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Substrata effects on bacterial biofilm development in a subsurface flow dairy waste treatment wetland

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Abstract Biofilm development on two distinct rock substrata was investigated both *in vitro* and in a subsurface flow wastewater treatment wetland in order to determine the effect of hydrophobicity on initial bacterial adsorption, tertiary biofilm development and microbial population structure. Two commonly used wetland rock types, slag (a hydrophobic by-product of the steel smelter industry) and greywacke (a more hydrophilic sedimentary rock) were evaluated. *In vitro* investigations of initial microbial adsorption trends showed that the more hydrophobic slag displayed rapid bacterial adsorption rates compared to greywacke. Mean microbial adsorption rates of a mixed wetland bacterial population over 5 hours, described using a first order kinetics model, were 1.3×10^{-12} m/sec for slag and consistently lower at 8.7×10^{-13} m/sec for greywacke. Pristine rock studs of the two substrata were also exposed to wetland microbial communities during a six week field trial using confocal scanning laser microscopy to determine tertiary biofilm structure and fluorescent *in situ* hybridisation to investigate bacterial populations. During the first five weeks of growth CSLM analysis revealed that 75% of biofilms on slag were thicker and had greater coverage compared with those grown on greywacke. After six weeks of growth over 50% of the tertiary biofilms were structurally very similar on both rock types and only 25% of those grown on slag were larger than those on greywacke. *In situ* hybridisation analysis of bacterial populations revealed very little difference in population structure between biofilms grown on slag and those grown on greywacke. Eubacteria were present as a very high proportion of total bacteria throughout biofilm development (74.3%). The beta subgroup was the most populous of the *Proteobacteria* (31.4%) followed by the gamma subgroup (13.4%)and the alpha subgroup (1.3%).

The results of this study suggest that slag, as a more hydrophobic substratum, promotes the initial adsorption of bacteria during early biofilm growth and better supports mature biofilm structures when used in wetlands. This study has implications for the design and construction of wastewater treatment wetlands.

Keywords Adsorption; confocal scanning laser microscope; FISH; hydrophobicity; population structure

Nomenclature

$d\Gamma/dt$	change in bacterial surface concentration over time (cells m^{-2} sec^{-1})
c_o	concentration of bacteria in the bulk solution (cells m^{-3})
κ_s	bacterial kinetic constant (m sec^{-1})
Γ	bacterial concentration on the surface (cells m^{-2})
ad	bacterial adsorption
des	desorption

Introduction

All biofilm development begins with the complex process of single bacterial cells attaching to a submerged surface. The major factors governing the initial adsorption of bacterial cells to a surface are electrostatic charges, van der Waals' forces and the hydrophobicities of both the bacterial cell and the surface (van Loosdrecht *et al.*, 1989; Bos *et al.*, 1996). The second stage of bacterial attachment to surfaces involves biological and chemical responses, such as the production of extracellular polysaccharides and the attachment by pilins and polymers (Ward and Berkeley, 1980). From this stage a biofilm may begin to attach larger particulate matter and hence develop a pronounced tertiary structure. Once a mature

tertiary structure develops in a biofilm its shape is dictated by such processes as predation by microbes and invertebrates, erosion by shear forces (Pederson, 1990), abrasion by particulate matter (Blenkinsopp and Lock, 1994) and sloughing (Rubio and Wilderer, 1987).

In waste treatment wetlands, function is dependent on the inter-relationship between microbial populations (predominantly in the form of biofilms) and the substrata, be that plants or inorganic material. The aim of this research was to fully characterise the early stages of biofilm development on specific substrata commonly used in wetland construction. In order to do this both *in vitro* and *in situ* experiments were performed using the rock substrates with differing hydrophobicities, greywacke and slag, as test substrata.

