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ORIGINAL PAPER

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# Microbial sensors for naphthalene using *Sphingomonas* sp. B1 or *Pseudomonas fluorescens* WW4

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**Abstract** Amperometric biosensors for naphthalene were developed using either immobilized *Sphingomonas* sp. B1 or *Pseudomonas fluorescens* WW4 cells. The microorganisms were immobilized within a polyurethane-based hydrogel, which was used for a microbial biosensor for the first time. Both strains were shown to be equally suited for the quantification of naphthalene in aqueous solutions. The biosensors were tested in a flow-through system and a stirred cell (batch method). In both systems a linear response down to the detection limit was obtained. Measurements in the flow-through system gave sensitivities of up to 1.2 nA $mg^{-1} l^{-1}$ and a linear range from 0.03 mg/l to 2.0 mg/l. The response time ($t_{95}$) was 2 min and the sample throughput six per hour; the repeatability was within ± 5 %. With the batch method, sensitivities of between 3 nA $mg^{-1} l^{-1}$ and 5 nA $mg^{-1} l^{-1}$ and a linear range of 0.01–3.0 mg/l were obtained; the response time was between 3 min and 5 min. The sensors reached an operational lifetime of up to 20 days. The sensitivity of both sensors for naphthalene was, in most cases, more than four times higher than for various other substrates.

## Introduction

Polycyclic aromatic hydrocarbons (PAH) represent the largest group of cancerogenic compounds present in the environment. PAH are produced by pyrolysis of organic-carbon-based material, which accounts for their ubiquity (Blumer and Youngblood 1975; Sims and Overcash 1983). They are a main constituent of coal tar and therefore accumulate around contaminated areas of closed-down gasworks and coking plants. As PAH adsorb to organic matter in the soil, they are quite persistent in the environment (Karickhoff et al. 1979; Means et al. 1980). Owing to its high water solubility (30 mg/l), naphthalene is often found in aqueous extracts of contaminated soils as a main component.

Degradation of PAH in soil and water is mainly performed by several specialized aerobic microorganisms (Cerniglia 1984; Gibson and Subramanian 1984). The initial formation of a diol by a dioxygenase is followed by cleavage of the aromatic ring. Further cleavage and oxidation reactions may lead to complete mineralization, especially of PAH containing up to three aromatic rings (Lingens 1988). *Sphingomonas* sp. B1 (formerly *Beijerinckia* sp. B1) and *Pseudomonas fluorescens* WW4 are two bacterial strains that are able to grow on mineral medium with PAH as the sole source of carbon (Gibson et al. 1973; Weißenfels et al. 1989).

In higher organisms PAH are oxidized by monooxygenases to form water-soluble and excretable compounds for detoxification. However, in some cases these chemical modifications may lead to highly mutagenic and cancerogenic compounds (Cerniglia 1984). The cancerogenic potential and the ubiquity of PAH have given rise to many efforts to determine their presence.

Conventional analysis of PAH by high-performance liquid chromatography (HPLC) has a low detection limit and makes possible the discrimination between single components, but is expensive and time-consuming with regard to both sample preparation and sample throughput. As biosensors based on microorganisms specialized in the degradation of PAH do not need sample preparation and allow a higher sample

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throughput, they may be useful for the rapid screening of a large number of samples. Several microbial sensors for the determination of xenobiotics have already been described, e.g. for phenol (Neujahr and Kjellén 1979), chlorinated phenols (Riedel et al. 1993), benzene (Tan et al. 1994), polychlorinated biphenyls (Beyersdorf-Radeck et al. 1993) and 3-chlorobenzoate (Riedel et al. 1991). The common transducer of the cited microbial sensors is a Clark-type oxygen electrode which detects increased respiratory activity of the microbial cells in the presence of analyte. As microbial degradation of PAH is performed under $O_2$ consumption, the same transducer principle should be applicable for a microbial naphthalene sensor.

