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CHANGES OF BIOFILM PROPERTIES IN RESPONSE TO SORBED SUBSTANCES – AN FTIR–ATR STUDY

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ABSTRACT

Biofilms play an important role as sorbents in the process of distribution of pollutants in surface water systems. The sorption properties of biofilms will influence the kind and the amount of sorbed substances. The heterogeneity of biofilms provides different sorption sites which exhibit a different sorption preference and capacity. As dynamic systems, biofilms will respond physiologically to their environment. Thus, the sorption of one substance may lead to a change in the composition of the EPS or other biofilm components and further alter the original sorption properties. In this paper, the influence of toluene on a biofilm was investigated. As a suitable method, FTIR-ATR spectroscopy was applied. The method is non destructive and allows the observation of biofilm formation and behaviour on line and *in situ*. A biofilm was allowed to form in ATR flow-through cells. The test strain was genetically engineered and contained a bioluminescent reporter gene which was switched on when toluene was metabolized. Thus, the degree of toluene degradation activity could be observed with great sensitivity. The FTIR spectrometer contained three flow-through cells which could be operated in parallel: one was run with sterile medium only, one with medium and bacteria, and one with medium, bacteria and toluene. This arrangement allowed the discrimination of the biofilm response from other effects. The IR spectrum showed specific bands of proteins, polysaccharides, phosphoryl compounds and other groups of molecules. A significant increase of EPS-polysaccharide formation was observed at a toluene level of 5 mg L^{-1}. At 15 mg L^{-1}, significantly more carboxyl groups were formed. Thus, the effect of the lipophilic organic pollutant toluene increased the amount of negatively charged groups and, consequently, the sorption capacity for metal cations. This result indicates that biofilms respond in a complex manner to different sorbates and alter their environmental properties.

KEYWORDS

Biofilms; degradation of toluene; FTIR-ATR spectroscopy; sorption capacity.

INTRODUCTION

Biofilms are complex microbial systems and respond to nutritional conditions. Not only the composition of the microbial population can change (Pujol and Canler, 1992) but physiological changes are also possible in response to degraded and/or sorbed substances. If a biocoenosis is exposed to a biodegradable organic

pollutant, it is of interest to know if and how much the activity may change and which concomitant physiological alterations occur. However, it is difficult to detect such interactions. The introduction of Fourier transform infrared spectroscopy into microbial ecology allowed the investigation of adhesion and physiology of biofilm organisms *in situ*, non-destructively, on line and in real time (Nichols *et al.*, 1985). As a model system in our investigations, the interaction of toluene with *Pseudomonas putida* was chosen. As a member of the BTEX group, toluene belongs along with benzene, ethyl benzene and xylol to an important group of pollutants and has been included in the U.S. EPA priority list of pollutants which have an impact on human health (Sittig, 1985). Toluene can be biologically degraded under aerobic and anaerobic conditions and, therefore, does not constitute a recalcitrant compound. In order to detect toluene degradation in such a small system as represented by a biofilm on an FTIR-ATR crystal, it was necessary to use an extremely sensitive probe. This can be obtained using bioluminescence. Thus, a strain was used in which the *lux* operon and the TOL plasmide pWWO were fused (Burlage *et al.*, 1989; kindly supplied by B. Burlage, Center for Environmental Biotechnology, University of Knoxville, TN). Here, a reporter gene was introduced which allowed the quantitative detection of toluene degradation activity, measured by emitted light. The aspects of toluene degradation are reported elsewhere (Schmitt *et al.*, in prep.), while the paper presented here focused on the physiological biofilm response to the exposure of toluene.

MATERIALS AND METHODS

3-channel FTIR spectrometer

An RFX-30 FTIR interferometer (Laser Precision Analytical) was used to design a three channel FTIR-spectrometer. It was equipped with three ATR flow-through cells resulting in parallel spectra for comparison. The design of a flow-cell is depicted in Fig. 1; further details are given by Nivens and Schmitt (1993).