Static incubation systems (Pringle and Fletcher, 1983, Sjollema *et al*., 1989) were used in this study as an *in vitro* mimic of the processes occurring during the initial biofilm development in a subsurface flow wastewater treatment wetland. Mixed planktonic wetland microbiota were exposed to the wetland rock substrata and were periodically examined in order to establish the amount of bacterial adsorption to those surfaces. From this adsorption kinetics could be calculated and used, together with knowledge of the substratum properties, to characterise these early stages of growth.

In order to develop an understanding of latter stages of biofilm maturation, and further refine our understanding of the impact of substratum characteristics, field trials were undertaken in a subsurface flow wastewater treatment wetland. Biofilms from this system were grown and then analysed using fluorescent *in situ* hybridisation (FISH) and confocal scanning laser microscopy (CSLM) to investigate biofilm structure and population composition over time.

Materials and methods

Wetland field site

A prototype, subsurface flow, dairy effluent treatment wetland in South Auckland, New Zealand was used for all field trials. The treatment system is one anaerobic pond receiving dairy shed effluent followed by an aerobic pond. Effluent from this pond is discharged into a subsurface flow wetland (Figure 1) consisting of two unplanted trenches filled with slag. The effluent from the wetland was discharged to a stream. The wetland had an average retention time of 12.5 days.

Four sampling points were established in the wetland as shown in Figure 1. A slide holder, within a perforated outer casing, was placed in the wetland at each of the sampling locations. Test slides, with attached rock studs, were then placed vertically at 15 cm and 55 cm depths in each of the holders.

Substratum preparation

Samples of greywacke (a sedimentary clastic rock) and slag (the residue of iron production from iron sand), were sliced into three millimetre thick slices using a Hillquist rock saw and

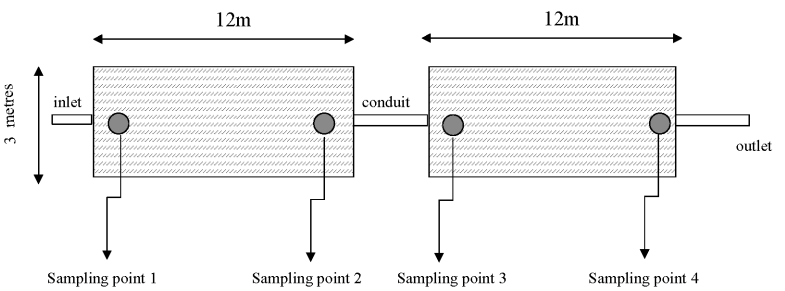

Figure 1 Schematic diagram of wetland together with biofilm sampling sites

grinder. The rock sections were attached to glass slides (25 × 40 mm, Biolab Scientific) using Hillquist epoxy resin and allowed to cure for three hours at 70°C. The surfaces of the rocks were ground to a uniform surface roughness, rinsed in water, air dried and stored in sterile covered beakers.

In vitro experiments

Characterisation of rock surface properties. Comparison of substratum roughness and elemental composition was performed using a Philips Model 505 Scanning Electron Microscope (SEM) with an attached Energy Dispersive X-ray analysis (EDX) unit. Microanalysis was performed on freshly prepared rock substrata by focusing the electron beam on a 0.4 mm^2 area of the rock surface with a penetration depth of 1 μm, 15° sample tilt, accelerating voltage of 20 kV. Five spatially separate areas of each rock sample were examined at 1,250 × magnification. Each sample was analysed for a total of 100 seconds using a probe current of 1nA. Semi-quantitative estimations were generated for individual elements using the IMIX software package (Princeton GammaTech, USA) from which percentage composition analyses for predetermined elements were obtained.

Determining hydrophobicity. A sessile water droplet (10 μl) was placed on the test substrata in a modification of the technique used by Pringle and Fletcher (1983). The angles of the water droplet were photographed and enlarged and the contact angles were determined. If both angles agreed to within 5° it was assumed that surface roughness or heterogeneity had not modified the angle of the water droplet.

