Evaluation of a FIA operated amperometric bacterial biosensor, based on Pseudomonas putida F1 for the detection of benzene, toluene, ethylbenzene and xylenes (BETEX)

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ABSTRACT

Recently, the development and optimisation of a flow injection analysis (FIA) operated bacterial biosensor based on the aerobic catabolism of *Pseudomonas putida* ML2 was reported in the literature (Layon Y.H. e al. 2004a; Lanyon Y.H. e al. 2004b). Adapted from these reports, it was investigated whether operating parameters and procedures of the benzene biosensor could be directly applied to a new system based on a different bacterial strain for the detection of the whole benzene, toluene, ethylbenzene and xylenes range. Cells of the investigated bacterial strain, *Pseudomonas putida* F1, were immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen electrode. *P. putida* F1 aerobically degrades benzene, toluene and ethyl-benzene (Gibson D.T. e al. 1968). The BTE biosensor in kinetic mode (Flow Injection Analysis) displayed a linear range of 0.02 – 0.14 mM benzene (response time: 5 min, base line recovery time: 15 min), 0.05 – 0.2 mM toluene (response time: 8 min, base line recovery time: 20 min), and 0.1 – 0.2 mM ethylbenzene (response time 12 min, base line recovery time: 30 min), respectively. Due to the differences in sensitivity, response and baseline recovery times for BTE, it was possible to differentiate each compound in mixtures of these VOCs. No response for Xylenes could be obtained since

they cannot be completely metabolised by this bacterial strain. However, it was reported that the range of compounds degradable by *P. putida* F1 can possibly be expanded by cultivating the cells on different carbon sources (Choi E.N. e al. 2003). The sensor showed good intra- and inter-assay reproducibility, and all obtained results were comparable with those reported in the literature. The demonstrated reproducibility and the simplicity and ease of use as well as the portability for *in situ* measurements, the biosensor could be suitable as a reliable initial warning device for elevated BTE levels in indoor and outdoor environments.

INTRODUCTION

The emissions of volatile organic compounds (VOCs) in the atmosphere represent one of the major causes to the air quality deterioration and environmental pollution. Among these VOCs, benzene, toluene, ethylbenzene, and xylenes (BTEX hereafter) are produced in huge amounts and are used in fuels, as solvents, and as starting materials for the production of plastics, synthetic fibers, and pesticides (Budavari, 1996). At room temperature and atmospheric pressure, these compounds are sufficiently vaporised to pose a significant health hazard to humans (Fruscella William 2002). A large amount of BTEX is therefore released into the atmosphere during manufacture, transportation, use, and disposal every year. BTEX vapors are corrosive and toxic substances and are on the EPA Priority Pollutant List (EPA, 1996), and in the top 100 chemicals on the Priority List of Hazardous Substances published by the Agency for Toxic Substances and Disease Registry (ATSDR, 2003). Exposure to BTEX primarily occurs via inhalation (WHO 2002); hence individuals working in industries producing or using these petrochemicals are exposed to the highest levels of these toxic aromatic hydrocarbons. Acute exposure to high levels of BTEX has been associated with skin and sensory irritation, central nervous system depression, and effects on the respiratory system. Prolonged exposure has similar effects and is additionally adversely affecting the kidney, liver and blood systems (Fruscella William 2002, Ozokwelu Dickson E. 2000,

Cannella William J. 2000). Due to the proven different degrees of toxicity, permissible exposure levels for each single BTEX-compound were established. The current European permissible exposure levels for benzene, toluene, ethyl-benzene and xylenes are reported in Table 1.

The conventional method for monitoring human BTEX exposure in the workplace includes the trapping of BTEX vapours on charcoal adsorption tubes, desorbtion with an appropriate solvent (e.g. carbon disulfide) and subsequent determination of the VOCs employing gas chromatography (WHO 2002, Healt and Safety Executive 1997). Classical chromatographic methods, such as GC-analysis, provide high accuracy and precision. These techniques however, are often time-consuming, require highly sophisticated equipment, are expensive, and are usually lab based. For the routinely performed detection of BTEX in air, alternative, faster, more economical and portable in-situ detection devices, such as biosensors, would therefore be desirable.