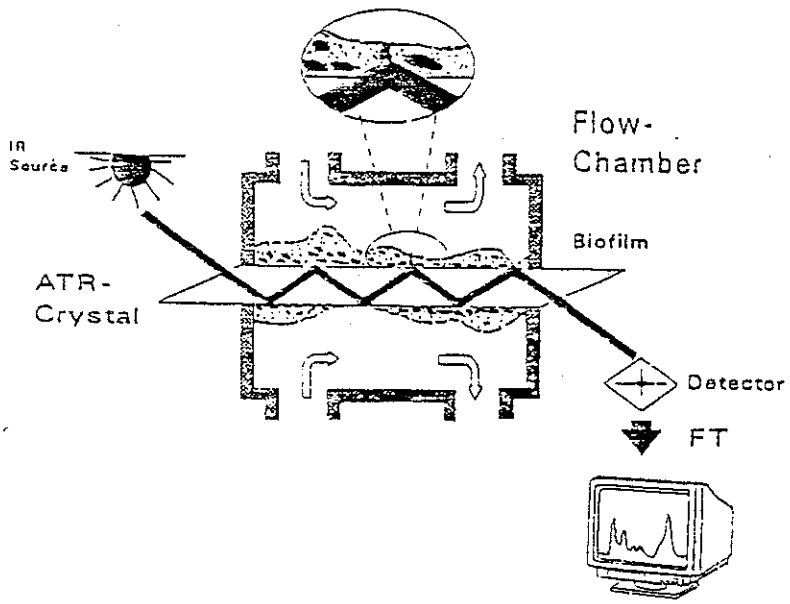

Figure 1. Design of an ATR flow-through cell.

The flow-chamber volume was 1.7 mL. As an internal reflection element, a germanium ATR crystal was used (50 x 20 x 3 mm) with 19 active reflections inside. Further details of FTIR-ATR spectroscopy are given by Griffiths and de Haseth (1986) and Schmitt *et al.* (1995).

System design (Nivens and Schmitt, 1994). The system contained three channels. In channel I, the sterile medium was used as a control. Channel II contained a biofilm of a *Pseudomonas putida* strain and was used to obtain the signal of the resulting toluene-free biofilm. In channel III, the procedure was the same as in channel II but toluene was added in defined concentrations. In channel III, the detection of bioluminescence was achieved by fiber optics; the signal was magnified by a photomultiplier. Sterilization of the system was carried out with ethylene oxide. The complete three-channel arrangement is given in Fig. 2.

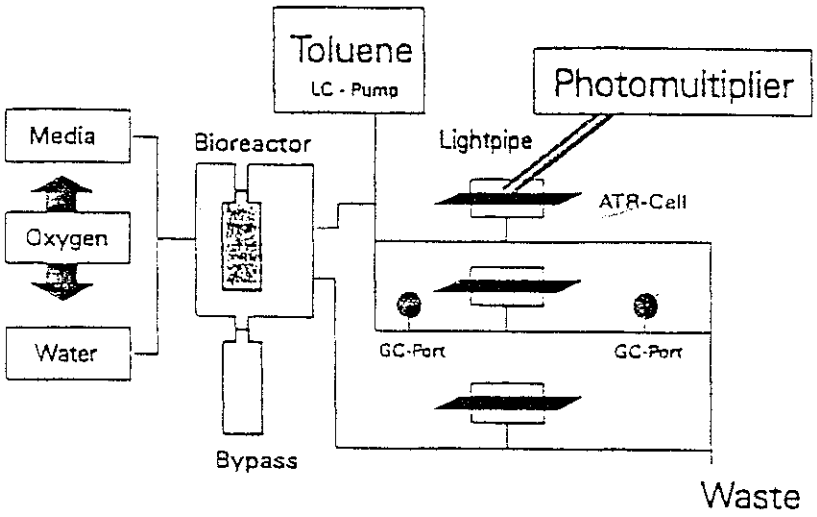

Figure 2. Test system with 3 ATR units.

Spectra acquisition. 256 sample interferograms were coadded, taken with a resolution of 4 cm^{-1}. The Norton-Beer function was used for apodisation. If necessary, baseline correction was carried out. At the start of the experiments, background spectra of water and the gas phase with water vapour were recorded.

Measurement of bioluminescence. Bioluminescence was measured by a digital Oriel photometer with a photomultiplier (Oriel Stratford, Conn.). It was connected to the ATR cell with a liquid light quide of 1.5 m length. The light was determined directly as electrical current by the induced photoelectric effect. The sensitivity was 1×10^{12} A.

Toluene concentration. The determination of toluene concentration was performed directly before and after the ATR cell via ports which led to flow-through head space vials. The head space samples were analyzed with a Shimadzu 9A gas chromatograph.