## Materials and methods

### Chemicals

Naphthalene (puriss.), phenanthrene (purum) and anthracene (puriss.) were obtained from Fluka (Neu-Ulm, Germany). Organic solvents (HPLC grade) used for extraction and as substrates were from Roth (Karlsruhe, Germany). The prepolymer was a research product from the Bundesforschungsanstalt für Landwirtschaft (Braunschweig, Germany) and is now available from SensLab (Leipzig, Germany). All other chemicals were purchased at analytical grade from Sigma (Deisenhofen, Germany). The organic salts contained sodium as counterion. The aqueous naphthalene solution needed for sensor measurements was prepared by dissolving 30 mg naphthalene in 1 l 30 mmol/l potassium phosphate buffer (pH 6.9) via ultrasonication. As naphthalene evaporates and adsorbs on vessel walls (Futoma 1981), its concentration in solution had to be monitored by extraction with hexane and fluorescence measurements (excitation wavelength = 280 nm, emission wavelength = 335 nm; luminescence spectrometer LS 50B, Perkin Elmer, Überlingen, Germany).

### Microorganisms and culture

The strain *Sphingomonas* sp. B1 (DSM 6900) was isolated from polluted stream water (Gibson et al. 1973) and *Pseudomonas fluorescens* WW4 from soil contaminated with PAH (Weißenfels 1989). The cells were grown on a rotary shaker at 30 °C in a mineral medium containing the following per litre of demineralized water: 3.06 g $Na_2HPO_4 \cdot 2H_2O$, 1.52 g $KH_2PO_4$, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, 5 mg EDTA, 2 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.03 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg $H_3BO_3$, 0.2 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuCl_2 \cdot 2H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, pH 6.9. Naphthalene (0.1 %), added in solid form, was used as sole carbon source. The growth of the cultures was monitored by means of a Klett photoelectric colorimeter (Manostat, New York, USA). The degradation of naphthalene was observed by extraction of the total bacterial cultures with dichloromethane at different states of growth and subsequent HPLC analysis (2.1 mm Vydac $C_{18}$ column, $H_2O$/acetonitrile gradient).

The microorganisms were grown under the same conditions on the other substrates using concentrations listed in Table 1.

### Immobilization

The microorganisms were immobilized using a bisulphite-blocked hydrophilic isocyanate prepolymer (Vorlop et al. 1992). The cells were grown on naphthalene and harvested at the end of exponential growth. The culture was filtered with an 8-μm capillary filter (AE 99, Schleicher & Schnell, Dassel, Germany) to remove naphthalene particles. The filtrate containing the cells was then centrifuged at 5000 rpm. Subsequently the cells were washed with 30 mmol/l potassium phosphate buffer (pH 6.9) and finally resuspended in the same buffer up to an absorbance ($A_{436}$) of 100.

The immobilization procedure was carried out similarly to previously described methods (Muscat et al. 1995; Kotte et al. 1995). In brief, 250 mg prepolymer (33.4 %) was mixed with an equal quantity of 30 mmol/l potassium phosphate buffer (pH 6.9). Poly(ethyleneimine) (2.5 % w/v in water) was added in 10-μl amounts under constant mixing to adjust the pH between 6.4 and 6.5. The pH was controlled with a Biotrode pH electrode (Hamilton, Darmstadt, Germany). After centrifugation to remove polymerized particles, 160 μl solution was mixed with 40 μl cell suspension ($A_{436} = 100$). Samples containing 5 μl of this mixture were spread onto a 7-mm$^2$ spot of a gas-permeable polyethylene membrane (thickness 10 μm, Metra, Radebeul, Germany) and incubated for 1 h at 30 °C in a water-vapour-saturated atmosphere to complete the polymerization to the hydrogel.

### Microbial sensors

An oxygen electrode (Pt cathode with a diameter of 0.5 mm and Ag/AgCl reference anode; PGW Medingen, Dresden, Germany) was first covered with the gas-permeable membrane onto which the immobilized microorganisms were adhering and then with a capillary membrane (diameter of pores 0.6 μm, thickness 10 μm; Oxyphen, Dresden, Germany) (Fig. 1). The sensor was installed in a flow-through system (BSB-Modul, PGW Medingen, Dresden, Germany) with an alternating flow of 30 mmol/l potassium phosphate buffer (pH 6.9) and analyte solution or in a stirred measuring cell containing 2 ml of the same buffer (batch system). For the amperometric detection in the stirred cell, an EP30 electrochemical detector (Biometra, Göttingen, Germany) was used. In both systems measurements were taken at 25 °C, and the change in current in the presence of analyte was followed at −0.8 V against Ag/AgCl.