Biosensors incorporate a biological sensing element (e.g. microorganisms), which is either intimately connected to or integrated within a suitable transducing system (Turner A.P.F. e al. 2000). The sensing element specifically recognises the species under investigation; the transducer quantitatively converts the biochemical signal into an electronic signal that can be suitably processed and converted to an output. Therefore, analytes may be detected by using the assimilation capacity of the microorganism as an index of the respiration activity or of the metabolic activity (Mulchandani A., Rogers K.R. 1998). Such microbial biosensors based on respiratory activity have had several applications in environmental monitoring over the past years (Tan H.M. e al. 1994, D'Souza S.F. 2001). Recently, a FIA operated amperometric bacterial biosensor, based on *Pseudomonas putida* ML2, for detection of benzene in workplace air was reported by Lanyon *et al.* (Lanyon Y.H. e al. 2004a, Lanyon Y.H. e al. 2004b). This sensor was constructed by immobilising the bacterial cells between two cellulose

acetate membranes, and subsequent fixation of the membranes onto an amperometric Clark type dissolved oxygen electrode.

Starting from the reports by Lanyon *et al.* (Lanyon Y.H. e al. 2004a, Lanyon Y.H. e al. 2004b), the applicability of an amperometric bacterial biosensor for the detection of the whole BTEX range in air was investigated in this work. The sensor was based on the same principles as the aforementioned benzene-sensor, but utilising a different bacterial strain, *Pseudomonas putida* F1.

Pseudomonas putida F1 (*Pp*F1) is a fluorescent soil bacterium that can assimilate toluene, benzene, ethylbenzene, phenol, and other aromatics as sole carbon and energy sources (Gibson D.T. et al., 1968). Like other *pseudomonads*, many of its induced enzymes are nonspecific and its metabolic pathways contain a high degree of convergence allowing for the efficient utilization of a wide range of growth substrates (Hutchinson and Robinson, 1988).

The enzymic pathway responsible for converting these aromatic hydrocarbons to TCA cycle intermediates is called the toluene degradation (tod) pathway (Finette et al., 1984; Gibson et al., 1990). The tod pathway consists of several enzymic reactions; for example (see Scheme 1), toluene is first transformed into *cis*-toluene dihydrodiol through the insertion of a molecule of oxygen catalyzed by a multicomponent toluene dioxygenase (Finette et al., 1984; Gibson et al., 1990; Yeh et al., 1977). *cis*-Toluene dihydrodiol is then dehydrogenated to form 3-methylcatechol, which is cleaved at the *meta* position by insertion of a second dioxygen molecule (Scheme 1). Then the reaction products are converted to tricarboxylic acid cycle (TCA cycle) intermediates (Lau et al., 1994; Zylstra and Gibson, 1989).

Benzene, toluene and ethylbenzene can be degraded by *P. putida* F1 via the same pathway (Scheme 1). Further studies on the substrate specificity of the tod pathway have revealed that n-propylbenzene, n-butylbenzene, cumene and biphenyl are degraded to only ring-fission dead-end products whereas *p*-xylene is only converted to 2,6-dimethylcatechol, (Cho et al.,

2000; Gibson et al., 1974, Yu et al., 2001), revealing the limitations of the corresponding enzymes in channeling substrates into TCA cycle intermediates (Furukawa et al., 1993).

Scheme 1: Pseudomonas putida F1 catabolyc pathways for benzene, toluene, ethylbenzene and p-xylene.

During the aerobic assimilation of BTE, an increase in respiration rate and corresponding oxygen consumption mainly due to the dioxygenases catalysis occurs and can be used as basis for the detection of these VOCs in solution. Xylenes, the last member of the BTEX range, can so far not be degraded by this bacterial strain. However, Choi *et al.* (Choi E.N. e al. 2003) reported that the range of compounds which can be metabolised by *Pseudomonas putida* F1 can possibly be expanded by cultivating the cells with different aromatic hydrocarbons (e.g xylenes) as growth-additives. Pre-existing metabolic pathways can be altered by natural adaptation of the *tod*-degradation pathway and hence, the novel metabolic capabilities of the bacterial cells might be subsequently exploited in a biosensor. A sensing device based on this principle might therefore be employed as an early warning device for the presence of toxic VOCs in air.

EXPERIMENTAL

Reagents

Bacterial culture constituents: Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Acros Organics (Fisher Scientific UK Ltd, Loughborough). Tryptone and agar were purchased from Oxoid S.p.A. (Milan, Italy). Magnesium sulphate ($MgSO_4.7H_2O$) and iron sulphate ($FeSO_4.7H_2O$) were purchased from Fluka (Milan, Italy). Ammonium sulphate $(NH_4)_2SO_4$ and benzene were purchased from Sigma (Milan, Italy).

Buffer components (KH_2PO_4 and $K_2HPO_4.3H_2O$) and potassium chloride (KCl) used for the electrolyte solution were purchased from Merck (Milan, Italy).