RESULTS

Characteristical regions in the spectra

The advantages of the modern FTIR spectrometers, the software and elaborated mathematical and statistical algorithms for analyzing IR-spectra allows the detection of differences small enough to distinguish different bacterial strains. This is already used for bacterial identification (Naumann *et al.*, 1988; Schmitt *et al.*, 1995). Figure 3 shows the FTIR-ATR spectrum of *P. putida*. The regions which are characteristic for the distinction of different microorganisms are indicated. Table 1 relates these wavenumbers to chemical functional groups of biomolecules and bacteria (after Jackson and Mantsch, 1993; Naumann *et al.*, 1988; Casal *et al.*, 1988; Singh and Fuller, 1991; Nivens and Schmitt, 1993).

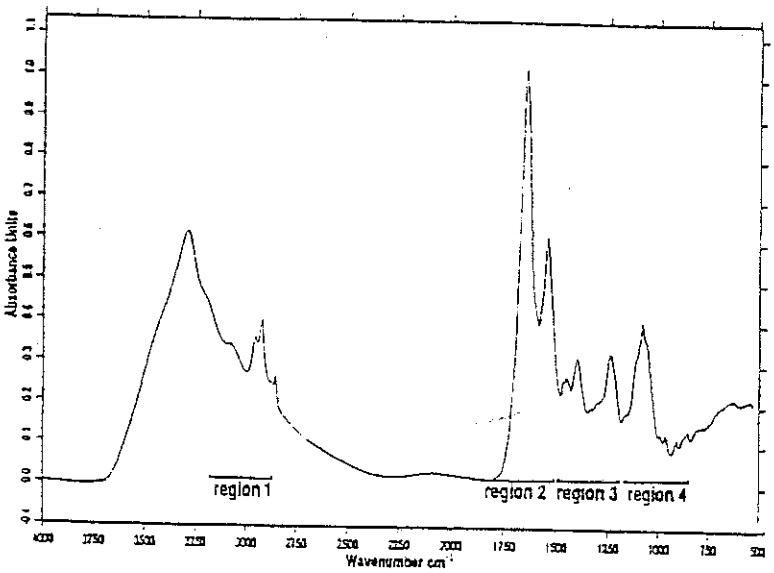

Figure 3. FTIR spectrum of *P. putida*. Region 1 = fatty acids; region 2 = amide I and II (proteins); region 3 = mixed region; region 4 = polysaccharides.

Table 1. Position of characteristical IR bands

Region	Wavenumber	Band assignment
1	3400	O-H of H_2O
fatty	2956	CH_3 asymm. stretch
acid	2920	CH_2 asymm. stretch
region	2870	CH_3 symm. stretch
	2850	CH_2 symm. stretch
2	1745/1735	>C=O Ester, fatty acids
protein	1705	>C=O Ester, carboxylic groups
region	1652-1648	Amide I (C=O), folded and helical structures of proteins
	1550-1548	Amide II, N-H, C-N
3	1460-1454	C-H from CH_2
mixed	1400-1398	C-O from carboxylate ions
region	1303	Amide II (C-N)
	1240	P=O from phosphate
	1222	P=O
4	1114	C-O-P, P-O-P, ring vibrations
polysacch.	1085	ring vibrations
region	1052	C-O, C-O-C from polysaccharides

Development of biofilm in the ATR cell

The *P. putida* cells were introduced into the sterile ATR flow-through cell as a suspension from a continuous suspended culture. After two hours, the supply with the suspension from a reactor was replaced by mineral medium only. Thus, after this time the biofilm growth is observed exclusively while the supply of suspended

cells which can attach is cut off. The time-resolved spectrum could be obtained non-destructively. A typical example of stacked spectra, representing biofilm growth, is given in Fig. 4. After one hour already, the cells can be observed beginning to attach to the crystal surface. This is first indicated by the occurrence of the amide I and II bands. Within 24 hours a rapid increase in cell numbers on the surface takes place until a plateau phase is reached, which is set optically by the depth of penetration in ATR-spectroscopy (Harrick, 1967). Further increases are slower and some times reverts into a decrease, indicating detachment of cell material from the surface. This could be confirmed microscopically. When the cell number per square unit exceeded 4 x 10^7 (as determined by epifluorescence microscopy), detachment started. The course of the spectra suggests that this is a discontinuous process. The spectra demonstrate that the intensity and composition of the bands between 1200 cm^{-1} and 900 cm^{-1} vary considerably, according to the physiological response of the biofilm. These regions are mainly correlated with the formation of EPS. This means that the physical and chemical properties of the EPS (Christensen and Characklis, 1990) may change more than expected during biofilm growth.