Static incubation. Samples of dairy wastewater effluent were collected from the outlet of the oxidation pond, chilled and processed within three hours of collection. Bacterial concentrations in the effluent were estimated by counting 20 fields using a gridded haemocytometer and an Olympus BH2 microscope (Olympus Optical Company, Tokyo, Japan) with a 100 × oil immersion lens.

For the static incubation system glass slides, acting as structural support for the test rock surfaces and control surfaces, were force-fitted vertically in 45 ml sterile centrifuge tubes (Boehringer Mannheim GmbH, Mannheim, Germany). Forty millilitres of the wetland effluent was then added to the incubation chamber and stored in the dark at 25°C. Substrata were removed at varying times and a 1 μg/ml concentration of 4',6-diamidino-2-phenylindole (DAPI) was used to stain the substratum for 5 minutes and bacteria were then enumerated using epifluorescent microscopy.

In situ hybridisation

All biofilm samples, after a set period of growth in the wetland, were fixed, hybridised and washed according to the procedure described by Manz *et al*. (1992). Briefly, samples were fixed for 1 hour in 4% paraformaldehyde and lysed in 0.1% non-ionic detergent (Nonidet P-40, Sigma) for three minutes. Two lysis steps was completed and samples were then dehydrated in three-minute steps in 50%, 80% and 99% ethanol.

Specific rRNA-targeted oligonucleotides were used to identify the alpha, beta and gamma subgroups of the *Proteobacteria* and the eubacteria. Probes BET (GCC TTC CCA CTT CGT TT), GAM (GCC TTC CCA CAT CGT TT) and EUB (GCT GCC TCC CGT AGG AGT) were 5′-aminolinked with the carbocyanine dye CY3 (excitation 552 nm. Biological Detection systems, Pittsburg, USA). Probe ALF (CGT TCG (C/T)TC TGA GCC AG) was 5′-aminolinked with the carboxyfluorescein (excitation maximum 488 nm). All probes were manufactured by MWG-Biotech (Ebersberg, Germany). Each biofilm sample was hybridised according to the protocol of Manz *et al*. (1992). The slides were air-

dried and counter-stained with 1 μg/ml DAPI (4,6-diamindino-2-phenylindole) (Sigma) for 10 minutes in the dark before being rinsed with distilled water as described by Porter and Feig (1980).

Epifluorescent microscopy. Hybridised bacteria were enumerated using a Zeiss Universal microscope with an excitation wavelength of 350 nm. Samples were magnified using a 63x oil immersion lens with a 2 × or 1.25 image enhancer and a 10 × stage magnifier. Twenty fields of vision from triplicate samples were analysed concurrently and results expressed as average number of cells/mm^2.

Confocal scanning laser microscopy. Biofilm structural analyses were performed on the untreated biofilms using the technique described in Silyn-Roberts and Lewis (1997). Biofilms were stained using 0.01% acridine orange. Separate transects, each increasing 1 μm in height above the previous, were individually analysed using UTHSCSA *ImageTool* software program.[1] The resultant image was then analysed by greyscale thresholding. These data were then graphed to give an averaged image of the biofilm coverage versus height.

Results and discussion

Substratum characteristics

Hydrophobicity, element composition and surface roughness analyses determined that hydrophobicity was one of the major differences between the two rock types. Both rock types had similar surface roughness and elemental composition (Table 1) while hydrophobicity was significantly stronger on the slag surface than on the greywacke. Overall, these findings suggested a comparison might be made of the two substrata where hydrophobicity was the governing difference between the two rock types.

Initial adsorption of wetland bacteria to substrata

Effluent from the oxidation pond (7 × 10^4 cells/mm^3) was used to inoculate static incubation systems. Adsorption data for the first two hours were extremely variable with low cell numbers and standard deviations higher than 10% and were discarded. Beyond two hours a cell adsorption preference to the more hydrophobic slag was apparent (Figure 2).