Bacterial strain and culture conditions.

P. putida F1 was maintained on agar plates containing a minimal medium (see below) and supplied with toluene vapor (0.2 mmol liter^{-1}). For the preparation of seed culture, a colony of *P. putida* F1 was inoculated into the same minimal medium supplied with toluene as the sole carbon source and incubated overnight at 30°C following previous methodologies reported by Lanyon (Lanyon Y.H. e al. 2004a). One litre of minimal medium contained the following constituents: KH_2PO_4 (1.02g), K_2HPO_4 (3.06g), $(NH_4)_2SO_4$ (1g), Tryptone (0.2g), $MgSO_4.7H_2O$ (0.4g), and $FeSO_4$.7H2O (0.04g). The bacterial cultures were incubated in an incubator shaker (Gerhardt Thermoshake, Königswinter, Germany) for 18 hours at 30°C and 150 rpm to obtain cells in the stationary phase of growth. Sub-cultures of *P.putida* F1 were prepared on a monthly basis and preserved on minimal medium agar plates (same constituents as the liquid medium with the addition of 15g agar per litre).

Instrumentation

All experiments were conducted using a battery operated portable Idronaut Oxyliquid model 23 dissolved oxygen probe and meter (Idronaut s.r.l., Brugherio (MI), Italy) as the transducer, and measuring device. An atmospheric air saturated potassium phosphate buffer (50mM, pH 7) carrier stream was pumped through a custom-made flow cell (containing the dissolved oxygen probe) using a peristaltic pump (Gilson Minipuls3, Gilson Medical Electronics, Paris, France). A Rheodyne model 7125 syringe loading sample injector (Rheodyne, CA, USA) was employed to introduce standard or sample solutions into the carrier stream. All biosensor experiments were performed at room temperature (25-30°C).

Immobilisation

The immobilisation was conducted according to the optimised procedure reported by Lanyon *et al.* (Lanyon Y.H. e al. 2004b). *P.putida F1* cells were harvested by centrifugation (Eppendorf, MiniSpin Plus) of 1 mL of an 18 h culture (2415 g, 10 min, room temperature). The supernatant was discarded and the pellet (0.9 mg mean dry weight) was re-suspended in 100 µL of potassium phosphate buffer (50 mM, pH 7). The suspension was pipetted onto the centre of a cellulose acetate membrane (13 mm diameter, 0.2 micron) (Sartorious, Antella (FI), Italy) which was placed on top of a filter flask. After the buffer had naturally filtered through, a second cellulose acetate membrane (25 mm diameter, 0.2 micron) was placed on top of the first to sandwich the bacterial cells between the two membranes. The bacterial membranes were subsequently transferred onto a gas permeable polyethylene membrane (approximately 30 mm x 30 mm), placed onto the cap of the dissolved oxygen probe and fixed using a rubber o-ring. Each bacterial membrane was used for as long as the sensitivity

and reproducibility of the sensor response persisted. When no experiments were performed the sensor (the cap of the dissolved oxygen probe with the immobilised bacterial cells fixed onto it) was stored at 4°C in a 1 mM benzene solution prepared in potassium phosphate buffer (50 mM, pH 7).

Measurement Scheme

The endogenous respiration rate of the immobilised *P. putida* F1 cells is represented by the steady state baseline of the oxygen meter. From the phosphate buffer dissolved oxygen diffuses continuously through the dialysis membranes to the surface of the platinum working electrode of the oxygen probe. The working electrode is kept at a fixed potential (-600 mV) against an Ag/AgCl reference electrode, at which oxygen is reduced and a current is produced. After the injection of benzene, the hydrocarbon diffuses through the membranes and is degraded by the immobilized bacteria causing an increase in their respiration rate and oxygen consumption. Consequently, the concentration of dissolved oxygen near the surface of the oxygen probe drops causing a decrease in the sensor output signal until a new steady state or “peak” is obtained. The drop in concentration of dissolved oxygen and the corresponding decrease in sensor signal are proportional to the concentration of benzene injected. All results are presented as percentage decrease from the original steady state baseline, B_x/B_o (%), where: B_o is the original baseline and B_x is the secondary baseline or peak (Lanyon Y.H. e al. 2004a, Lanyon Y.H. e al. 2004b).