## Results

### Bacterial growth

Both *Sphingomonas* sp. B1 and *P. fluorescens* WW4 grew on mineral medium containing 1 mg/ml naphthalene as sole source of carbon with generation times of about 3.5 h, as illustrated in Fig. 2. Maximum

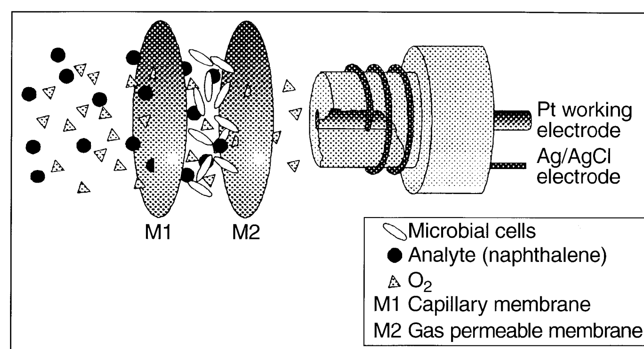


**Fig. 1** Schematic set-up of the biosensor

naphthalene degradation rates of 4 $mg l^{-1} day^{-1}$ were observed in the logarithmic growth phase of the bacteria. HPLC analysis proved that naphthalene was completely degraded in the bacterial cultures, whereas naphthalene concentrations in sterile control tests remained unchanged.

To investigate whether growth of naphthalene-cultivated *Sphingomonas* sp. and *P. fluorescens* cells on other substrates can be induced, naphthalene-grown cultures in their exponential phase were inoculated into mineral medium containing various substrates as sole carbon sources. After a few hours of lag time, both strains could be grown on most tested substrates with different generation times (Table 1). With these data a comparison between growth behaviour and sensor characteristics was possible.

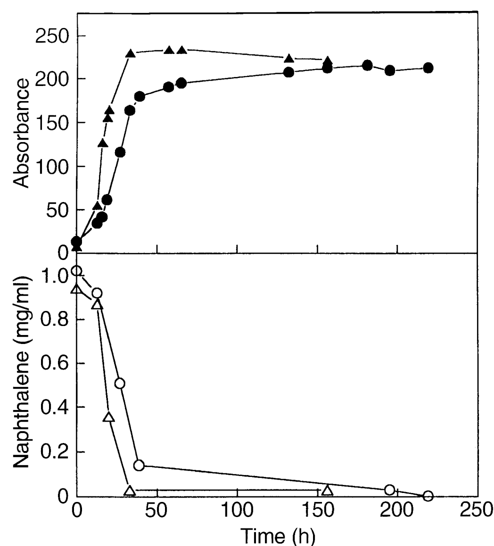


**Fig. 2** Naphthalene degradation and growth of *Sphingomonas* sp. (○, ●) and *Pseudomonas fluorescens* (△, ▲). Growth conditions: mineral medium pH 6.9 containing 1 mg/ml naphthalene as sole carbon source, rotary shaker at 120 rpm and 30 °C. Absorbance: Klett-Summerson colorimeter scale

## Sensor response to naphthalene

Both *Sphingomonas* sp. and *P. fluorescens* showed an identical performance in the determination of naphthalene in aqueous solution. Figure 3 depicts a typical response curve obtained in the flow-through system. The current decreases for 2 min during which the analyte solution is passed through the system. The assimilation of naphthalene by the immobilized microorganisms reduces the oxygen flow to the electrode. When contact of the sensor with analyte is stopped, the current increases again. The maximum recovery time was 8 min allowing a sample throughput of six per hour.