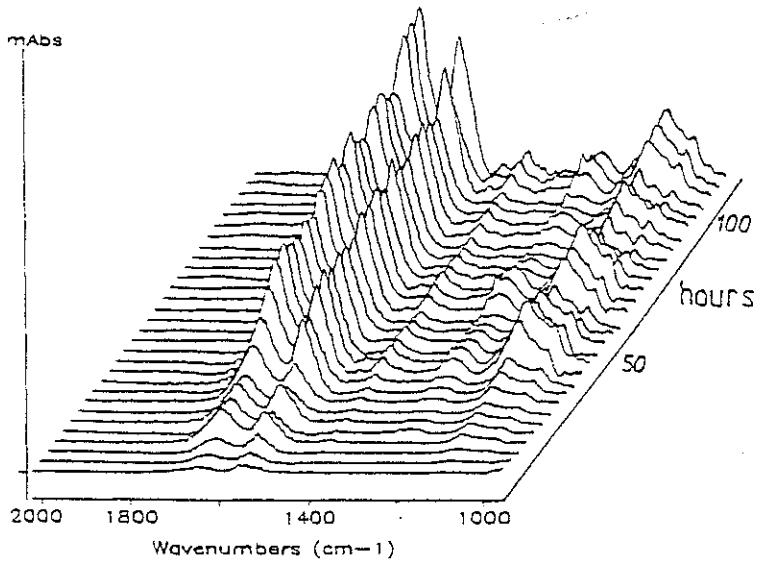

Figure 4. Time dependent development of FTIR spectra in an ATR cell during adhesion and biofilm growth of *Pseudomonas putida*.

Physiological biofilm response to toluene

A biofilm had been established in the ATR cell, which developed in the presence of various concentrations of toluene. These experiments had to be carried out subsequently for different toluene concentrations. As a control, the biofilm spectrum without toluene was taken. The toluene concentrations were 5 and 15 ppm. The spectra were taken for 120 hours. Figure 5 shows the spectra after biofilm growth had reached the plateau phase. The effect of 5 ppm toluene is clearly visible as a change of the polysaccharide region compared to the toluene-free biofilm. At 15 ppm toluene, significantly more carboxyl groups are formed and could be attributed to the EPS by extraction and FTIR comparison spectra (not shown). These effects were reproducible.

Toluene was degraded aerobically by *P. putida* (Burlage *et al.*, 1989; Rose and Tempest, 1990). As mentioned before, the strain contained a *lux* operon as reporter gene which indicated toluene-degrading activity. The intensity of bioluminescence could be quantified at a high sensitivity with the experimental set up as depicted in Fig. 2. This led to the establishment of a correlation between the nutritional status in terms of toluene concentration and the degradation activity. The degradation performance was correlated with the amide II band as an indicator for protein concentration or biomass. Oxygen concentration proved to be the most important parameter for the degradation activity. At 172 hours, the oxygen supply was cut off, which resulted in an immediate decrease of bioluminescence because of its oxygen dependency. The fluctuations of

bioluminescence however correlate with fluctuations of the protein concentration. The latter are caused by sloughing off events. After these events, the degradation activity is always increased, suggesting that sloughing may both improve the diffusion of substrates into the biofilm and trigger the physiological activity of biofilm cells.