RESULTS AND DISCUSSION

Preliminary tests of the biosensor in batch mode revealed that it was possible to detect benzene, toluene and ethylbenzene. No detectable responses for xylenes could be obtained

although p-xylene was reported to be at converted to a dead-end product: 2,6-dimethylcatechol (Yu et al., 2001). These findings were in accordance with those reported by Choi *et al.* (Choi E.N. e al. 2003) and Gibson *et al.* (Gibson D.T. e al. 1968). Due to the promising results in batch mode, all subsequent experiments were performed in the beneficial kinetic (FIA) mode. The use of a FIA system with a bacterial biosensor brings forth different advantages with respect to the batch system, including an increased simplicity of operation and a very accurate analysis of a greater number of samples over a given period (Lanyon Y.H. e al. 2004a). The sensitivity and reproducibility of the sensor response to different concentrations of BTEX were examined to establish linear ranges and detection limits of the biosensor. Furthermore, different parameters possibly affecting the sensor response were investigated and compared with results reported in the literature in order to optimise the performance of the system. In these experiments, (i) effects of the solvent employed, (ii) effects of different flow rates and injection volumes, (iii) effects of membrane cell loadings, (iv) effects of different growth-additives, and (v) lifetime of the bacterial membranes were determined.

Effect of N-, N-Dimethylformamide (DMF)

Due to the poor solubility of toluene, ethyl-benzene and xylenes in water, all BTEX stock solutions had to be prepared in an organic solvent. The employed solvent had to be miscible with the water-based sample carrier stream (potassium phosphate buffer) and did not interfere with the biosensor response or the viability of the bacterial cells. The most compatible solvent for use with a previously reported microbial biosensor for detection of benzene in workplace air was N, N-Dimethylformamide (DMF) (Lanyon Y.H. e al. 2004b). DMF was also reported to be used as an alternative desorbtion solvent for the extraction of VOCs following the sampling of workplace air on charcoal tubes (Johansen I., Wendelboe J.F. 1981). Hence, such

a sample-eluent could be directly analysed with the biosensor to qualitatively and quantitatively determine the different VOCs sampled in air. Due to these beneficial properties, the suitability of DMF as possible solvent for use with the BTEX sensor was investigated in this work. Figure 1 shows the effects of increasing concentrations of DMF on the response of the BTEX-biosensor. It was found that a concentration of 2% DMF did not cause a significant decrease from the baseline. A similar maximum concentration of DMF was reported by Lanyon *et al.* (Lanyon Y.H. e al. 2004b). A 2% concentration of DMF allowed 100 µL of BTEX stock solutions (BTEX in pure DMF) to be diluted in 5 mL of phosphate buffer; the volume required to flush and fill a 1 mL injection loop of the FIA system.

Effect of Flow Rates and Injection Volumes

In order to achieve greatest sensitivity at reasonable fast response and baseline recovery times, different flow rates and injection volumes were investigated. In the literature a flow rate of 0.5 mL min-^{1} was reported to be optimum for the use with similar biosensing systems [1,16]. In this work, flow rates between 0.25 mL min^{-1} and 1 mL min^{-1} were investigated. In Table 1 a summary of the biosensor response to BTE (each 0.1 mM, a concentration which caused a significant decrease from the baseline in batch mode) can be seen. At the lowest flow rate investigated (0.25 mL min^{-1}), the highest sensitivities for benzene, toluene and ethylbenzene were obtained. However, the increased sensitivity was always coupled with longer response and base line recovery times. On the contrary, at the highest flow rate tested (1 mL min^{-1}), low response and baseline recovery times could be obtained. However, the sensitivity of the biosensor to benzene and toluene was extremely low and it was impossible to detect ethylbenzene.

In addition to flow rates, also the effect of different injection loop volumes was investigated. According to the literature, a 1 mL injection loop was reported to be the most advantageous

volume (Lanyon Y.H. e al. 2004a). Starting from this value, higher and lower injection volumes were tested at a flow rate of 0.5 mL min^{-1} to determine the optimum injection loop volume for the BTEX biosensor. A summary of the results obtained can be seen in Table 2. Increasing the injection volume had similar effects on the biosensor response as decreasing the flow rate and vice versa (results not displayed).

The BTEX sensor showed the highest sensitivity to each BTEX compound at very low flow rates (1mL min^{-1}) and high injection volumes (3mL). However, with the increasing sensitivity also the response time and base line recovery time increased. Hence, a suitable balance was to be found to assure sufficient sensitivity to be able to detect low concentrations of BTEX at reasonable response and baseline recovery times. Taking into account the results reported in the literature (Lanyon Y.H. e al. 2004a, Lui J. e al. 2000) and the experimental results obtained, a flow rate of 0.5 mL min^{-1} and an injection volume of 1 mL were found to be the most suitable operating condition s for the FIA system employed in this work.