As within the flow-through system the biosensor is supplied with oxygen via injection of air into the flow of buffer or analyte solution, resulting in aerosol formation, the volatile naphthalene is likely to evaporate. The extent of naphthalene losses was investigated by fluorescence measurements of samples collected from the flow-through system at the site of the biosensor. It was found that, independent of sample concentration, $25 \pm 1\%$ of the naphthalene reached the biosensor.

Using the batch method, the response time for reaching 95% of the steady-state signal ($t_{95}$) was between 3 min and 5 min (data not shown). The recovery time was about 20 min, because of the refilling of the stirred cell with fresh buffer after each measurement.

**Table 1** Comparison between growth data and sensor sensitivities. *ND* not determined

| Substrate | *Sphingomonas* sp. | | | *Pseudomonas fluorescens* | | |
|---|---|---|---|---|---|---|
| | Lag time (h) | Generation time (h) | Relative sensitivity (%)[a] | Lag time (h) | Generation time (h) | Relative sensitivity (%)[a] |
| Naphthalene 0.1% | 0 | 3.5 | 100 | 0 | 3.5 | 100 |
| Phenanthrene 0.1% | 0 | 11 | ND | 0 | 31 | ND |
| Anthracene 0.1% | 0 | 156 | ND | No growth | – | ND |
| Glucose 0.4% | 1.5 | 1.9 | 6 | 5 | 3.0 | 3 |
| Fructose 0.4% | 2 | 1.7 | 0.2 | 5.5 | 2.7 | 10 |
| Sodium acetate 0.1% | 4.5 | 2.8 | 22 | 5 | 2.9 | 67 |
| Sodium pyruvate 0.2% | 0 | 23 | 17 | 1.5 | 1.7 | 56 |
| Citric acid 0.2% | No growth | – | 0 | 11 | 2.4 | 0.2 |
| Disodium succinate 0.2% | 2 | 1.7 | 13 | 1.5 | 2.0 | 22 |
| Sodium salicylate 0.1% | 8 | 6.0 | 60 | 4.5 | 2.3 | 25 |
| Sodium benzoate 0.1% | 6 | 4.7 | 20 | 12 | 10.2 | 2 |
| Sodium glutamate 0.2% | 2 | 8.6 | 27 | 1.5 | 1.7 | 15 |
| Yeast extract 0.5% | 1.5 | 1.5 | 17 | 1.5 | 1.7 | 32 |