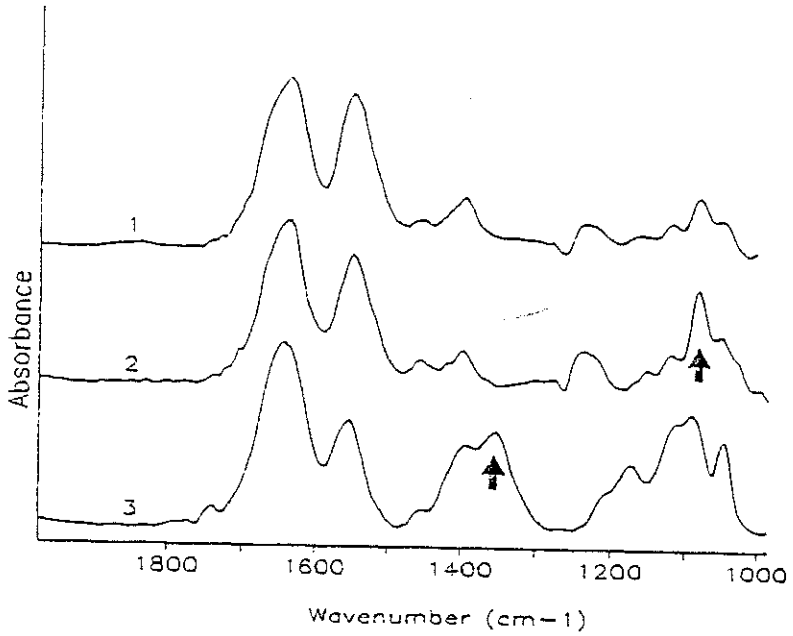

Figure 5. FTIR-spectra of biofilms after 100 hours of development. 1 = biofilm without toluene; 2 = 5 ppm toluene; 3 = 15 ppm toluene. Arrows: increases of the polysaccharide peaks at 5 ppm toluene and of the carboxylic group peak at 15 ppm toluene.

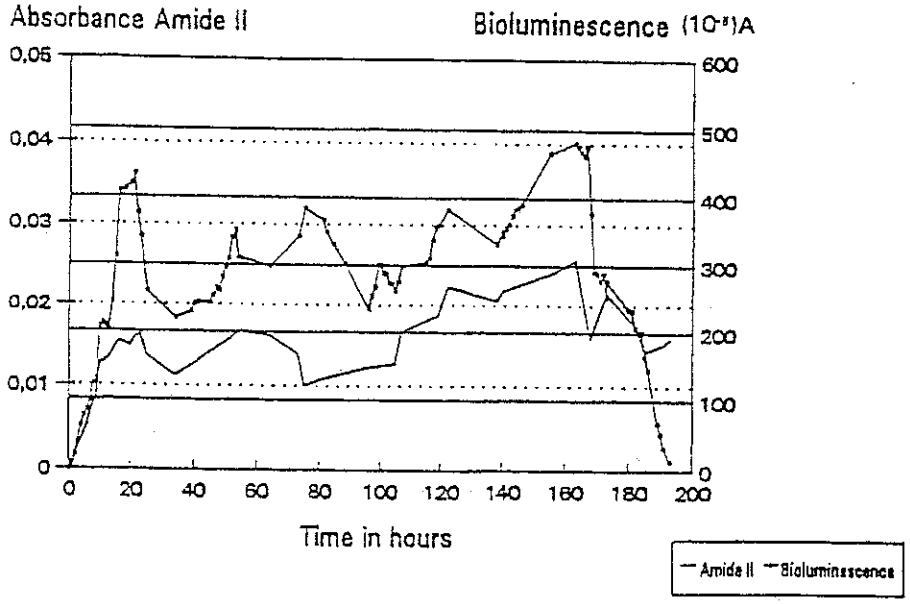

Figure 6. Correlation of toluene-triggered bioluminescence of *P. putida* and intensity of the amide II band as a quantitative marker for biomass.

DISCUSSION

The results of this study demonstrates that the FTIR-ATR technique is suitable for the detection of physiological details and changes in biofilms *in situ*, non-destructively, at high sensitivity, and time resolved. Thus, chemical information about the processes in the biofilm is provided.

Of particular interest is the observation that the biofilm responds to toluene in different ways. At low concentrations such as 5 ppm, an increased formation of polysaccharides is noted. This can be interpreted as a signal from additional EPS, as indicated by the position of the IR-band at 1085 cm^{-1}. As the strain is capable to metabolize toluene, the polysaccharides may be able to sorb toluene from the water phase; similar effects have been observed when EPS from phenanthrene-degrading organisms influenced the transport of this pollutant (Dohse and Lion, 1994). In addition, at the level of 15 ppm toluene, polysaccharides change their structure and an increase in carboxylic groups is observed. A plausible ecological explanation is still lacking. However, the results are remarkable in terms of sorption: an organic substrate triggers the formation of carboxylic groups, thus, increasing the amount of cation binding sites in the EPS. Such groups provide an extended ion exchange capacity and are known to bind to cations such as metals (Geesey and Jang, 1989).

Of course, these data cannot be extrapolated uncritically to mixed populations and other contaminants. However, they reveal that nutrient conditions, EPS structure, and sorption properties in biofilms are interrelated in a complex way. Given the appropriate conditions, an increase or a decrease in ion exchange sites may be possible. This is an interesting dynamic process which can contribute to a better understanding of the role of biofilms as sorbents and sink, in the distribution and as a potential source of pollutants in the aquatic environment (Flemming, 1995). Physiological responses may change the sorption capacity of sediment biofilms significantly. This has to be considered when it is attempted to quantify the sorption and remobilization potential of natural biofilms.

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