Effect of the Growth additive (Carbon Source)

The Aim of this investigation was to find out whether the different growth-additives influence the sensitivity of the biosensor response to any of the BTEX compounds. *Pseudomonas putida* F1 cells were grown in the presence of benzene, toluene and ethyl benzene. In addition, the bacterial cells were grown in the presence of xylenes to establish if stable mutants with the ability to also degrade this VOC can be cultivated and the detection array of the biosensor could possibly be expanded.

In Table 4 the response of the biosensor to all BTEX compounds is summarised. A concentration of 0.1 mM (a concentration which caused a significant response in the static mode) of each hydrocarbon was injected. It can be seen that the biosensor showed a response to benzene, toluene and ethylbenzene. No response could be obtained for xylenes when the

bacterial cells were cultivated with BTE as growth additives. Regarding the sensitivity, response time and baseline recovery time one can see in Table 4 that there is no significant difference if the cells are grown on benzene or toluene. If the bacteria were grown on ethylbenzene, however, the performance of the biosensor was impaired (i.e. the sensitivity to BTE decreased and the baseline recovery time was longer). This decrease of sensitivity might be due to the fact that the number of viable cells immobilised on the membrane is smaller if cells are grown in presence of ethylbenzene, indicating that there may have been a toxicity effect. Cell loading experiments with this culture revealed that if increasing volumes of culture aliquots were immobilised the sensitivity increased accordingly. The growth conditions for *Pseudomonas putida* F1 have been optimised for toluene and benzene as growth substrates [4] and toluene was reported to be the most common additive (§Gibson D.T. e al. 1968). In order to optimise the growth conditions for ethylbenzene, more work would be required to determine the growth curve in the presence of this hydrocarbon and to assess after which incubation time the optimum amount of viable cells can be obtained. In addition, the harvested cells were not stable and died over night; i.e. also the applied storage method (0.1 mM benzene-solution, 4°C) would have to be changed. As for benzene and toluene the growth conditions are well established and no significant advantages are expected if the bacteria are grown in the presence of ethylbenzene, cells for all other experiments were grown in the presence of toluene (10 mM) following procedures described by Choi *et al.* (Choi E.N. e al. 2003) and Lanyon *et al.* (Lanyon Y.H. e al. 2004a).

Effect of Cell Loading

The response of a respiration activity-type microbial biosensor is strongly dependent on the number of viable cells immobilised on the membrane (Prescott L.M. e al. 1993. Higher cell loadings should therefore result in higher sensor sensitivities. Cell loadings (of bacterial cells

grown with toluene as carbon source) from 0.5 mg up to 2 mg (dry weight) were investigated and indeed it was found that the membrane with the highest cell loading showed the highest sensitivity to BTE (with a concentration of 0.1 mM each). However, the gas permeability through the microorganism-immobilized membrane has to be considered if an oxygen electrode-based sensor is used (Karube Isao, Chang M.E.S.M. 1991). Lanyon *et al*. (Turner A.P.F. e al. 2000) as well as Mulchandani and Rogers (Mulchandani A., Rogers K.R. 1998) reported that too high cell loadings cause a high diffusion resistance, which results in a very low signal. For the biosensor used in this work, bacterial pellet dry weights of more than 1.5 mg restricted the oxygen passage through the membrane and caused a decrease in sensor sensitivity. These findings are in accordance with those reported in the literature (Lanyon Y.H. e al. 2004a, Im Lee J., Karube I. 1996).

Apart from diffusion resistance, the stabilisation time (the time taken after immobilisation before the sensor is ready for use) also has to be considered in the construction of a bacterial biosensor. The stabilisation time for the membrane showing the highest sensitivity in this work (1.5 mg pellet dry weight) was around 3 hours, compared to only 0.5 hours for the membrane with the lowest number of cells immobilised (0.5 mg pellet dry weight). As biosensors are by definition rapid monitoring devices, a stabilisation time of 3 hours is unreasonable. Therefore, a sensible balance between sensitivity and stabilisation time needed to be found. Lanyon *et al.* (Lanyon Y.H. e al. 2004a) reported that a culture volume of 1 mL (0,9 mg pellet dry weight) was regarded to be optimal for the benzene biosensor. The same pellet dry weight was found to be most advantageous also for the sensor investigated in this work. Using a 1 mL immobilisation volume (0.9 mg pellet dry weight), the biosensor showed a satisfying sensitivity to BTE (see Table at a reasonable stabilisation time (around 1 hour).