[a] Selected sensor data from Table 2

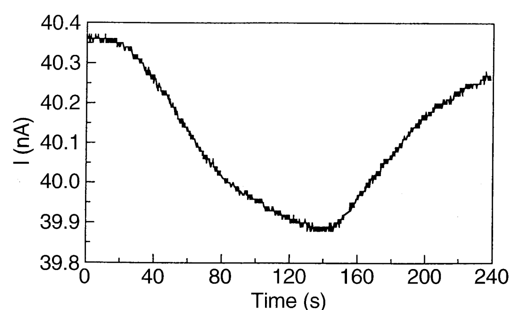


**Fig. 3** Current/time curve of the *Sphingomonas*-sp.-containing sensor for naphthalene in the flow-through system

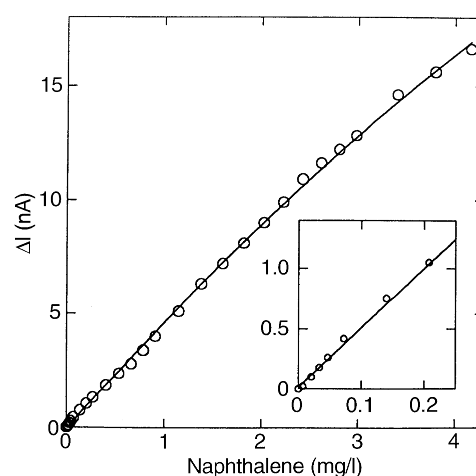


**Fig. 4** Calibration curve for the determination of naphthalene with *P. fluorescens* in the batch system

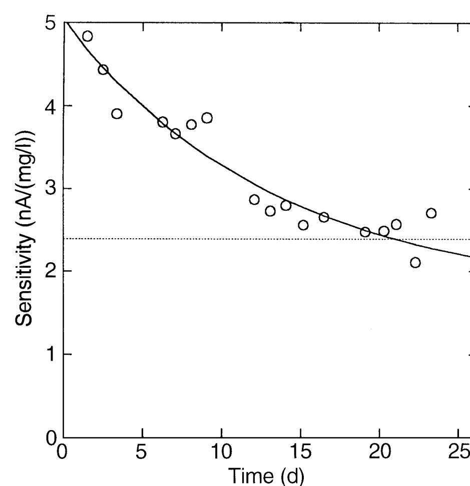


**Fig. 5** Operational lifetime of a *P. fluorescens*-containing sensor in the batch system (··· 50 % of initial sensitivity)

## Calibration

When calibrated with naphthalene, the sensors showed identical parameters with both strains. In all cases the biosensors gave a linear response down to the detection limit.

Measurements in the flow-through system gave sensitivities of up to 1.2 nA $mg^{-1} l^{-1}$, a background noise of $\pm$ 0.01 nA, and a linear range of 0.03–2.0 mg/l. The repeatability was within $\pm$ 5 % of the mean value in a series of ten samples when the test solution contained 1.5 mg/l naphthalene (data not shown).

In the batch method, calibration curves were obtained by successive addition of naphthalene stock solution to the stirred cell. Thus, sensitivities of between 3 nA $mg^{-1} l^{-1}$ and 5 nA $mg^{-1} l^{-1}$, a background noise of $\pm$ 0.01 nA, and a linear range from 0.01–3.0 mg/l were obtained (Fig. 4). The repeatability was $\pm$ 5 %, like that observed in the flow-through system.

With both systems, the measuring range could be extended to 6 mg/l by using second-order calibration curves.

## Functional stability

To investigate the functional stability of the biosensors, freshly prepared sensors were installed in the measuring systems and subsequently calibrated daily with naphthalene solution. During intervals between measurements the sensor remained in the system and was supplied with buffer. The biosensors showed an operational lifetime of up to 20 days during which their sensitivity decreased to 50 % of the initial value (Fig. 5). In the same period, the upper limit of the linear range decreased to about 1 mg/l and, with second-order calibrations, an extension up to 2 mg/l was possible.

## Selectivity

The selectivity of the naphthalene biosensors was determined by taking calibration curves in the batch system for a variety of substrates and calculating the sensitivities within the linear range in relation to that for naphthalene (Table 2). Owing to successive additions, the biosensors were exposed to the respective substrate for up to 2 h. After each substrate, the calibration curve for naphthalene was determined and, in all cases, found not to be influenced by the preceding substrate measurement. To most substrates, the *Sphingomonas*-sp.-containing sensor was less sensitive than that with *P. fluorescens*. *Sphingomonas* sp. reached maximum cross-sensitivities of 60 % for salicylate, and *P. fluorescens* 160 % for ethanol, both organisms having sensitivities below 1 % for some substrates. Some of the relative sensitivities were checked in the flow-through system and agreed with those obtained in the batch system.

The reponse times ($t_{95}$) for the various substrates were between 3 min and 20 min. The substrate calibrations were linear down to the detection limit and, in

**Table 2** Selectivity of *Sphingomonas*- and *Pseudomonas*-containing sensor for naphthalene and various substrates. *ND* not determined