A simple model was used to describe the kinetic process of bacterial adsorption to the substratum surface using a static system (Duhkin and Rosenberg, 1995).

$$\frac{d\Gamma}{dt} = \kappa_{ad} c_o - \kappa_{des} \Gamma \qquad (1)$$

Adsorption kinetics were then determined from the gradient of the regression line together

Table 1 Substrata characteristics used for biofilm development studies

Substratum	Water contact angle (s)	Surface roughness	Elemental content (in order of decreasing % composition)
Greywacke	44° ± 7°	Planar surfaces 50 to 200 μm^2 in size	Iron, calcium, aluminium, silicon and magnesium
Slag	77° ± 8°	Planar surfaces 10 to 100 μm^2 in size	Titanium, calcium, magnesium and silicon

[1] Developed at the University of Texas Health Science Centre at San Antonio, Texas, *ftp://maxrad6.uthscsa.edu*

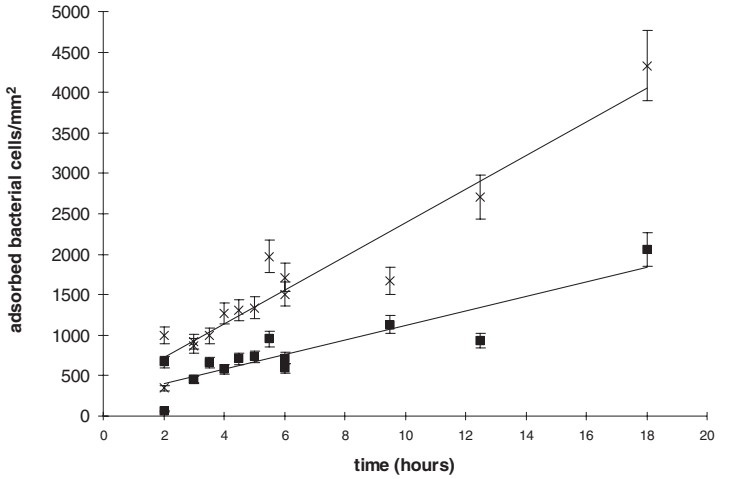

Figure 2 Average adsorption of wetland bacteria, over 18 hours, onto the rock substrata slag (x) and greywacke (+)

with the initial concentration of the bacteria in the liquid suspension. It was assumed that: a) adsorption was the only transfer process and, as such, was the rate-limiting transfer process; b) surface bacterial concentrations were small i.e. $\Gamma \cong 0$.

Thus, for initial adsorption, Eq. (1) becomes:

$$\frac{d\Gamma}{dt} = \kappa_{ad} c_o \tag{2}$$

Integrating over the initial adsorption period, assuming no significant change in the bulk cellular concentration, Eq. (2) becomes:

$$\Gamma_t = \kappa_{ad} c_o t \tag{3}$$

Eq. (3) was then used to determine the rates of adsorption of bacterial cells onto substrata, given the initial concentration of the bacterial suspension and the change in bacterial concentration on the surface over five hours. It was found that the kinetics of bacterial adsorption to greywacke was 8.7×10^{-13} m/sec (R^2 value of trendline = 0.80) while slag was 1.3×10^{-12} m/sec (R^2 value of trendline = 0.92).

The differences of initial adsorption of bacterial cells onto surfaces of different hydrophobicity agree with those of Fletcher and Floodgate (1976), Fletcher and Loeb (1979), and Pringle and Fletcher (1983) for static systems. Kalmbach *et al*. (1997) also made similar observations in *in vivo* experiments with drinking water pipes.

Effect of substratum on biofilm growth over six weeks in the wetland

Biofilms grown on slag and greywacke for six weeks at each site in the wetland were analysed for biofilm tertiary structure using CSLM and for bacterial community structure using *in situ* hybridisation.

Bacterial community composition. Figure 3 presents the results of *in situ* hybridisation of bacterial cells within growing biofilms at a the site adjacent to the inlet of the wetland (site 1 at 15 cm depth). These data are representative of those found at the other wetland sites and depths in the wetland. No statistically significant effect of substratum could be found on population dynamics.

