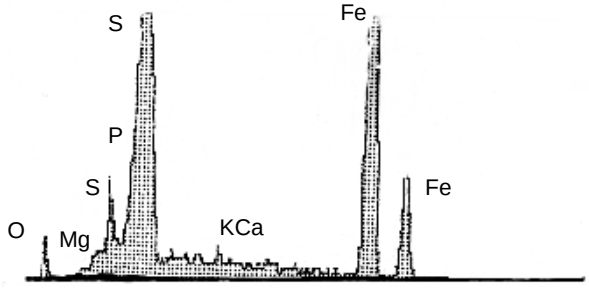

FIG. 5 X-ray microanalysis emission spectra of dehydrated specimen.

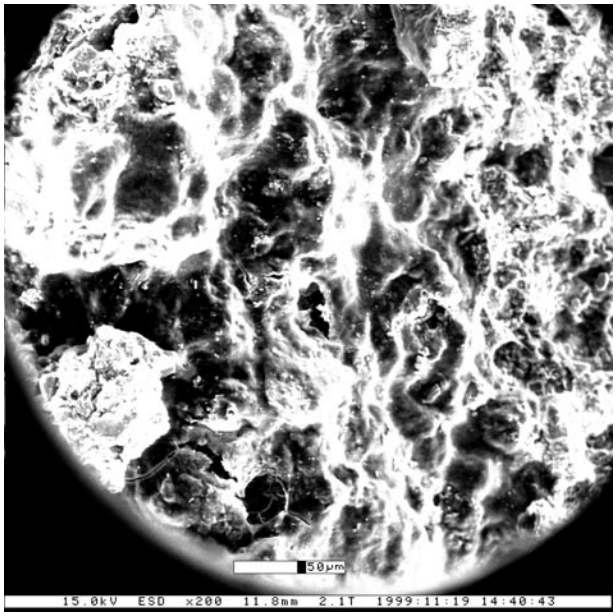

FIG. 6 Environmental scanning electron microscope image of dehydrated specimen (phenol+BOD nutrient) (marker 50 µm).

Landfill design and construction is primarily based upon permeability experiments using mono or combined chemical testing. Landfills are biological reactors, and as such this is the first modified permeability test to incorporate a biological influence.

Differences due to the influence of biological activity are seen in the form of a biofilm formed on the clay liquid phase interface of the seeded microcosms. The microcosms permeated with phenol + BOD nutrients are depicted in Figure 4a to c; this sample was then dehydrated as depicted in Figure 6. A similar effect could have been produced in Figure 4 had the sample been exposed to excess water. When the sample was dehydrated, however, the sample contained a surface crust.

The hydrated gel (biofilm) viewed in Figure 4 could not have been due to excess water, as this would have evaporated when viewed dehydrated. The fact that a crust was observed must have been due to the addition of bacteria, which was the only difference between the nonseeded and seeded samples. The addition of bacteria has clearly demonstrated that a biofilm exists because, when the biofilm is hydrated, a surface crust remains.

Microorganisms that colonise surfaces produce polymers and form a gel matrix that is central to the integrity of the biofilm (Consterton and Geesey 1986). The surface topography of the seeded microcosms consists of a surface covering of a gel matrix, interbedded with nodules. Only within the seeded microcosms can the biofilm and nodules be seen. The normal macro- and micropore structure of the clay matrix is now modified because of pore plugging by the biofilm, at the surface interface.

X-ray microanalysis of biological specimens in the ESEM provides a potentially useful complement to conventional procedures (Sigee *et al.* 1993). In this study, it provided information on elemental composition of the nodules. X-ray microanalysis emission spectra identified iron and sulphur as the main elements in the nodules. Background elemental composition was determined from analysis of the control specimen.

These nodules are indicative of mineralisation processes often seen in wetlands, which are of a similar anaerobic ecological form to the microcosms. Ferris *et al.* (1989), when discussing biofilms in acidic and neutral pH environments, state that the extracellular polymeric material surrounding the cells typically exhibited a fibrous structure and often contained an abundance of iron rich precipitates. Clumps of acicular crystalloids developed under acidic conditions. These were noted as containing sulphur under acidic conditions. This would form a sulphurous iron clump, which is what was probably observed within the microcosms.

Conclusions

Environmental microscopy allowed immediate visualisation of the surface in its natural wet anaerobic state. This

is as close as practicable to obtaining an in-situ investigation of the microcosms.

Pore plugging as seen in other disciplines has been observed in the inoculated microcosm using ESEM imaging.

Biological activity contributed to the differences in observed surface morphology.

X-ray microanalysis provided identification of the sulphurous iron nodules, which reveals information regarding the ecological conditions and reactions occurring in the inoculated microcosms.

By examining the specimens during their natural wet anaerobic state in the ESEM, a greater understanding of microbial interactions upon the clay / leachate (liquid phase) can be obtained. This, in turn, will lead to a better assessment of the clay liners in containing waste and its associated pollution.

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