| Substrate | *Sphingomonas* sp. | | | *P. fluorescens* | | |
|---|---|---|---|---|---|---|
| | Relative sensitivity (%)[a] | Standard error | Upper limit of linear range (mg/l) | Relative sensitivity (%)[a] | Standard error | Upper limit of linear range (mg/l) |
| Naphthalene | 100 | 5 | 3 | 100 | 5 | 3 |
| Carbohydrates | | | | | | |
| Glucose | 6 | 3 | 10 | 3 | 2 | 15 |
| Fructose | 0.2 | 0.1 | 50 | 10 | 6 | 2 |
| Organic acids | | | | | | |
| Gluconate | 0.025 | 0.005 | > 2000 | ND | ND | ND |
| Acetate | 22 | 2 | 5 | 67 | 5 | 3 |
| Pyruvate | 17 | 2 | 2 | 56 | 3 | 6 |
| Citric acid | 0 | 0.01 | – | 0.2 | 0.05 | 100 |
| Succinate | 13 | 2 | 5 | 22 | 4 | 8 |
| Salicylate | 60 | 20 | 1.5 | 25 | 4 | 4 |
| Benzoate | 35 | 5 | 0.5 | 2 | 0.5 | 2 |
| Amino acids | | | | | | |
| L-Glutamic acid | 27 | 2 | 10 | 15 | 1 | >7 |
| $\beta$-Alanine | 0 | 0.01 | – | 30 | 3 | 2 |
| Alcohols | | | | | | |
| Methanol | 0.02 | 0.01 | 500 | 0.3 | 0.1 | 300 |
| Ethanol | 15 | 4 | 3 | 160 | 10 | 1 |
| Glycerol | 0.04 | 0.01 | 1000 | 9 | 6 | 15 |
| Others | | | | | | |
| Yeast extract | 17 | 3 | 3 | 32 | 3 | 15 |
| Peptone | 8 | 3 | 15 | 25 | 6 | 20 |
| Acetone | 0.01 | 0.005 | 1500 | 0.01 | 0.005 | >2500 |

[a] the sensitivity (nA $mg^{-1} l^{-1}$) for the substrate (concentration calculated without counterion) was determined within the linear range of the calibration curve and related to the corresponding sensitivity for naphthalene

most cases, reached maximum currents that were much lower than for naphthalene, as demonstrated for acetate and benzoate in Fig. 6. It should be noted that the linear ranges for the various substrates were not monitored throughout the operational lifetime of the sensors and may vary during a period of measurement, like those observed for naphthalene.

A remarkable feature of the biosensors was the ability to detect naphthalene when other substrates were present at saturation concentration. This was demonstrated in the batch system for most of the analytes listed in Table 2. In no case was the sensitivity significantly different from that measured with only naphthalene present.

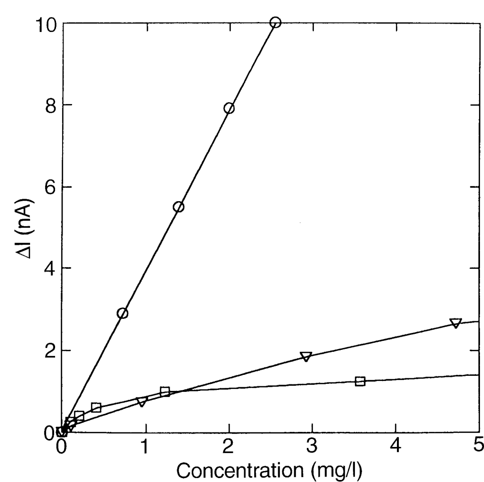


**Fig. 6** Calibration curves of the *Sphingomonas*-sp.-containing sensor for naphthalene (○), acetate (▽) and benzoate (□) in the batch system

## Discussion

This study provides a simple and rapid method for the determination of naphthalene in aqueous solutions by means of a biosensor containing either immobilized *Sphingomonas* sp. or *P. fluorescens* cells. Our sensors reached lower detection limits than most microbial sensors for xenobiotics described so far. A sensor for phenol containing *Rhodococcus* sp. P1, for example, reached a linear range of 0.5–2.5 mg/l (Riedel et al. 1993), and a linear calibration of benzoate with *Pseudomonas putida* 87 was possible between 0.5 mg/l

and 20 mg/l (Riedel et al. 1991). For both of these sensors poly(vinyl alcohol) was used as the immobilization matrix. The low detection limit (0.01 mg/l) reported in this paper will probably be due to the high naphthalene-degradative capacities of the microorganisms as well as to the low background noise during operation (less than 0.01 nA). This can be attributed to the homogeneous diffusion properties of the polyurethane-based hydrogel matrix, which was used for the first time for a microbial biosensor.