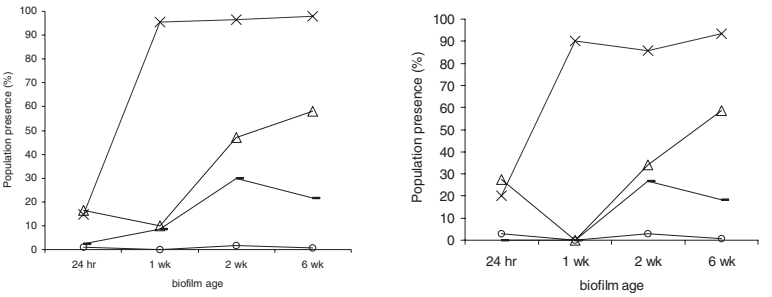

Figure 3 Changes in bacterial populations on slag (left) and greywacke (right) grown at 15 cm depth at Site 1: Eubacteria (x), alpha (o), beta (Δ) and gamma (–)

Trends were observed in the overall wetland population dynamics. Eubacteria were present as a very high proportion of total bacteria throughout biofilm development (overall average of 74.3%). The beta subgroup was the most populous of the *Proteobacteria* (31.4%) followed by the gamma subgroup (13.4%) and the alpha subgroup (1.3%). Very similar proportions of *Proteobacteria* have been observed in other aquatic systems such as

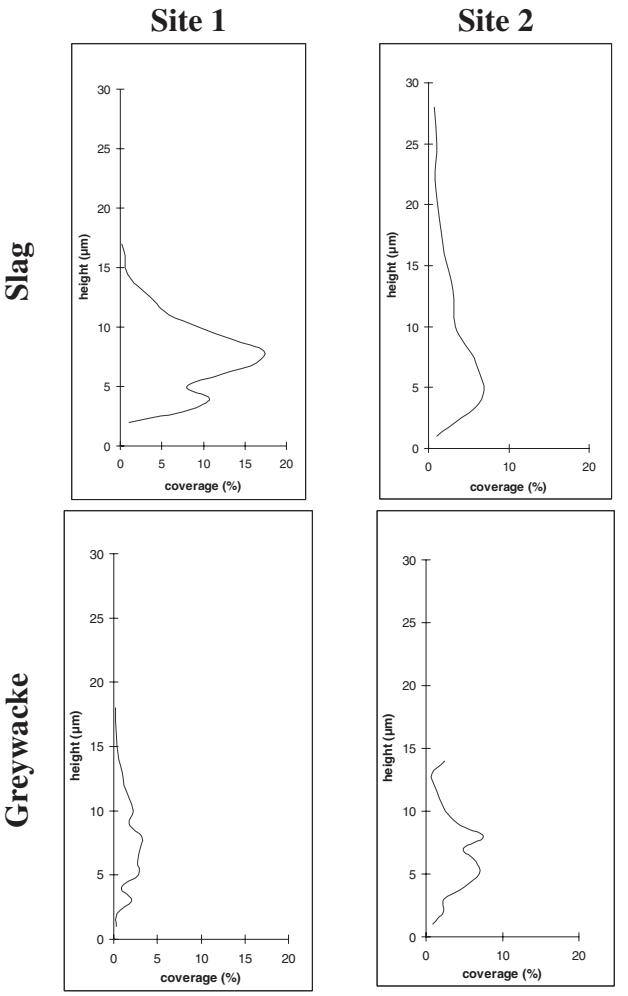

Figure 4 Comparison of four-week-old biofilms grown on greywacke and slag rock substrata at 15 cm depth throughout the wetland

activated sludge (Snaidr *et al*., 1997) and lakes (Alfreider *et al*., 1996), while similar proportions of eubacteria have been observed in drinking water (Kalmbach *et al*., 1997). An interesting facet of these results is the speed with which these bacterial subpopulations establish in biofilm matrices.