BTEX Linear Detection Ranges

After evaluating the optimum operating parameters of the biosensor, the linear detection range for each single BTEX compound was determined. Utilising an immobilisation volume of 1 mL of bacterial culture, a flow rate of 0.5 mL min^{-1} and an injection volume of 1 mL linear detection ranges between 0.01 - 0.16 mM benzene (slope y = -323x, R^2 = 0.991), 0.04 – 0.2 mM toluene (slope: y = -188x, R^2 = 0.987) and 0.1 – 0.2 mM ethylbenzene (slope: y = -82.5x, R^2 = 0.994) were obtained. The sensor did not display response to xylenes. A summary of the calibration curves for BTE is shown in Figure 1. A similar linear detection range for benzene was reported in the literature for a sensor based on a different bacterial strain, *Pseudomonas putida* ML2 (Turner A.P.F. e al. 2000). The BTEX biosensor displays different sensitivities (Figure 1) response and baseline recovery times (Table 3) for benzene, toluene and ethylbenzene. In a further experiment BTE were injected at the same time in different mixtures at different concentrations (results not displayed). Due to the differences in sensitivity, response and baseline recovery time, it was possible to determine which compounds were present in the mixture by interpreting the different peak shapes displayed on the chart recorder. Injection of benzene resulted in the greatest decrease from the original baseline (due to the steepest slope with respect to toluene and ethylbenzene: y = 323x, R^2 = 0.991) with the steepest slopes of the peaks due to the fast response time (5 min) and base line recovery time (15 min). A lesser decrease from the baseline was, with less steep slopes of the peaks was obtained when toluene (slope: y = -188x, response time: 8 min, base line recovery time: 20 min) was injected. Ethylbenzene showed the least high peaks with the flattest slopes (slope: y = -82.5x, response time: 12 min, base line recovery time: 30 min). In addition to the qualitative properties of the biosensor, the sensor response (i.e. the decrease from the original baseline) to the mixtures of BTE always equaled the sum of the sensor responses to each single compound.

Stability and Reproducibility

Culturing bacterial cells is a very time and material consuming process. Hence it is beneficial to prepare a bacterial membrane once and use it for as long as the sensor sensitivity and reproducibility persist. With the bacterial cells employed in this work good intra-assay reproducibility over one day of continuous use could be achieved In addition, the sensor displayed good inter-assay reproducibility. No decrease in sensitivity could be observed over 2 weeks of continuous use. During the time when the sensor was not in use, the bacterial

membrane was stored at 4°C in the fridge in a 1mM benzene solution. As no decrease in sensor response occurred over the period of investigation; also the applied storage method proved to be efficient. Utilising the same storage method, Lanyon *et al*. (Lanyon Y.H. e al. 2004a) reported that a reproducible sensor response was obtained over a period of 28 days. A similar storage method was also employed by Schmidt *et al.* (Schmidt A. e al. 1996), reporting a lifetime of 25 days, however, with a sensitivity loss of 70% within first 7 days of use. Stable and reproducible readings over a period of 3 months were obtained by Tan *et al.* (Tan H.M. e al. 1994) with storage of the membrane (left on the oxygen probe) at room temperature in a 1.35 mM substrate solution. However, these results did not prove to be reproducible, as Lanyon *et al*. (Lanyon Y.H. e al. 2004a) reported a significant decrease in sensor sensitivity after 3 days of use and storage under those conditions. Additionally, in the literature (Lui J. e al. 2000) evidence can be found that storage at room temperature generally causes similar decreases in sensor response. Apart from the storage conditions, also the frequency of use of a bacterial membrane might influence its lifetime; numerous measurements have implied high stability, whereas a low analysis number and/or long pauses in between implied low stability (Mulchandani A., Rogers K.R. 1998).

Interfering Compounds

In industry air, the BTEX compounds will usually occur in a mixture with various other VOCs. Hence, it was important to assess if the sensor was selective to BTE. According to the literature (Choi E.N. e al. 2003), *Pseudomonas putida* F1, does not only degrade benzene, toluene and ethyl-benzene, but also other aromatic hydrocarbons; e.g. different benzene derivates such as isopropyl benzene, see introduction). However, the oxidation rate and hence the oxygen uptake under the current operation conditions of the biosensor are too low to cause a significant decrease from the original baseline. Therefore, the sensor can be regarded as specific to benzene, toluene and ethyl benzene.