The main advantage of a flow-through system, a high and automated sample throughput, is impaired by a reduced sensitivity and poorer detection limit for naphthalene. This is due to evaporation of naphthalene caused by aerosol formation in this measuring system. The extent of the decrease in sensor sensitivity correlates to 25 % of the naphthalene reaching the biosensor, as found by fluorescence measurements.

However, as detection limits in the stirred cell have been calculated for naphthalene concentrations present in the batch volume and not in the added sample, the flow-through system may still be considered the more promising tool for process control and screening of aqueous samples. The linear range of our biosensors corresponds to the concentrations found in aqueous extracts of contaminated soil (up to 1 mg/l) and matches PAH threshold values for the remediation of ground water stated in the List of the Netherlands (0.04 mg/l, VROM/NL 1988).

Another important feature of a biosensor is its selectivity, and we therefore investigated whether growth data allow prediction of sensor characteristics and vice versa. In this respect Rothe and Hertel (1993) reported that the sensor response allows predictions about biodegradative capacities of the microorganisms. They examined the relative sensor sensitivities of phenol-induced cells for a variety of aromatic compounds and postulated that a sensor signal of more than 40 % in relation to phenol coincides with biodegradability in submerged culture. In contrast to this, the elaborate experiments conducted in our study do not reveal such a correlation. Although *Sphingomonas* sp., for example, had a very low sensor response for fructose, the strain could be grown on this substrate with short lag and generation times; similarly, the highest relative sensitivity of *Sphingomonas* sp. observed for salicylate coincides with a long lag time and slow growth (Table 1). This is most probably due to sensor signals and growth data representing two different physiological processes. The latter show the biodegradability of a certain substrate as the sole source of carbon, i.e. whether and with how much delay the enzymes necessary for the synthesis of all essential components for growth and cell division can be induced by this substrate. The former only show whether and to what extent an oxygen-consuming reaction (transport into the cell or assimilation) takes place within the response time during which the cells are exposed to a substrate; in short, sensor data reflect the actual enzyme equipment of the cells. However, conclusions with regard to biodegradability may be possible when examining similar substrates that cause oxygen-consuming reactions by enzymes actually present in the cells.

Although in the batch system the biosensors were continuously exposed to increasing substrate concentrations for up to 2 h because of successive additions and, in some cases, long response times as well, no adaptation to the substrate used could be observed. The naphthalene response was in no way influenced by the preceding substrate measurements.

Moreover, even in the presence of high or saturation concentrations of other substrates the detection of naphthalene was possible without any significant influence on sensitivity. Likewise, in the presence of salicylate at saturation concentrations, salicylate being an intermediate of naphthalene degradation, the sensitivity of both sensors for naphthalene stayed the same. This proves that most probably only the first oxidation and cleavage steps of the polyaromatic system are responsible for the oxygen consumption observed. The observed additivity of the signals is quite a useful sensor characteristic for detecting naphthalene besides other analytes in high concentrations.

The selectivity of the *Sphingomomas*-sp.-containing sensor for naphthalene is generally better than that of the sensor with *P. fluorescens*. Moreover, the ability of *Sphingomonas* sp. to grow on phenanthrene and anthracene (Gibson et al. 1973) may mean that the naphthalene biosensor also detects these PAH, as they are mostly degraded by the same sequence of enzymes. This was underlined by growth experiments carried out in this work, where naphthalene cultures grew on phenanthrene or anthracene without lag times, longer generation times being attributed to a slower solution rate of these PAH (Table 1). It remains to be determined whether the biosensors developed here may not only be used for the detection of naphthalene as a marker for PAH contamination in aqueous samples, but also for the analysis of other PAH.

The biosensors presented in this work are not superior to HPLC analysis in terms of detection limit and discrimination of single components, but sensor sensitivity and selectivity data show that the biosensors are principally suited for environmental naphthalene measurements. As analysis in aqueous solutions is possible with a high sample throughput, the biosensors will be particularly suited for the rapid screening of a large number of samples.

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