Biofilm tertiary structure. Biofilms grown in the wetland were observed to develop from monolayers through to complex tertiary structures of varying height and coverage over six weeks. Figure 4 below shows the results of CSLM used to determine the tertiary structure of four week old biofilms grown throughout the wetland. There is a distinct difference, in terms of biofilm height and coverage, between the two different rock types. The most prominent trend observed was that biofilms grown on slag appeared to contain more mass, both in terms of biofilm thickness and coverage than that on greywacke. These results are similar to those found by Kalmbach *et al*. (1997), where hydrophobic surfaces (polyethelene) attracted over five times as many bacterial cells as the hydrophilic (glass) for up to 50 days. The results of this wetland study indicate much the same results, with higher bacterial adsorption and subsequent growth on the hydrophobic slag.

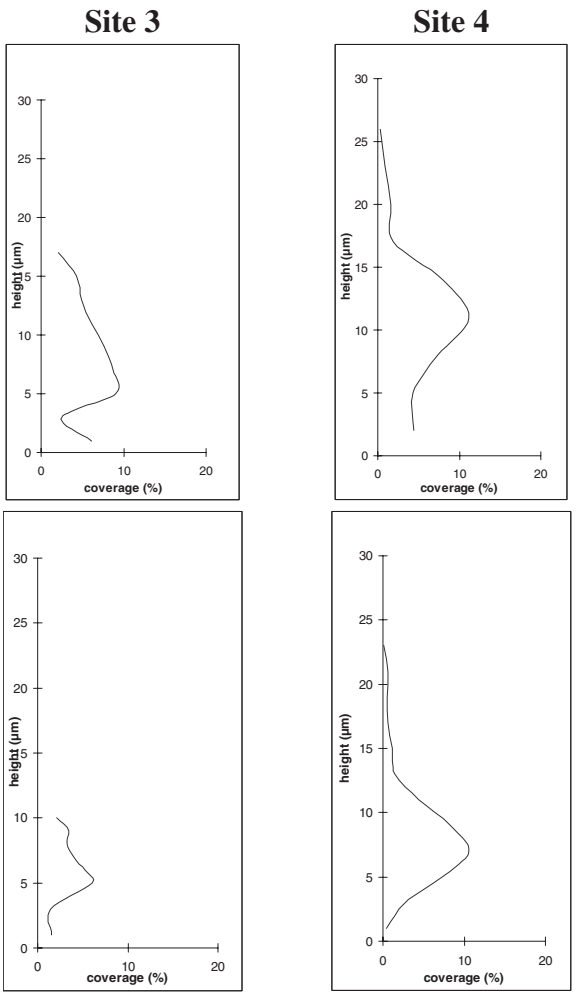

Figure 4 *Continued*

However, by the sixth week, over 70% of biofilm structures on greywacke and slag were similar in terms of both maximum height and maximum coverage. Only 12% of biofilms grown on slag were larger than those grown on the greywacke. This suggested that older biofilm structure was dependent on factors other than substratum.

Conclusions

While other studies have shown that substratum hydrophobicity is a major factor in the adsorption of isolated bacterial cells, this study is unique in that it demonstrates the extrapolation of these previously published findings to the wider context of adsorption of complex microbial communities to rock substrata. In wastewater treatment systems such as wetlands, where biofilm growth is promoted, the use of hydrophobic material may assist establishment of biofilms and subsequently enhance growth rates. Despite the complexity of the experimental operating parameters, trends were established in terms of initial bacterial adsorption to substrata, biofilm tertiary structure and microbial population dynamics. In this instance, it was shown that the more hydrophobic substratum, slag, promoted greater biofilm development for prolonged periods.

This study has provided a profile of microbial population changes and biofilm development and has implications for improvement of overall wetland performance and relevance to wetland design, operation and use.

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