CONCLUSION

The experiments revealed that the bacterial strain, *Pseudomonas putida* F1, can be used as biological element in a FIA operated amperometric biosensor for the detection of benzene,

toluene and ethylbenzene. No response for xylenes could be obtained and no stable clone could be isolated after the cell culturing experiments employing xylenes as carbon sources. It was found that all the optimised conditions (cell-culturing, construction, and FIA parameters) for the previously developed amperometric benzene biosensor (Lanyon Y.H. e al. 2004a, Lanyon Y.H. e al. 2004b) could be directly applied to this new biosensor yielding satisfactory results regarding the sensitivity and reproducibility of the sensor. Furthermore, it was possible to distinguish single BTE compounds in mixtures, due to the significant differences in peak shapes. IF the FIA-system could be further automated and if appropriate software could be developed for the system, it might also be possible to quantitatively differentiate between benzene, toluene and ethyl benzene in mixtures as the sensor response to a mixture of BTE equaled the sum of the sensor responses to each single compound. If the sensor should be operated as an early warning device for the presence of BTEX in workplace air, future work should initially focus on cultivating stable clones of *Pseudomonas putida* F1 that are able to degrade xylenes in addition to BTE. The earlier described natural adaptation of the *tod*-degradation pathway of *P. putida* F1 is possible. However, it is a very slow process and it might be beneficial to try to introduce the additional metabolic capability of xylenes degradation by genetic engineering techniques.

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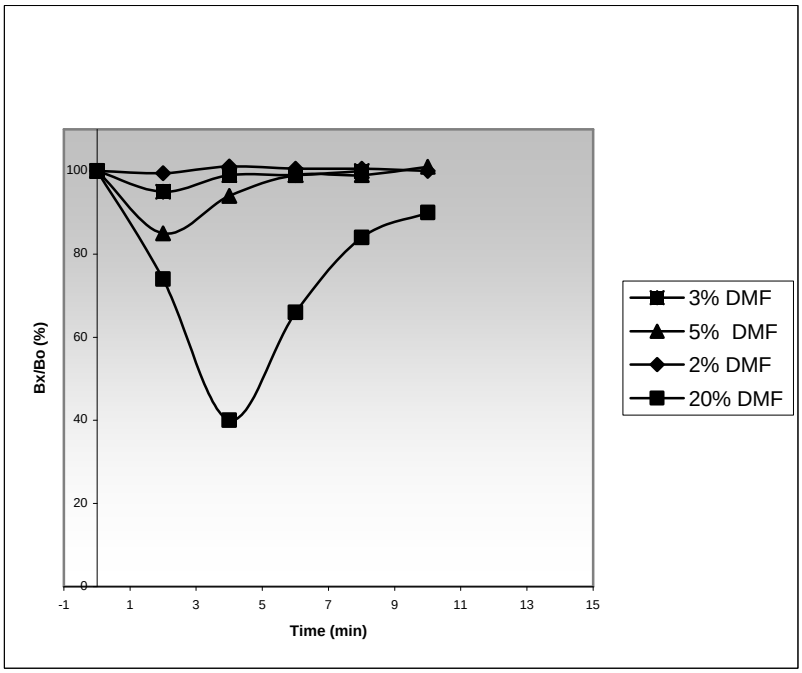

Figure 1: The effect of increasing concentrations of N,N-Dimethylformamide (DMF) on the biosensor response. FIA conditions: flow rate: 0.5 mL min^{-1}; injection loop: 1 mL. Bx/Bo (%), where Bx is the sample/standard maximum sensor output signal, and Bo is the original baeline.

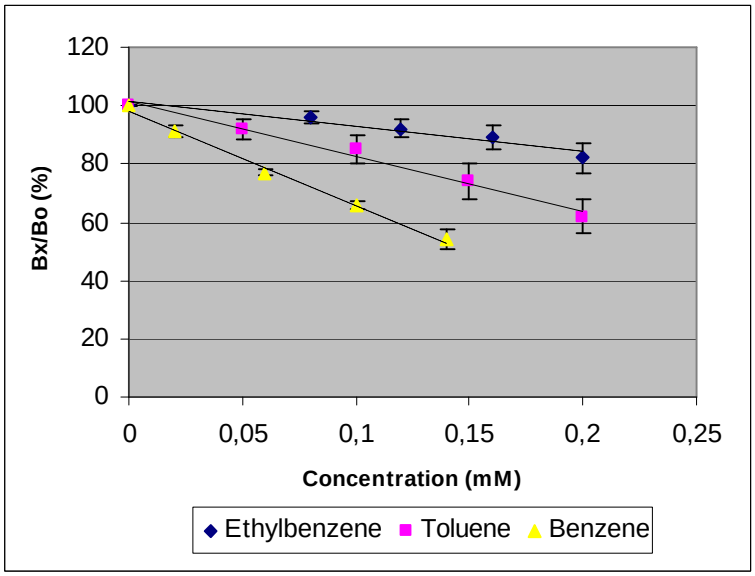

Figure 1: Calibration curves for benzene (concentration range 0.02 – 0.14 mM), toluene (concentration range 0.05- 0.2 mM) and ethylbenzene (concentration range 0.1 – 0.2mM) in potassium phosphate buffer (50 mM, pH 7). *Bx/Bo (%)*: percentage decrease from the original baseline, where *Bx* is the sample/standard baseline and *Bo* is the original baseline. Standard error bars: standard deviation from the mean based on three repetitions

Table 1: Current permissible exposure limits to benzene, toluene, ethylbenzene and xylenes. TWA: Time weighted average (HSE EH 40 2000)

	8h TWA (ppm)	**15 min TWA** (ppm)
Benzene	1	5
Toluene	50	150
Ethylbenzene	100	125
Xylenes	100	150

Table 2: Effect of different flow rates on the biosensor response. B=benzene, E=ethylbenzene, T=toluene. FIA conditions: injection loop: 1 mL. Bx/Bo (%): the percentage decrease from the original baseline, where Bx = sample/standard baseline and Bo = the original baseline.

Flow Rate	**Sensitivity to 0.1 mM BTE** **(Bx/Bo) /** CV (n = 3)		**Response Time**	**Base Line Recovery Time**
0.25 mL/min	**B**	**41%** / 1.8%	8 min	20 min
	T	**62%** / 2.2%	12 min	30 min
	E	**75%**/ 1.5 %	16 min	50 min
0.5 mL/min	**B**	**63%** / 2,1%	5 min	15 min
	T	**84%** / 3.5%	8 min	20 min
	E	**93%** / 3,2%	12 min	30 min
1 mL/min	**B**	**72%** / 3.3%	3 min	12 min
	T	**90%** / 2.9%	5 min	15 min
	E	**98%** / 1.5%	10 min	20 min

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Table 3: Effect of different injection volumes on the biosensor response. B=benzene, E=ethylbenzene, T=toluene. FIA conditions: flow rate: 0.5 mL min^{-1}. Bx/Bo (%): the percentage decrease from the original baseline, where Bx = sample/standard baseline and Bo = the original baseline.

Injection Volume	**Sensitivity to 0.1 mM BTE (Bx/Bo) / CV (n = 3)**		**Response Time**	**Base Line Recovery Time**
0.5 mL	**B**	**70%** / 2.2%	3 min	10 min
	T	**92%** / 3.6 %	6 min	15 min
	E	- / -	- min	- min
1 mL	**B**	**65%** / 2.9%	5 min	15 min
	T	**82%** / 2.5%	8 min	20 min
	E	**94%** / 2.7%	12 min	30 min
2 mL	**B**	**58%** / 3.3%	8	18
	T	**73%** / 2,4%	10	25
	E	**89%** / 3.1%	15	45

Table 4: Summary of sensor response to benzene, toluene, ethylbenzene and xylenes employing *Pseudomonas putida* F1 cells grown on different carbon sources. B = benzene, T = toluene, E = ethylbenzene, X = o, m, p- xylenes. FIA conditions: flow rate: 0.5 mL min^{-1}; injection loop: 1 mL. B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = the original baseline.

Carbon Source	**Culture Aliquot**	**Sensitivity to 0.1 mM BTEX** **Bx/Bo / CV (n = 3)**		**Response Time**	**Base Line Recovery Time**
Benzene	**1 mL**	**B**	**65%** / 1.9%	5 min	15 min
		T	**83%** / 1.8%	8 min	20 min
		E	**93%** / 2.2%	12 min	30 min
		X	- / -	-	-
Toluene	**1 mL**	**B**	**65%** / 2.9%	5 min	15 min
		T	**82%** / 2.5%	8 min	20 min
		E	**94%** / 2.7%	12 min	30 min
		X	- / -	-	-
Ethylbenezene	**2 mL**	**B**	**72%** / 3.1%	3 min	12 min
		T	**90%** / 2.6%	5 min	15 min
		E	**98%** / 1.1%	10 min	20 min
		X	- / -	-	-
Xylenes		No cells could be harvested. Afer one month of incubation a slight cell growth could be obtained, however, yet no stable clone could be isolated.			

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